MOLECULAR CLONING AND CHARACTERIZATION OF THE *SQUALENE SYNTHASE* FROM GRAIN AMARANTH

A THESIS SUBMITTED TO

THE UNIVERSITY OF TRANS-DISCIPLINARY HEALTH SCIENCES AND TECHNOLOGY

FOR THE PARTIAL FULFILLMENT OF THE AWARD OF THE DEGREE OF
M.Sc. LIFE SCIENCES (AYURVEDA BIOLOGY)

BY

AMAN THAKUR

UNDER THE GUIDANCE OF

Dr. GAURAV ZINTA (SENIOR SCIENTIST)
CSIR-INSTITUTE OF HIMALAYAN BIORESOURCE TECHNOLOGY,
PALAMPUR, HIMACHAL PRADESH, INDIA

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THE UNIVERSITY OF TRANS-DISCIPLINARY HEALTH SCIENCES AND TECHNOLOGY
Private University Established in Karnataka by ACT 35 of 2013
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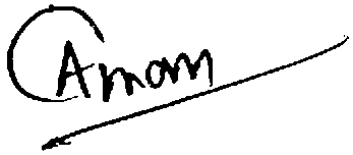
DECLARATION BY THE CANDIDATE

I declare that this thesis "**Molecular Cloning and characterization of the *Squalene synthase* from grain amaranth (*Amaranthus sp.)***" submitted for the award of Master of Science to THE UNIVERSITY OF TRANS-DISCIPLINARY HEALTH SCIENCES AND TECHNOLOGY, Bengaluru, is my original work conducted under the supervision of **Dr. Gaurav Zinta** at CSIR-Institute of Himalayan Bioresource Technology, Palampur, H.P. I confirm that no part of the work reported herein has been submitted for a degree or examination at any other university. References, funding and material obtained from other sources have been duly acknowledged, and no part of this dissertation has been plagiarised.

Place: Palampur, H.P



Signature of the Candidate

Date: 07-July-2023

Name of candidate: Aman Thakur

Reg. No.: 2021MscAB01

CERTIFICATE FROM THESIS SUPERVISOR (S)

This is to certify that the work incorporated in this thesis **"Molecular cloning and characterization of the *Squalene synthase* from grain amaranth (*Amaranthus sp.)*"** submitted by **Aman Thakur** was carried out under my supervision and co-supervised by a Ph.D. scholar in my lab. No part of this thesis has been submitted for a degree or examination at any other university. References, help and material obtained from other sources have been duly acknowledged. I confirm the originality of the work and that there is no plagiarism in any part of the thesis.

Name: Dr. Gaurav Zinta

Designation: Senior Scientist,
Biotechnology Division,
CSIR-Institute of Himalayan Bioresource Technology,
Palampur, H.P

Roles: Supervisor

Signature,

Date: 04-07-2023

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Summary

Grain Amaranth is a highly nutritious crop that is widely cultivated for its edible leaves and seeds. *squalene synthase* is important enzyme involved in the biosynthesis of *squalene*, which is a key precursor for the synthesis of plant sterols and triterpenoids.

In this study, we cloned and characterized the *Squalene synthase* (SQS) gene from grain Amaranth. The full-length cDNA sequence of the gene was obtained by PCR amplification and sequencing. The gene was found to be 1300 bp long, encoding a protein of 555 amino acids. Phylogenetic analysis revealed that the grain Amaranth *Squalene synthase* belongs to the same clade as other plant SQS.

To check the expression pattern of the gene, we performed qRT-PCR analysis in different tissues of grain Amaranth. The results showed that the gene was highly expressed in the mature leaf.

We successfully cloned and characterized the SQS from grain amaranth. Furthermore, this work would provide important insights into the biosynthesis of squalene and lay a foundation for the functional validation of *SQS* in grain amaranths.

Personal Reflection

Squalene synthase is an enzyme involved in the biosynthesis of cholesterol and other lipids. Cloning the *Squalene synthase* (SQS) gene involves isolating and amplifying the DNA sequence that codes for this enzyme. This process typically involves a series of technical steps, such as RNA extraction, cDNA synthesis, PCR amplification, DNA sequencing, and gene expression analysis, among others.

To successfully clone any gene such as SQS, researchers would require a strong foundation in molecular biology and genetics, as well as a range of technical skills, such as pipetting, gel electrophoresis, and genetic transformation techniques, etc. Additionally, a good understanding of bioinformatics is essential for analyzing and interpreting the sequencing data.

In addition to technical skills, successful gene cloning also requires strong social skills, including collaboration, communication, and problem-solving abilities. Plant molecular biology work often involves working in a team, sharing data and resources, and communicating results effectively. It also involves troubleshooting and adapting to unexpected challenges, which requires creativity, flexibility, and a willingness to learn from mistakes.

Overall, gene cloning requires a combination of technical and social skills, as well as a passion for scientific discovery and a commitment to advancing knowledge in the field of molecular biology.

ABBREVIATIONS:

Abbreviation	Definition
SQS	Squalene synthase
HIV	Human immunodeficiency virus
HMG Co-A	Hydroxy methyl glutaryl-coenzyme
IPP	Isopentenyl diphosphate
CO_2	Carbon dioxide
cDNA	Complementary DNA
CDS	Coding sequence
BLAST	Basic Local Alignment Search Tool
OE	Overexpression
PCR	Polymerase chain reaction
qPCR	Quantitative polymerase chain reaction
pET	Bacterial Recombinant Protein Vector
LBB	Luria-Bertani (LB) Broth
LBA	Luria-Bertani (LB) Agar
MS media	Murashige and Skoog medium
IAA	Indole-3-acetic acid
BAP	6-benzylaminopurine

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1. INTRODUCTION:

Squalene is a naturally occurring triterpenoid compound with a unique and diverse range of biological activities. It is an intermediate in the biosynthesis of cholesterol, steroid hormones, and other important molecules in humans and other animals. Squalene is also produced by plants and microorganisms, where it plays a role in membrane structure and function, as well as in the synthesis of secondary metabolites.

Squalene was first isolated from shark liver oil in the early 20th century, and since then, it has been extensively studied for its potential health benefits. It has been reported to exhibit antioxidant, anti-inflammatory, immunomodulatory, and anticancer activities, among others. In addition, squalene has been used in various industrial applications, such as in the production of cosmetics, food supplements, and biofuels.

The structure of squalene consists of a linear chain of 30 carbon atoms, with six double bonds arranged in a specific pattern. This unique structure gives squalene its characteristic properties, such as its high degree of unsaturation and its ability to form stable free radicals. The double bonds in squalene can react with oxygen and other free radicals, leading to the formation of various oxidation products, such as squalene mono hydroperoxide and squalene epoxide.

One of the key enzymes involved in the biosynthesis of squalene is *Squalene synthase* (SQS), which catalyzes the condensation of two molecules of farnesyl diphosphate to form squalene. This reaction is the first committed step in the biosynthesis of cholesterol and other sterols, as well as of other isoprenoids, such as carotenoids and ubiquinone. Squalene synthase is a membrane-bound enzyme that is highly conserved across different species, from bacteria to humans.

The regulation of *Squalene synthase* activity is complex and involves multiple factors, such as substrate availability, feedback inhibition, and post-translational modifications. For example, the availability of farnesyl diphosphate, the substrate for *Squalene synthase*, is regulated by the mevalonate pathway, which controls the synthesis of isoprenoids in cells. In addition,

Squalene synthase activity is inhibited by its end-product, squalene, as well as by other downstream metabolites, such as cholesterol and bile acids.

The importance of squalene and its biosynthetic pathway is underscored by the fact that mutations in *Squalene synthase* and other enzymes involved in the pathway can lead to various diseases, such as Smith-Lemli-Opitz syndrome, a rare genetic disorder characterized by developmental abnormalities and intellectual disability. In addition, the modulation of squalene synthase activity and squalene levels has been proposed as a potential therapeutic strategy for several diseases, such as cancer, cardiovascular disease, and neurodegenerative disorders.

Squalene was discovered in 1906 by the Japanese researcher Dr. Mitsumaru Tsujimoto, an expert in oils and fats at Tokyo Industrial Testing Station. He separated the unsaponifiable fraction from the shark liver oil *"kuroko-zame*" and discovered the existence of a highly unsaturated hydrocarbon. Ten years later, Tsuji Moto succeeded to obtain by fractional vacuum of the liver oil from two deep-sea shark species an unsaturated hydrocarbon, with the chemical formula C30H50, which he named "squalene" (Tsujimoto, 1916). The name came from the denomination of the sharks' family: Squalidae. The decreased risk for various cancers associated with high olive oil consumption may be due to the presence of squalene.

The traditional source for squalene is primarily from shark (*Centrophorus squamous*) and whale (*Physeter macrocephalus*) liver oil. Several studies have confirmed the health benefits of SQS in nutritional, medicinal, and pharmaceutical aspects. It is considered a potent chemopreventive and chemotherapeutic agent, which inhibits tumor growth in the colon, skin, lung, and breast, and it stimulates the immune system for the application of drugs in the treatment of diseases such as HIV, H1N1, leukemia, papilloma, and herpes, among others (Edo, 2022). Squalene is of high commercial importance; for example, 2500 tons with a commercial value of 93 million dollars were produced in 2013 and highly demanded by the cosmetics, food, and pharmaceutical industries (Huang et al., 2009).

1.1 Squalene structure and biosynthesis:

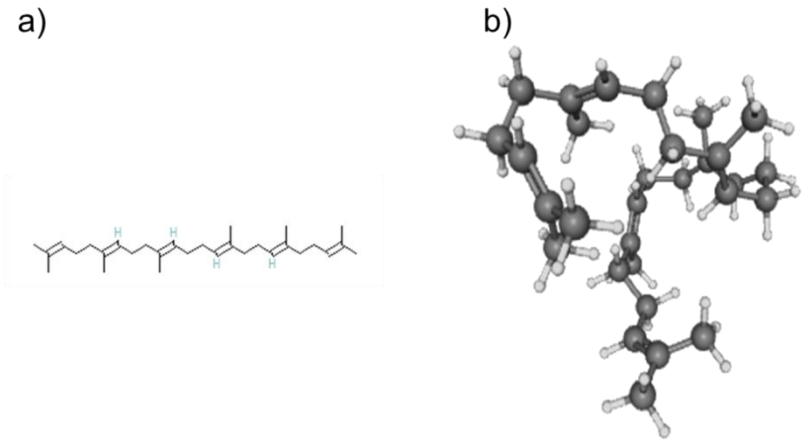


Figure 1: a: 2 D structure of squalene, b: 3 D structure of squalene

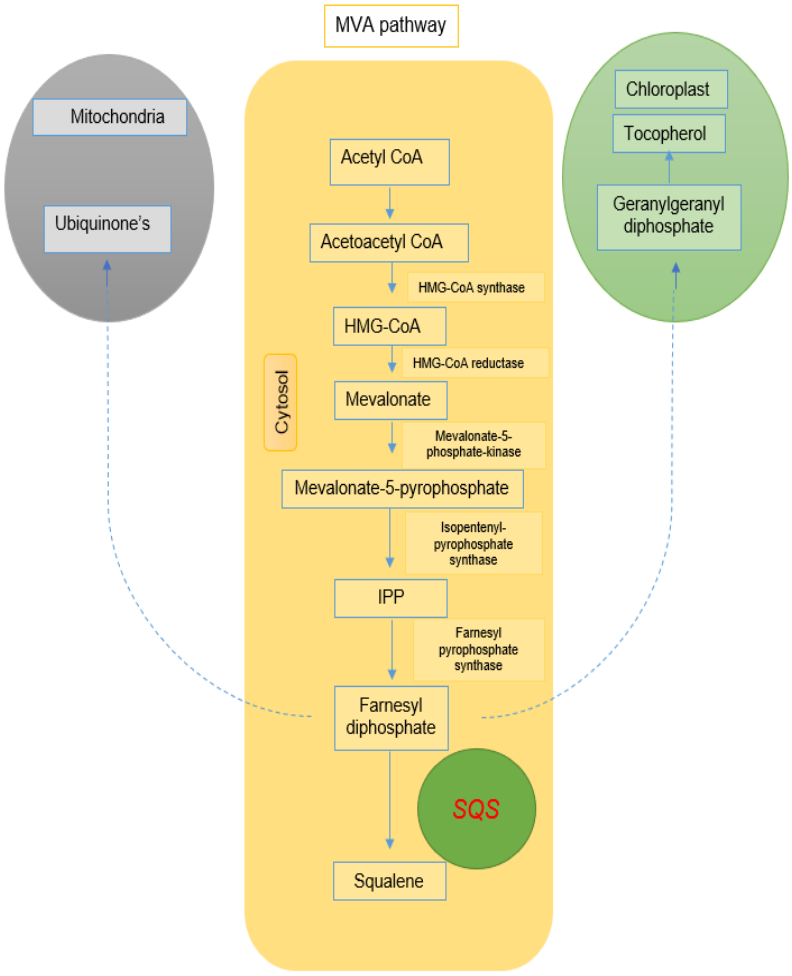
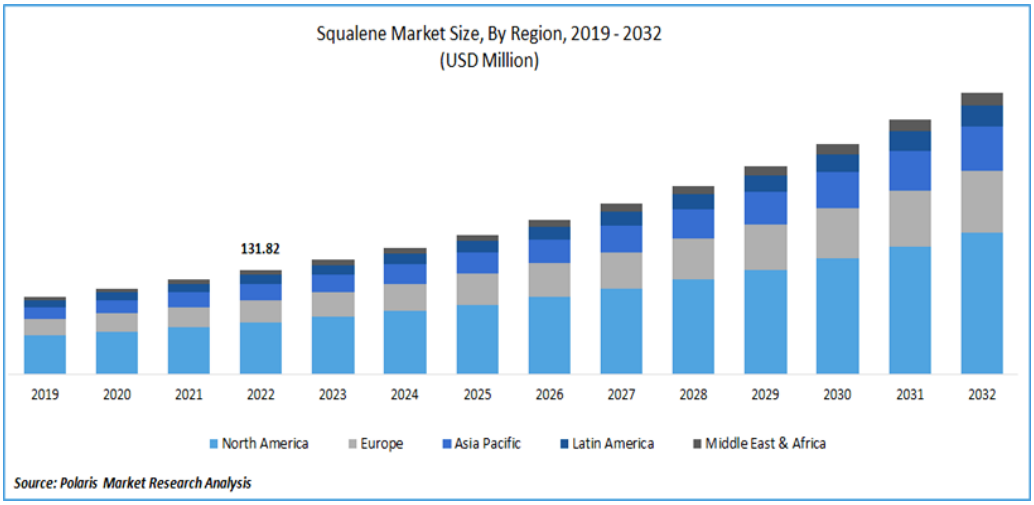


Figure 2: Routes of squalene biosynthesis via Mevalonate (MVA); Acetyl CoA: Acetyl coenzyme A; HMG-CoA: Hydroxymethyl glutaryl coenzyme A; IPP: Isopentenyl diphosphate; *SQS*: *Squalene synthase.*

1.2 Market size of squalene:



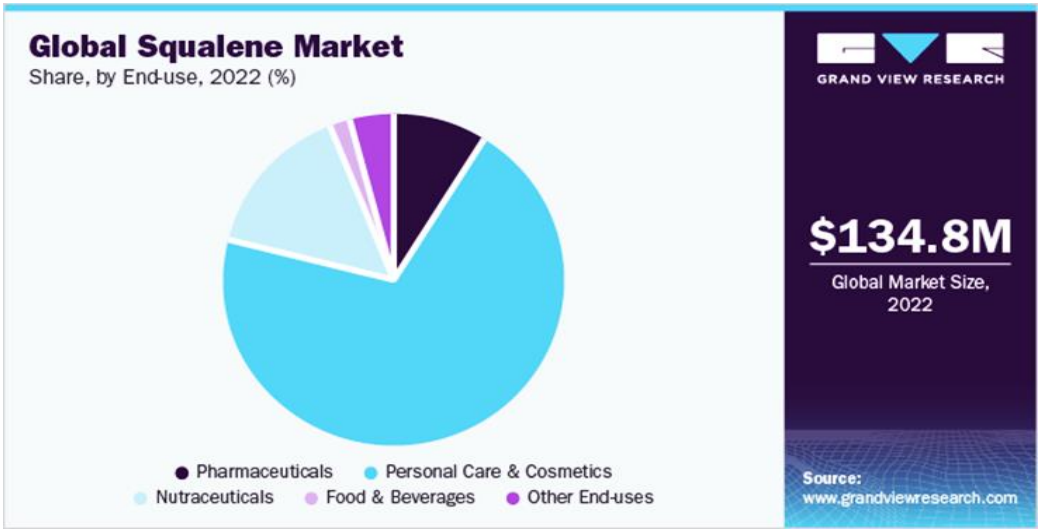


Figure 3: Market size of squalene oil by type, application and region. The global squalene market size was valued at USD 134.76 million in 2022 and is expected to expand at a compound annual growth rate (CAGR) of 10.9% from 2023 to 2030.

1.3 Why squalene from sharks is controversial:

Cosmetics industry accounts for an astonishing 90% of the global demand for shark liver oil. Estimated 1,900 tons of squalene used for hair conditioners, creams, lipsticks, foundations, sunscreens, and more some even audaciously labeled as cruelty free (Bloom association 2012.)

Today, the mass slaughtering of sharks for their prized liver oil is taking a major toll on certain populations (Gohil et al., 2019) . And when apex predators suffer, so does the health of the entire ecosystem (Estes et al., 2011).

Deep-sea sharks which are most desirable by the beauty industry are especially vulnerable because they have such long lifespans and, therefore, slow reproduction rates. For example, the leaf scale gulper shark that lives in the Atlantic, Indian, and Pacific Oceans doesn't reach sexual maturity until about 35 years old. In 2019, the International Union for Conservation of Nature (IUCN) elevated the species' listing from vulnerable to endangered (View, 2009).

Overfishing (for fins, meat, leather, and oil) is reportedly the leading reason why global populations of oceanic sharks and rays declined by 71% from 1970 to 2020 (Pacoureau et al., 2021).

Production of squalene oil from shark liver is controversial because it involves killing of sharks., which are already threatened by overfishing and other environmental pressures. Sharks are apex predators and play a crucial role in maintaining the health of ocean ecosystems. The practice of killing them for their liver oil, which is used in various industries including cosmetics, pharmaceuticals, and dietary supplements, has led to a decline in their populations and raised concerns about the sustainability and ethics of the industry.

Furthermore, there are alternatives to shark liver oil, such as squalene derived from plant sources or synthesized in a laboratory. These alternatives are considered more sustainable and ethical, and do not contribute to the decline of shark populations.

There have also been concerns about the safety and efficacy of shark liver oil. While some studies have suggested that it may have health benefits, such as boosting the immune system and reducing inflammation, others have found no significant effects or even potential risks associated with its use.

Overall, the controversy surrounding shark liver squalene oil highlights the need for more sustainable and ethical practices in industries that rely on natural resources, as well as the importance of considering the environmental and ethical implications of consumer choices.

1.4 Alternatives to animal squalene:

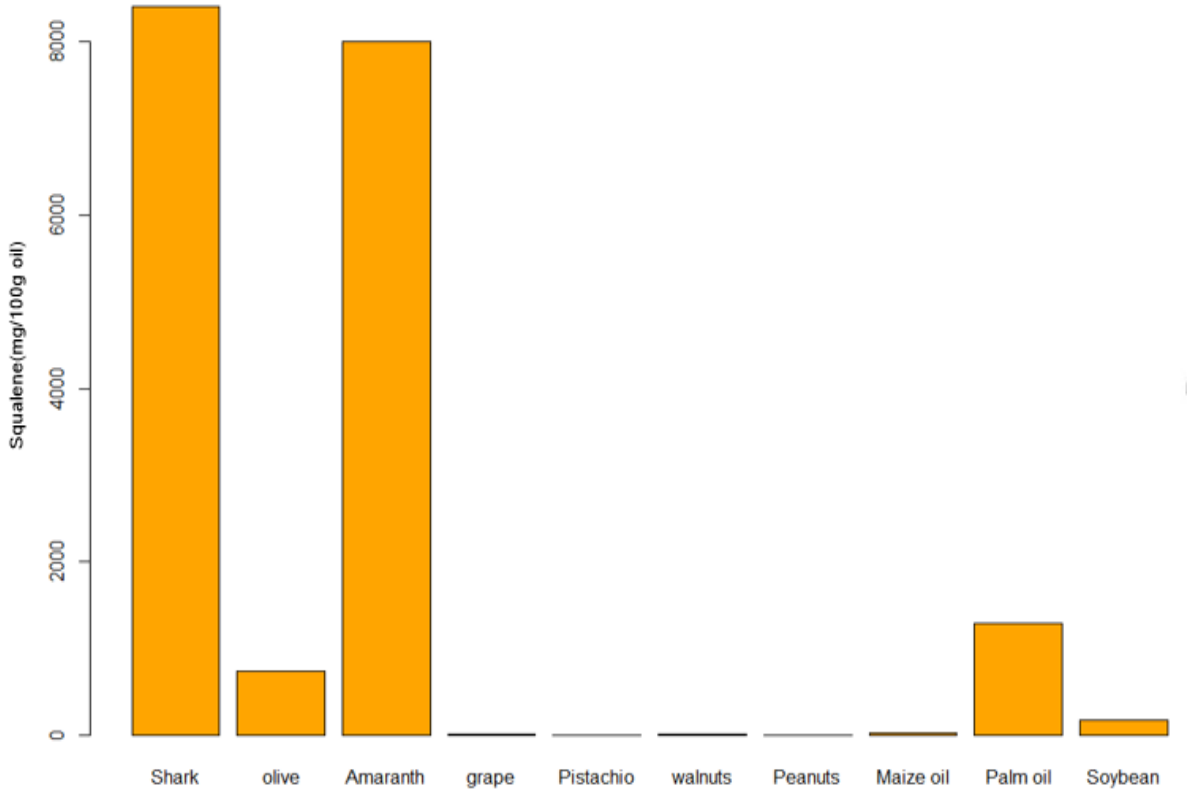


Figure 4: Alternatives of squalene plant-based as compare to shark liver oil in per 100g of oil.

Table 1: Alternatives of squalene and squalene content after extraction and purification.

Sources	Squalene (mg/100g oil)	References
Vegetables		
Olive	150-747	(Edo, 2022)(Mastralexi & Tsimidou, 2021), (Rosales-García et al., 2017)
Amaranth	6000-8000	(Wejnerowska et al., 2013), (Ariza Ortega et al., 2012)
Seed of grape	2.7-14.1	(de Souza et al., 2020)
Pistachio	1.1-2.2	(Rotondo et al., 2020)
Walnuts	0.9-18.6	(Maguire et al., 2004)
Peanuts	9.8	(Maguire et al., 2004)
Maize oil	10-27	(Lozano-Grande et al., 2018)
Sunflower oil	2.2-2.6	(Pramparo et al., 2005)
Palm oil	0.1-1300	(Nang Lau et al., 2005), (Goh et al., 1985)
Soybean	1.2-180	(Gunawan et al., 2008)
Animals		
Shark (Liver oil)	2300-8400	(Carroll et al., 1996), (Spanova & Daum, 2011), (Zhang et al., 2018)
Yeast and fungi		
Saccharomyces cereviceae	40	-
Aranthiochytrium sp.	900-6940	(Nakazawa et al., 2014)
Pseudozyma sp.	340.5	(Xu et al., 2016)

1.5 *Amaranthus hypochondriacus:*

Common name: Lady Bleeding, Prince-of-Wales feather.

Description: *Amaranthus hypochondriacus,* is an herbaceous annual or short-lived perennial plant with showy feathery fed flowers often grown as an ornamental. Amaranthus is dioecious (i.e., separate male and female individual plants).

Amaranth (*Amaranthus spp*.) is considered a pseudocereal because it neither belongs to the grass family nor contains gluten, which makes this seed attractive to people with celiac diseases (Coelho et al., 2018). The richest SQS source is shark liver oil, representing up to 40% of the liver weight. Since these animals are protected, plant sources should be explored (Naziri et al., 2016). SQS could be extracted from a plant with organic solvents.

Image (A)

Image (B)

Figure 5: Image (A) *Amaranthus spp*. Image (B) *Amaranthus hypochondriacus*

2. Material and method:

2.1 Plant material:

Amaranthus hypochondriacus were grown in the experimental field at the CSIR- Institute of Himalayan Bioresource Technology, Palampur, Himachal Pradesh which was used in this study. Fresh leaves were collected at the time of sampling and immediately frozen in liquid nitrogen and then kept at -80°C for later RNA extraction and cDNA synthesis.

2.2 Gene selection:

SQS *(squalene synthase)* gene are responsible for squalene production in *Amaranthus hypochondriacus* and other crops found from the already available literature. The CDS sequence of this gene from grain *Amaranthus hypochondriacus* was blasted on Phytozome against the genomic sequence. This confirms the presence of this gene in pseudocereals and their CDS sequences are collected from the database stored at the Phytozome.

2.3 Primer design:

CDS sequences of gene were obtained from the Phytozome and the forward and reverse primers were designed manually ApE A Plasmid Editor (Table1.) and ordered from Bio serve Biotechnology Pvt Ltd, Hyderabad.

Table 2: Gene-specific primers used in this study. O.E primers attached with specific restriction enzyme sequence at the 3' and 5' end.

Primer Name	**Direction**	**Sequence (5' ⟶ 3')**
Squalene synthase (F)	Forward	ATGGGGAGTTTAGGAGCG
Squalene synthase (R)	Reverse	TTAGTTACTAATCATTCTGTTGG
Squalene synthase O. E	Forward	CGGGATCCATGGGGAGTTTAGGAGCG
Squalene synthase O. E	Reverse	CGACGCGTGTTACTAATCATTCTGTTGG

Squalene synthase qPCR	Forward	ATGCGGGTTTGGAGGATCTT
Squalene synthase qPCR	Reverse	CATGCGACATTTTGGAATCTCAT
Squalene synthase (F)	Forward	CCGGGATCCATGGGGAGTTTAGGAGCG
Squalene synthase (R)	Reverse	CGGGATCCGTTACTAATCATTCTGTTGG

2.4 Vector selection:

For the cloning of *Squalene synthase* (SQS), the pJET1.2 blunt vector (Thermo Scientific) was selected respectively and for overexpression (*pCAMBIA 1305*) vector (Invitrogen, Waltham, U.S.A) and for protein expression pET-28a (+) were selected for the gene (Table 2).

Table 3: Vector-specific primers used in this study.

Primer name	**Direction**	**Sequence (5' ⟶3')**
Pjet1.2 blunt vector	Forward	CGACTCACTATAGGGAGAGCGGC
Pjet1.2 blunt vector	Reverse	AAGAACATCGATTTTCCATGGCAG
FLAG4 vector (CP3)	Forward	CCGATTCATTAATGCAGCTG
FLAG4 vector (CP2)	Reverse	CGGCAACAGGATTCAATCT
T7 Promoter (F)	Forward	TAATACGACTCACTATAGGG
T7 Terminator (R)	Reverse	GCTAGTTATTGCTCAGCGG

2.5 RNA extraction:

Frozen leaves and steams were ground with liquid nitrogen using a clean sterile mortar and pestle. 100mg of crushed samples from different plant tissues were weighed and stored in liquid nitrogen. Then, RNA was extracted using an RNA isolation kit (Sigma-Aldrich, U.S.A) according to the product instruction. After the extraction, the RNA concentration was quantified by the Nanodrop one microvolume (Thermo Scientific) and its integrity was examined by electrophoresis in 1.2% (w/v) agarose gel. The isolated RNA was stored at -20°C.

2.6 cDNA synthesis:

The RNA sample was then further used for the synthesis of cDNA using the Verso cDNA synthesis kit (Thermo Scientific) according to the product instructions. All the experiment was done on ice/mini cooler to avoid degradation of RNA.

2.7 qPCR analysis:

To check the tissue-specific expression of the gene SQS, we used a technique named qPCR (quantitative polymerase chain reaction). qPCR reaction contains SYBR Green master mix 16ul, SQS forward primer 1ul, SQS reverse primer, template(50ng/ul), water 10ul. And for reference, we took Actin as a housekeeping gene.

2.8 Cloning of *squalene synthase*:

The process of cloning starts with the amplification of the desired gene. The *Squalene synthase* gene was amplified by PCR. The PCR reaction was prepared in a volume of 25ul containing 1ul cDNA, 1ul *SQS* forward primer,1ul *SQS* reverse primer each, 10ul polymerase Master mix, and 12ul NFW. The PCR ran for 2 hr. and 30 min and the thermal cycle conditions involved: Denaturation at 95°C for 3 min, and 95°C for30 sec; Annealing at 53°C for 30 sec; Extension at 72°C for 2 min, and Final Extension at 72°C for 7min. After this PCR product was run on 1.2% agarose gel. After getting the band size of 1300bp, the gel was cut down and further processed for gel elution using a gel elution kit (Qiagen, Hilden, Germany) according to product instructions. Eluted product concentration was determined by nanodrop one Microvolume (Thermo Scientific). After this ligation process took place in which *SQS* eluted product was ligated with *pJET* vector in the ratio of 5:1 then ligated product was incubated at room temperature for 1 hour then at 4°C for 12-16 hours. After ligation, the transformation process occurs in which the ligated product was transformed into *DH5-α E. coli* competent cells. For this, 10ul of the ligated product was added to a vial containing 100ul competent cells and incubated for 30 min. in ice. After ice incubation, Heat shock treatment was given to the cells using a water bath at 42°C for 90 sec. Then again it was incubated on ice for 5 min. After that 4X volume of autoclaved LBB was added to a vial (inside the laminar

airflow) and incubated for 90 mins. In a shaking incubator for 37°C at 220 rpm. Plates containing LBA media with ampicillin antibiotic were taken. Vials were pellet down by centrifugation at 6,000 rpm for 3 min. excess 400ul supernatants were discarded and the pellet was gently mixed in the remaining 100ul supernatant. And spread over the ampicillin plate. Then the plate was incubated at 37°C for 12-16 hours.

After getting the colonies in ampicillin containing LBA plate colony PCR was performed to check the positively transformed colonies. Master plate were prepared from the primary plate. And each colony was streaked on a fresh ampicillin containing LBA plate. The two PCR reactions would be prepared from the same colony in which one PCR reaction would contain a gene-specific primer and another PCR reaction would contain vector-specific primer. For *SQS*, the one set of PCR reaction was in a volume of 25ul containing: Template from the bacterial colony (mix bacterial colony in 13ul NFW and incubated it at 98°C for 2mins before adding any other PCR reagent components, 1ul SQS forward primer and *SQS* reverse primer each and 10ul polymerase master mix , and another set of PCR reaction was in the volume of 25ul containing:Template from the bacterial colony(mix bacterial colony in 13ul NFW and incubated it at 98°C for 2 mins before adding any other PCR reagent components, 1ul *pJET* vector forward primer and *pJET* vector reverse primer reach and 10ul polymerase Master mix. The thermal cycle condition for 1 cycle was: Denaturation at 95°C for 3 min, and 95°C for30 sec; Annealing at 53°C for 30 sec; Extension at 72°C for 2 min, and Final Extension at 72°C for 7 min. Then PCR product was run on 1.2% agarose gel. Colonies numbers whose bands were observed at 1300 bp position were further processed for plasmid isolation. In this 5ml of autoclaved LBB was taken in the autoclaved test tube and 5ul of ampicillin antibiotic was added to each tube. A small portion of those colonies that showed the positive result was picked by the tips and added to the LBB media. For different colonies, different LBB tubes were taken. These tubes were then incubated at 37 °C for 12-16 hours. After incubation, these tubes were subjected to plasmid extraction using a HiGenoMB Himedia plasmid kit. According to the product instructions. Plasmid concentration was determined by a Nanodrop one microvolume (Thermo Scientific). The eluted plasmid was stored at-20°C.

After verification, another PCR reaction was set for the cloned SQS plasmid. For O.E *SQS*, the PCR reaction was in the volume of 25ul containing: 1ul template from SQS plasmid, 1ul SQS O.E forward primer and SQS O.E reverse primer each,10ul polymerase Master mix, and 12ul NFW. The thermal cycle condition was Denaturation at 95°C for 3 min, and 95°C

for30 sec; Annealing at 58°C for 30 sec; Extension at 72°C for 2 min, and Final Extension at 72°C for 7min. Then the PCR product was run on 1.2%(w/v) agarose gel. After getting the band at 1300bp, the gel was cut down and further processed for gel elution using a gel elution kit (Qiagen, Hilden, Germany) according to the product instruction. Eluted product concentration was determined by a Nanodrop One Microvolume (Thermo Scientific, Wilmington, U.S.A) before the ligation, these PCR products were subjected to restriction digestion along with the restriction digestion of the O.E vector *pCAMBIA 1305*. Both PCR product and vector were treated with double restriction digestion which contains 1ul plasmid, 1ul *BamHI* NEB restriction enzyme (Biolabs, New England),1ul *MluI* NEB restriction enzyme (Biolabs, New England) 5ul 10X reaction buffer, and NFW up to 50ul. They were incubated at 37°C for 6-8 hrs. and after that, they have incubated again at 65°C for 20 mins. To stop the reaction. Then subjected to PCR Purification using a PCR purification kit (Qiagen, Hilden, Germany) according to the product instructions. Plasmid concentration was determined by a Nanodrop one Microvolume (Thermo Scientific) after this ligation process takes place in which *SQS* PCR purified product and *pCAMBIA 1305* vector PCR purified product were mixed in the ratio of 5:1 along with 2ul 10X buffer and 1ul T4 DNA buffer. The ligated product was incubated at room temperature for 1 hour then at 4°C for 12-16 hours. After ligation, the transformation process occurs in which the ligated product was transformed into *DH5-α E. coli* competent cells. For this, the ligated product was added to a vial containing 100ul competent cells and incubated for 30 min. in ice. Then heat shock treatment was given using a water bath at 42°C for 90 sec. Then again it was incubated on ice for 2min. After that 4X volume of autoclaved LBB was added to a vial (inside the laminar airflow) and incubated for 90 mins. On a shaking incubator for 37°C after 90 mins. Plates containing LBA media with kanamycin antibiotics were taken. Vials were pellet down by centrifugation at 13,000 rpm for 1 min. excess 400ul supernatants were discarded and the pellet was gently mixed in the remaining 100ul supernatant. And spread over the Kanamycin plate. Then the plate was incubated at 37°C for 12-16 hours. Only those bacterial colonies would grow in which vector was inserted as *pCAMBIA 1305* vector showed resistance towards kanamycin antibiotics.

After getting the colonies to check the positive colonies we do colony PCR. Initially, master plates were prepared from the primary plates. And each colony was picked which would be used as a template for colony PCR. Take in mind that two PCR reactions would be prepared from the same colony in which one PCR reaction would contain a gene-specific primer and another PCR reaction would contain a vector-specific primer. For O.E SQS, the one set of

PCR reactions was in a volume of 25ul containing: 1ul template from the bacterial colony (Mix bacterial colony in 13ul NFW and incubate it at 98°C for 2mins before adding any other PCR reagent components),1ul O.E *SQS* forward and reverse primer and 10ul polymerase Master mix, and another set of PCR reactions was in the volume of 25ul containing: Template from the bacterial colony (Mix bacterial colony in 13ul NFW and incubated it at 98°C for 2mins. Before adding any other PCR reagent components, 1ul CP2 forward and CP3 reverse primer each and 10ul polymerase Master mix. The thermal cycle condition was 1 cycle (Denaturation for (95°C (3 min), and 95°C (30 sec); (Annealing for (58°C, 30 seconds); (Extension for 72°C, 2min), Final Extension for 72°C, 7min.) After completing the PCR, the PCR product was run under 1.2%(w/v) agarose gel. Colony numbers whose bands were observed at 1300bp position were further processed for inoculation. In this 5ml of autoclaved LBB were taken in the autoclaved test tube and 5ul of kanamycin was added to each test tube. A small portion of those colonies that showed the positive result was picked by the tips and added to the LBB media. For different colonies, different LBB tubes were taken These tubes were then incubated at 37°C for 12-16 hours. After incubation, these test tubes were subjected to plasmid extraction using a HiGenoMB Himedia plasmid kit. According to product instructions, plasmid concentration was determined by a Nanodrop One microvolume (Thermo Scientific, Wilmington, U.S.A) . to check whether the eluted plasmid contains the desired genes, they were subjected to restriction digestion, the eluted plasmid was treated with double restriction digestion which contain 1ul plasmid,1ul *BamHI* NEW restriction enzyme (Biolabs, New England) 1ul *MluI* NEB restriction enzyme (Biolabs, New England),5ul 10X reaction buffer, and NEW up to 50ul. They were incubated at 37°C for 6-8hrs and after that, they have incubated again at 65°C for 20mins to stop the reactions. The eluted plasmid was also subjected to DNA Sequencing for double confirmation.

After getting the positive confirmation of the plasmid. The eluted plasmid was then transformed in the *Agrobacterium tumefaciens* to make the construct. 1ug of plasmid was mixed in 100ul *Agrobacterium tumefaciens.* Incubated it on ice for 10mins and transferred the vial to liquid nitrogen for 3-5mins. Incubated it again at 37°C for 15mins. After incubation, 4X volume of autoclaved LBB was added to the vial (occurred in the laminar airflow) and incubated again at 28°C for 3 hours. After 180 mins, plates containing LBA media with rifamycin, kanamycin, and gentamycin antibiotics were taken. The vials sample were pellet down by centrifugation at 13,000 rpm for 1 min, excess 400ul supernatants were discarded and the

pellet was gently mixed in the remaining 100ul supernatant which was then spread over the kanamycin plate. The plate was incubated at 28°C for 48 hours. Then check the positive colony through colony PCR, Initially, master plates were prepared from the primary plates. And each colony was picked which would be used as a template for colony PCR. Take in mind that two PCR reactions would be prepared from the same colony in which one PCR reaction would contain a gene-specific primer and another PCR reaction would contain a vector-specific primer. For O.E SQS, the one set of PCR reactions was in the volume of 25ul containing: 1ul template from the bacterial colony(mix bacterial colony in 13ul NFW and incubated it at 98°C for 2mins before adding any other PCR reagent components), 1ul O.E *SQS* forward primer and O.E SQS reverse primer each and 10ul polymerase Master mix, and another set of PCR reaction was in the volume of 25 ul containing: 1ul template from the bacterial colony (mix bacterial colony in 13ul NFW and incubated it at 98°C for 2 mins before adding any other PCR reagent components) , 1ul CP3 forward primer and CP2 reverse primer each and 10ul polymerase Master mix, The thermal cycle condition was 1 cycle Denaturation at 95°C for 3 min, and 95°C for30 sec; Annealing at 53°C for 30 sec; Extension at 72°C for 2 min, and Final Extension at 72°C for 7min. Then the PCR product was run on 1.2%(w/v) agarose gel. Colony numbers whose bands were observed at 1300bp position were further processed for inoculation.

After getting the positive colonies then take LB media tubes and add antibiotics (Rifamycin, Kanamycin, and Gentamycin) also named Primary culture, picked the positive colony and put it on LB media tube containing all three antibiotics, and put it at 28°C for at least 2 days. After 2 days take out the primary culture from the Incubator and make secondary culture in an LB media flask containing rifamycin, kanamycin, and gentamycin antibiotics add 50ul primary culture to the secondary culture containing all three antibiotics, and put the flasks in 28°C for overnight. Next day take out the culture flasks from the incubator and pelletize the culture and remove the supernatant and dissolve the pellet in half M.S media and check the growth in a spectrophotometer. Agrobacterium-mediated gene delivery of tobacco was conducted using the *pCAMBIA2301* binary vector (11634 bp). This vector carries the kanamycin resistance gene of neomycin phosphotransferase (nptII), which acts as a selectable marker for plant selection, and the gusA reporter gene. In the left border (LB), the nptII gene is driven by the cauliflower mosaic virus 35S (*CaMV35S2*) promoter and *CaMV35S* terminator, whereas the intron—gusA in the right border (RB) is driven by the *CaMV35S* promoter and NOS-terminator. The effects of the factors in Agrobacterium-mediated gene transformation,

including Agrobacterium strains (*GV3101*), Agrobacterium cell density (OD600 = 0.6, 0.7, and 0.8), acetosyringone concentration (200, 300, and 400 µmol/L), and inoculation duration (immersion time) (10, and 20 min) were assessed. Pre-incubated (2 days) leaf disk explants (1 × 1 cm2) of 4-week-old in vitro-obtained plantlets of tobacco were inoculated into the Agrobacterium suspension, containing the binary vector, were blot dried on sterile filter paper and co-cultivated in a phytotron at 25 ± 1°C with 60 to 70% relative humidity for 48 h (in dark). The Murashige and Skoog medium (MS) supplemented with 0.1 mg/L indole-3-acetic acid (IAA) + 1 mg/L 6-benzylaminopurine (6-BAP) and 100 µM acetosyringone was used for co-cultivation. Explants were then transferred to the selective regeneration medium.

2.9 Transformation:

I. Explants preparation:

- ❖ Take healthy fully expanded leaves from 4–5-week-old tissue culture grown tobacco plant
- ❖ Cut into 0.6-0.8 cm squares (or can use a cork borer, which is about 1.0 cm diameter)

II. Bacterial suspension:

- ❖ Collect bacterial cells by a centrifuge
- ❖ Discard the liquid and suspend the bacterial pellet using half MS media
- ❖ Dilute the suspension to an O.D.600 of 0.5-0.8

III. Media used:

- ❖ Half M.S media containing acetosyringone concentration (200 mM)
- ❖ Full M.S media containing auxin (IAA) and cytokinin (BAP), antibiotics Hygromycin and cefotaxime

2.10 Transformation in *Arabidopsis thaliana*

Growing healthy Arabidopsis plants until they begin to bolt and produce floral infloresences.

- ❖ Germinate seeds and grow seedlings. There are two different methods (A and B, below) for this purpose. Generally, recommend the standard method (A). However, in

particular cases, such as with mutations that cause reduced viability and low germination frequency, we suggest that users follow the more laborious (B).

(A) Standard *Arabidopsis* germination procedure:

- Suspend seed in 0.05% agarose and keep them in darkness for 3 days at 4 °C to break dormancy. This short period of stratification might not be completely necessary but certainly allows uniform and maximal seed germination.
- Spread around 20–30 seeds on wet soil in each 4 in. * 4 in. pot.

(B) Alternative *Arabidopsis* germination procedure:

- Sterilize and place seeds on MS medium, referring to Steps 15–18 below. Stratify seeds by keeping them in darkness at 4 °C for 3 d.
- Germinate seeds and grow the seedlings in long-day conditions (16 h light/8 h dark, 20 1C) for 2 weeks, and then transfer them to wet soil (B12 seedlings/pot).
- Cover the pots with cling film till the germination stage.
- Grow plants in a growth chamber or a greenhouse under short days for 3-4 weeks, then move them to long-day conditions to induce flowering. Pots and trays must be well watered until the first two true leaves are developed in the case of procedure 1(A). For example, we place the pots in a tray and cover the pots with plastic domes to maintain high humidity for the first 2 weeks. In the case of procedure 1(B) we keep high humidity for the transferred seedlings for 3–4 d before removing the plastic covers. Healthy plants are a prerequisite for a successful transformation. Following these recommendations is important to obtain big plants that can produce a large number of seeds.

2.11 Transformation by floral dip

- Start preparing the Agrobacterium strain that harbors the gene of interest in a binary vector by inoculating a single Agrobacterium colony into 5 ml liquid LB medium containing the appropriate antibiotics for binary vector selection. Incubate culture at 28 1C for 2 d.

- Use this feeder culture to inoculate a 500-ml liquid LB with the appropriate antibiotics and grow the culture at 28 1C for 16–24 h. We exclusively use cells that grow to the stationary phase (OD B1.5–2.0).
- Collect Agrobacterium cells by centrifugation at 4,000g for 10 min at room temperature, and gently resuspend cells in 1 volume of freshly made 5% (wt/vol) sucrose solution with a stirring bar.
- Add Silwet L-77 to a concentration of 0.02% (vol/vol) (100 ml per 500 ml of solution) and mix well immediately before dipping. Transfer the Agrobacterium cell suspension to a 500-ml beaker.
- Invert plants and dip aerial parts of plants in the Agrobacterium cell suspension for 10 s with gentle agitation. We dip not only inflorescences but also the rosette to soak shorter axillary inflorescences. Remove dipped plants from the solution and drain the treated plants for 3–5 s. A film of liquid coating the plants should then be visible.
- Cover dipped plants with a plastic cover or wrap them with plastic film. Lay down the treated plants on their sides for 16–24 h to maintain high humidity.
- Remove the cover the next day.
- Collect dry seeds using a sieve mesh.

2.12 Screening of primary transformants

- Pour selection plates (150 x 150 x 25 mm) containing carbenicillin and the appropriate antibiotics (Hygromycin).
- Sterilize seeds by first treating them with 50 x volume of 70% ethanol for 1 min and mixing the seed suspension thoroughly.
- Continue the sterilization process by treating the seeds with a 50-x volume of 50% bleach/50% water/0.05% Tween for 10 min and vertexing the suspension vigorously every 2 min.
- Rinse the seed three times with sterile water. In the last washing step, the wash should appear clear and without any yellow color; if it does not, this indicates that residual bleach is still present in the wash.
- Re-suspend the sterilized seeds in sterile 0.05% agarose (40 ml seed per ml agarose) and spread the seed-agarose suspension onto selection plates. Plate 3–4 ml seed-

agarose mixture on each 150 x 150 x 25 mm plate. Dry plates under a laminar flow hood until agarose dries up and seeds become stable on the plate.

- ❖ Vernalize seeds by placing them at 4 1C for 3 d.
- ❖ Move plates to a tissue culture room or a growth chamber under continuous light (50–100 microeinsteins m–1 s–1) or long-day conditions. After 7–10 d, transformants should be readily distinguished as seedlings with healthy green cotyledons and true leaves and roots that extend into the selective medium.
- ❖ Transplant plantlets to water-saturated soil and cover the tray with a plastic film to maintain high humidity for 2 d.
- ❖ Timing:
  - Growth of *Arabidopsis* plants: 2 months.
  - Growth of agrobacteria and floral dipping transformation: 3 days.
  - Seed set and maturation of transformed seeds: 1 month.
  - Screening of primary transformants: 10-14 days.

2.13 Cloning of SQS *(squalene synthase)* in pET-28a (+) vector

The first round of PCR is an amplification of *squalene synthase* gene, the PCR reaction was in a volume of 25ul containing 1ul cDNA, 1ul SQS forward primer,1ul SQS reverse primer each, 10ul polymerase master mix, and 12ul NFW. Thermal cycle condition was (PCR which ran for , Denaturation at 95°C for 3 min, and 95°C for30 sec; Annealing at 53°C for 30 sec; Extension at 72°C for 2 min, and Final Extension at 72°C for 7min. After this PCR product was run on 1.2% agarose gel. After getting the band size of 1300bp, the gel was cut down and further processed for gel elution using a gel elution kit (Qiagen, Hilden, Germany) according to product instructions. Eluted product concentration was determined by nanodrop one Microvolume (Thermo Scientific). After this ligation process taken placed in which SQS eluted product was ligated with pET-28a (+) vector in the ratio of 5:1 then ligated product was incubated at room temperature for 1 hour then at 4°C for 12-16 hours. After ligation, the transformation process occurs in which the ligated product was transformed into *DH5-α E. coli* competent cells. For this, the ligated product was added to a vial containing 100ul competent cells and incubated for 30 min. in ice. Then heat shock treatment was given using a water bath at 42°C for 90 sec. Then again it was incubated on ice for 2min. After that 4X volume of autoclaved LBB was added to a vial (inside the laminar airflow) and incubated for

90 mins. On a shaking incubator for 37°C after 90 mins. Plates containing LBA media with ampicillin antibiotic were taken. Vials were pellet down by centrifugation at 13,000 rpm for 1 min. excess 400ul supernatants were discarded and the pellet was gently mixed in the remaining 100ul supernatant. And spread over the ampicillin plate. Then the plate was incubated at 37°C for 12-16 hours.

After getting the colonies in LBA containing ampicillin plate. The next step was colony PCR to check the positive response of single colonies. Master plates were prepared from the primary plate. And each colony was picked which would be used as a template for colony PCR. Take in mind that two PCR reactions would be prepared from the same colony in which one PCR reaction would contain a gene-specific primer and another PCR reaction would contain a vector-specific primer. For pET *SQS*, the one set of the PCR reaction was in a volume of 25ul containing: Template from the bacterial colony (mix bacterial colony in 13ul NFW and incubated it at 98°C for 2mins before adding any other PCR reagent components, 1ul *SQS* forward primer and *SQS* reverse primer each and 10ul polymerase master mix, and another set of the PCR reaction was in the volume of 25ul containing: Template from the bacterial colony(mix bacterial colony in 13ul NFW and incubated it at 98°C for 2 mins before adding any other PCR reagent components, 1ul pJET vector forward primer and pJET vector reverse primer reach and 10ul polymerase master mix. The thermal cycle condition for 1 cycle Denaturation at 95°C for 3 min, and 95°C for30 sec; Annealing at 53°C for 30 sec; Extension at 72°C for 2 min, and Final Extension at 72°C for 7min. Then PCR product was run on 1.2% agarose gel. Colony numbers whose bands were observed at 1300 bp position were further processed for inoculation. In this 5ml of autoclaved LBB was taken in the autoclaved test tube and 5ul of ampicillin antibiotic was added to each tube. A small portion of those colonies that showed the positive result was picked by the tips and added to the LBB media. For different colonies, different LBB tubes were taken. These tubes were then incubated at 37 °C for 12-16 hours. After incubation, these tubes were subjected to plasmid extraction using a HiGenoMB Himedia plasmid kit. According to the product instructions. Plasmid concentration was determined by a Nanodrop one microvolume (Thermo Scientific). The eluted plasmid was stored at-20°C.

Eluted product concentration was determined by a Nanodrop One Microvolume (Thermo Scientific, Wilmington, U.S.A) before the ligation, these PCR products were subjected to restriction digestion along with the restriction digestion of the pET-28a (+) vector. Both PCR

product and vector were treated with double restriction digestion which contain 1ul plasmid, 1ul *BamHI* NEB restriction enzyme (Biolabs, New England),5ul 10X reaction buffer, and NFW up to 50ul. They were incubated at 37°C for 6-8 hrs. and after that, they have incubated again at 65°C for 20 mins. To stop the reaction. Then subjected to PCR Purification using a PCR purification kit (Qiagen, Hilden, Germany) according to the product instructions. Plasmid concentration was determined by a Nanodrop one Microvolume (Thermo Scientific) after this ligation process takes place in which *SQS* PCR purified product and pET-28a (+) vector PCR purified product were mixed in the ratio of 5:1 along with 2ul 10X buffer and 1ul T4 DNA buffer. The ligated product was incubated at room temperature for 1 hour then at 4°C for 12-16 hours. After ligation, the transformation process occurs in which the ligated product was transformed into *DH5-α E. coli* competent cells. For this, the ligated product was added to a vial containing 100ul competent cells and incubated for 30 min. in ice. Then heat shock treatment was given using a water bath at 42°C for 90 sec. Then again it was incubated on ice for 2min. After that 4X volume of autoclaved LBB was added to a vial (inside the laminar airflow) and incubated for 90 mins. On a shaking incubator for 37°C after 90 mins. Plates containing LBA media with kanamycin antibiotic were taken. Vials were pellet down by centrifugation at 13,000 rpm for 1 min. excess 400ul supernatants were discarded and the pellet was gently mixed in the remaining 100ul supernatant. And spread over the Kanamycin plate. Then the plate was incubated at 37°C for 12-16 hours. Only those bacterial colonies would grow in which vector was inserted as pET-28a (+) vector showed resistance towards kanamycin antibiotics.

3. Results:

3.1 Selection of the tissues:

Figure 6: Image: (A) Inflorescence, (B) Young Leaf, (C) Mature Leaf, (D) Shoot, (E) Root selection of the tissues for RNA isolation and to check the expression through qPCR.

3.2 RNA Isolation:

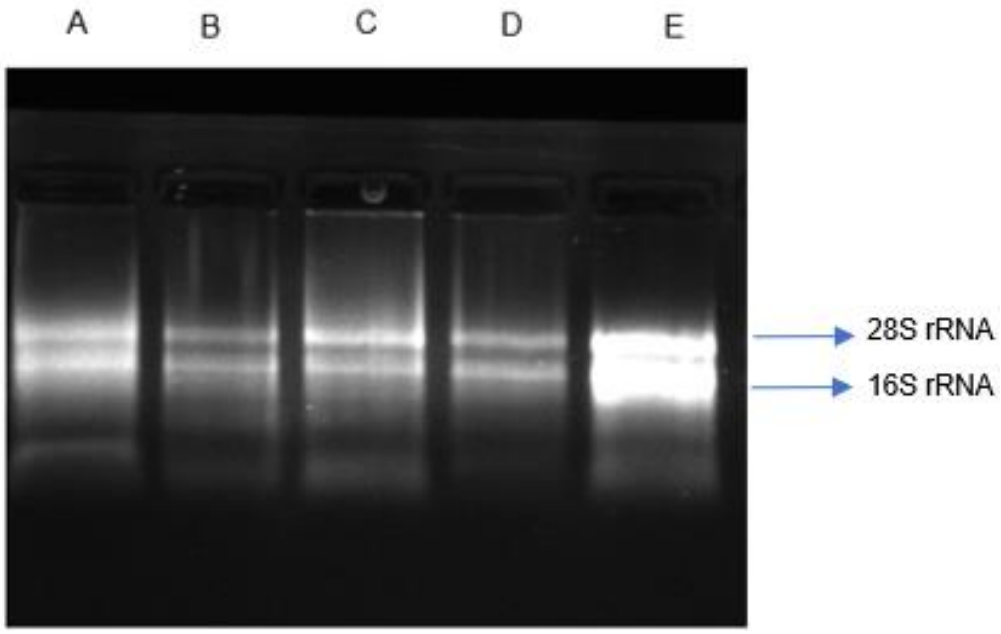


Figure 7: Image: RNA (A) Inflorescence, (B) Young Leaf, (C) Mature Leaf, (D) Shoot, (E) Root, RNA gel image of all the selected tissues.

3.3 qPCR results:

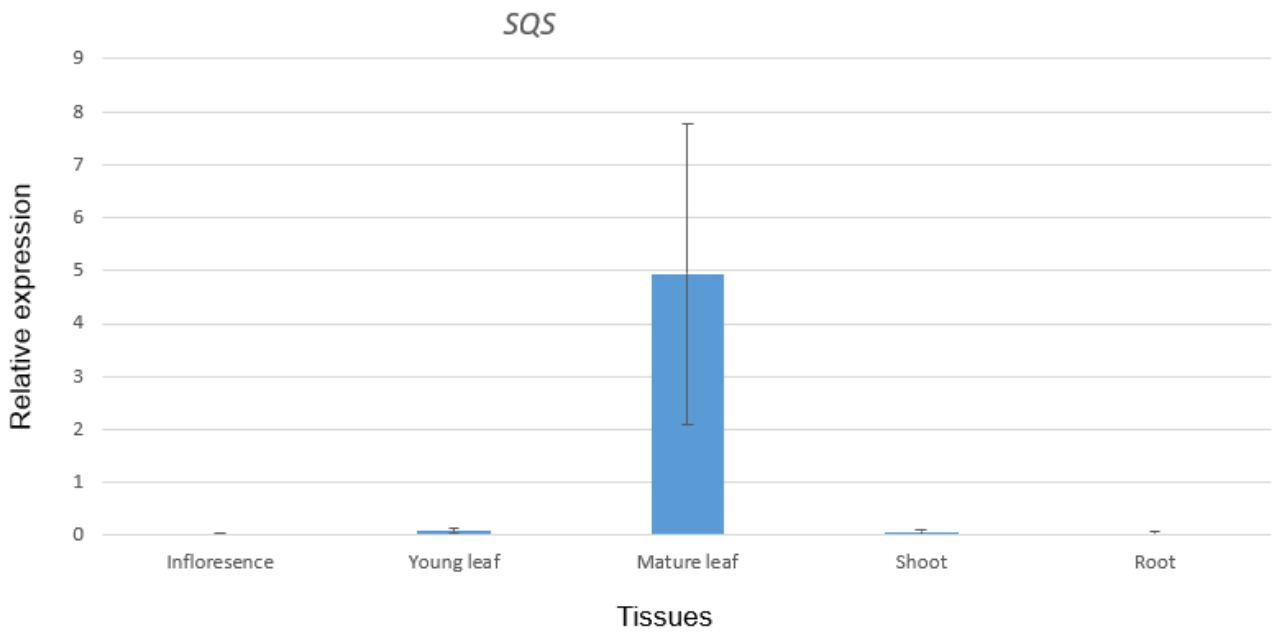


Figure 8: qPCR results shows that SQS is highly expressed in Mature leaf.

3.4 Amplification:

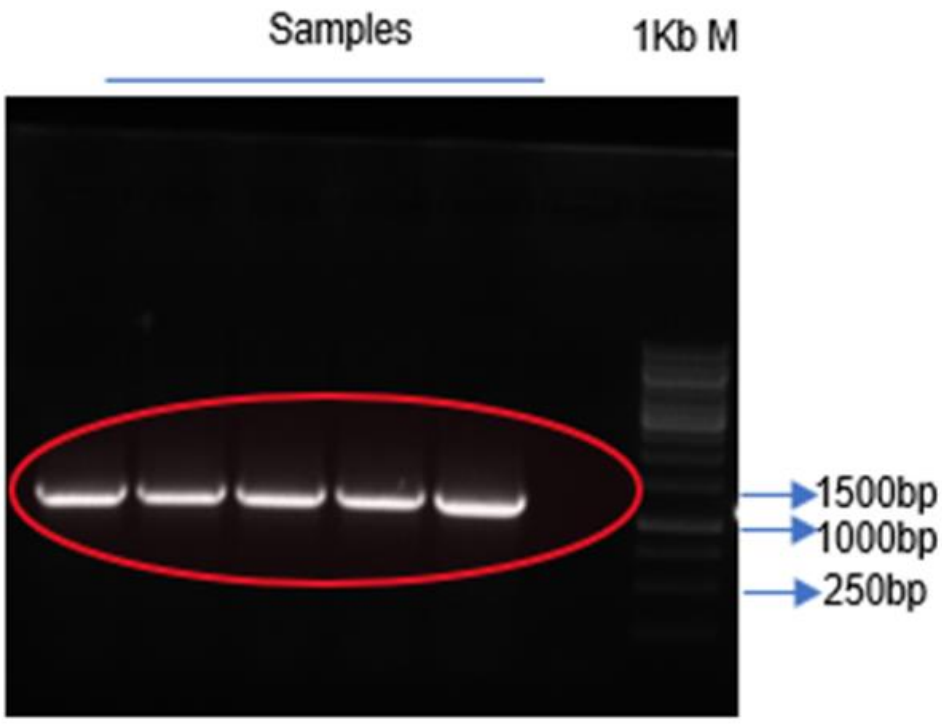


Figure 9: Red circle represents the amplification of the SQS gene(1300bp) from cDNA

3.5 Colony PCR:

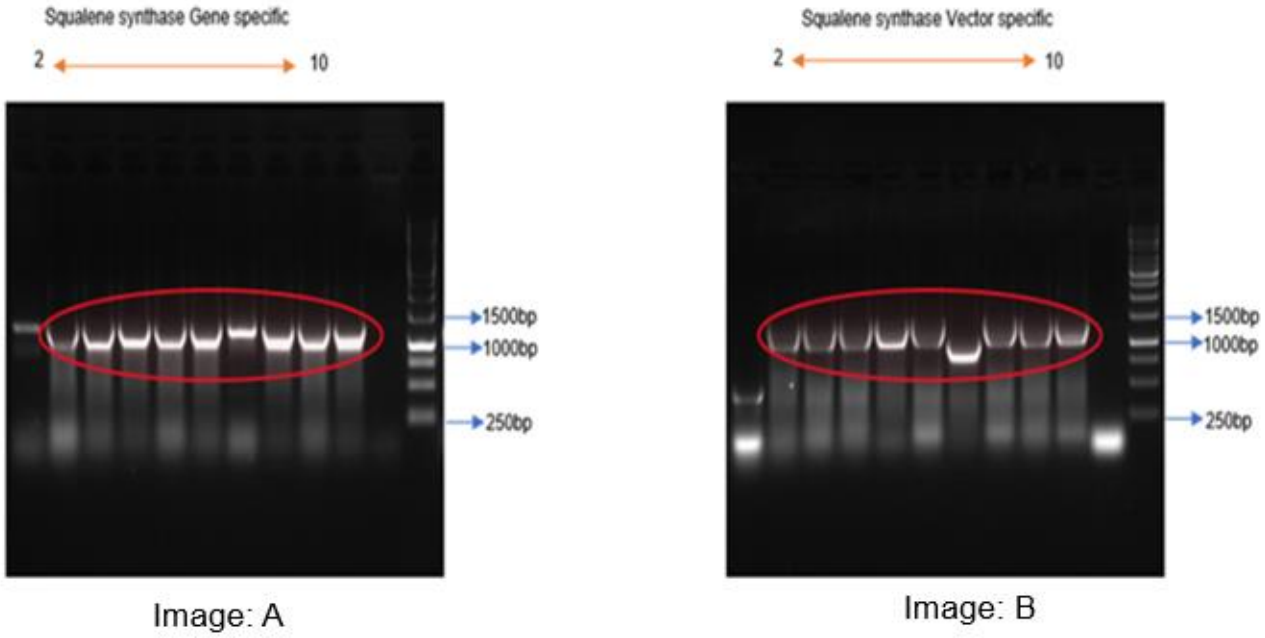


Figure 10: Colony PCR of SQS gene(1300bp) in Cloning vector, Image (A) Gene-specific and Image (B) Vector specific.

3.6 Plasmid from PCR:

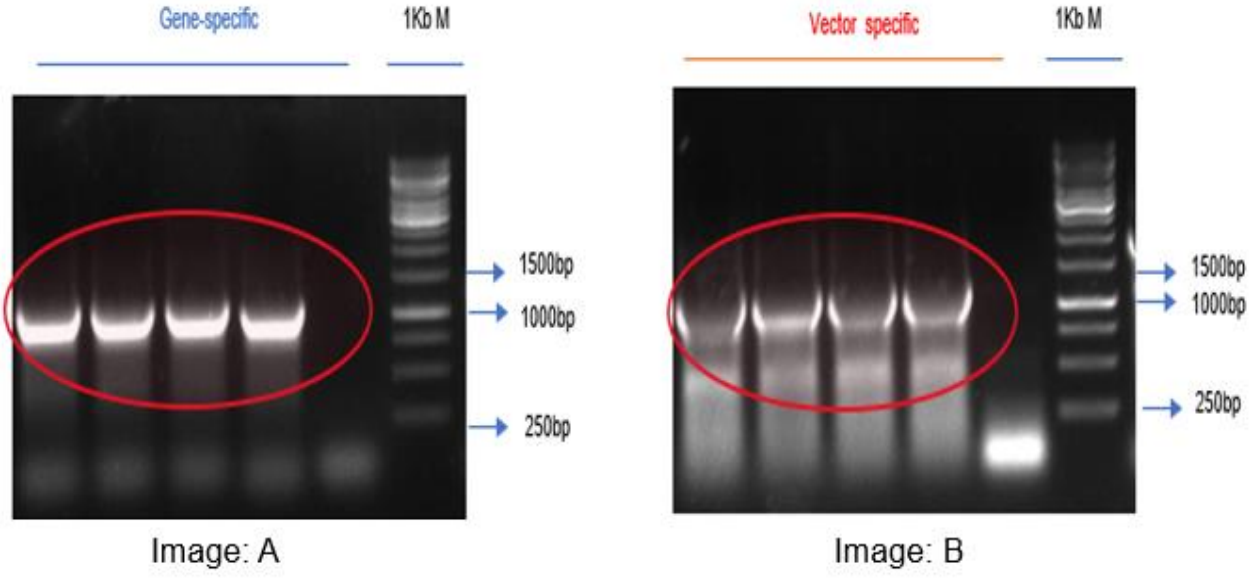


Figure 11: Plasmid PCR of SQS gene(1300bp) in Cloning vector, Image (A) Gene-specific and Image (B) Vector specific.

3.7 SQS(*squalene synthase*) sequence using forward primers:

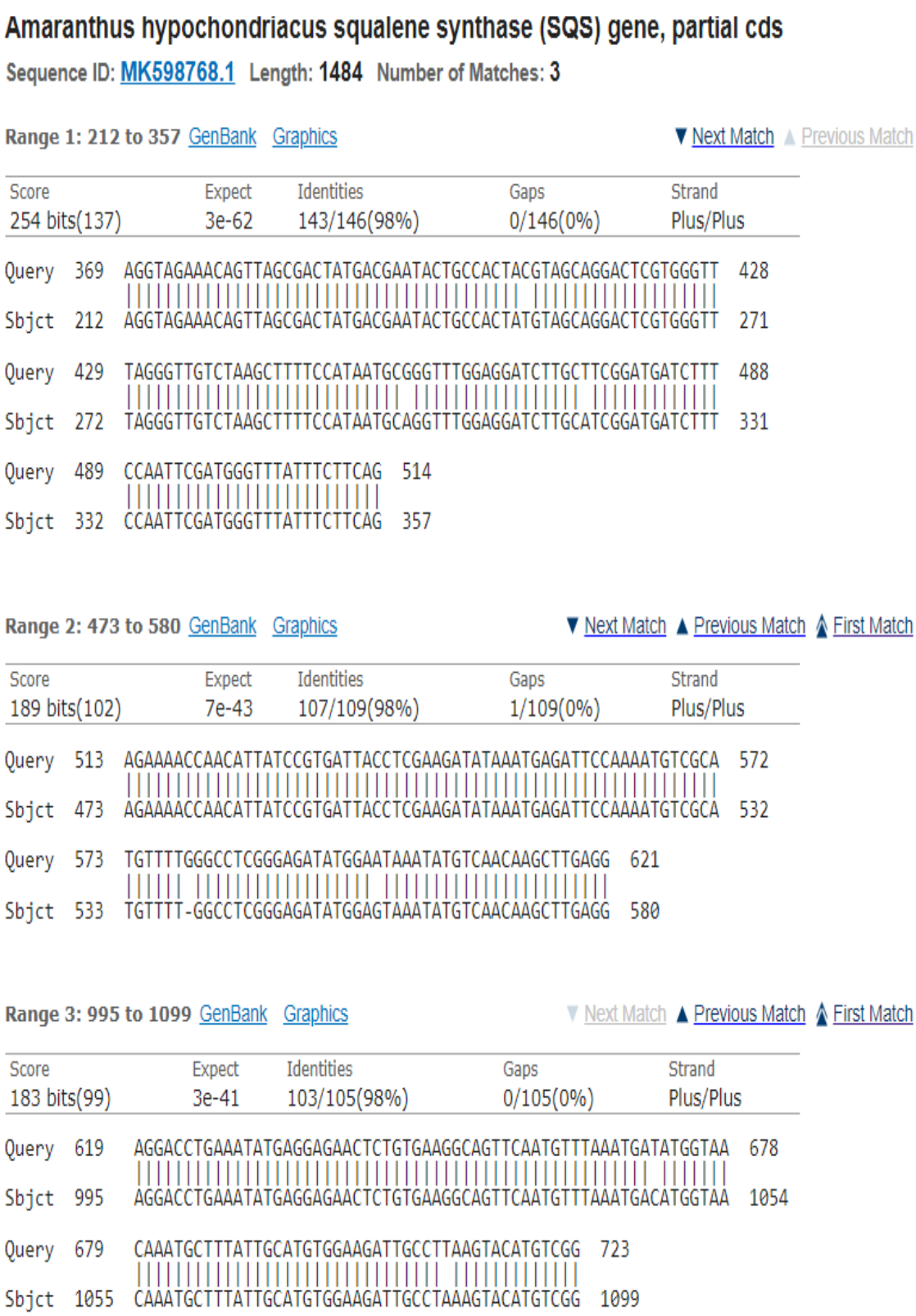


Figure 12: *squalene synthase* sequence using forward primers and BLAST

3.8 SQS(*squalene synthase*) sequence using reverse primers:

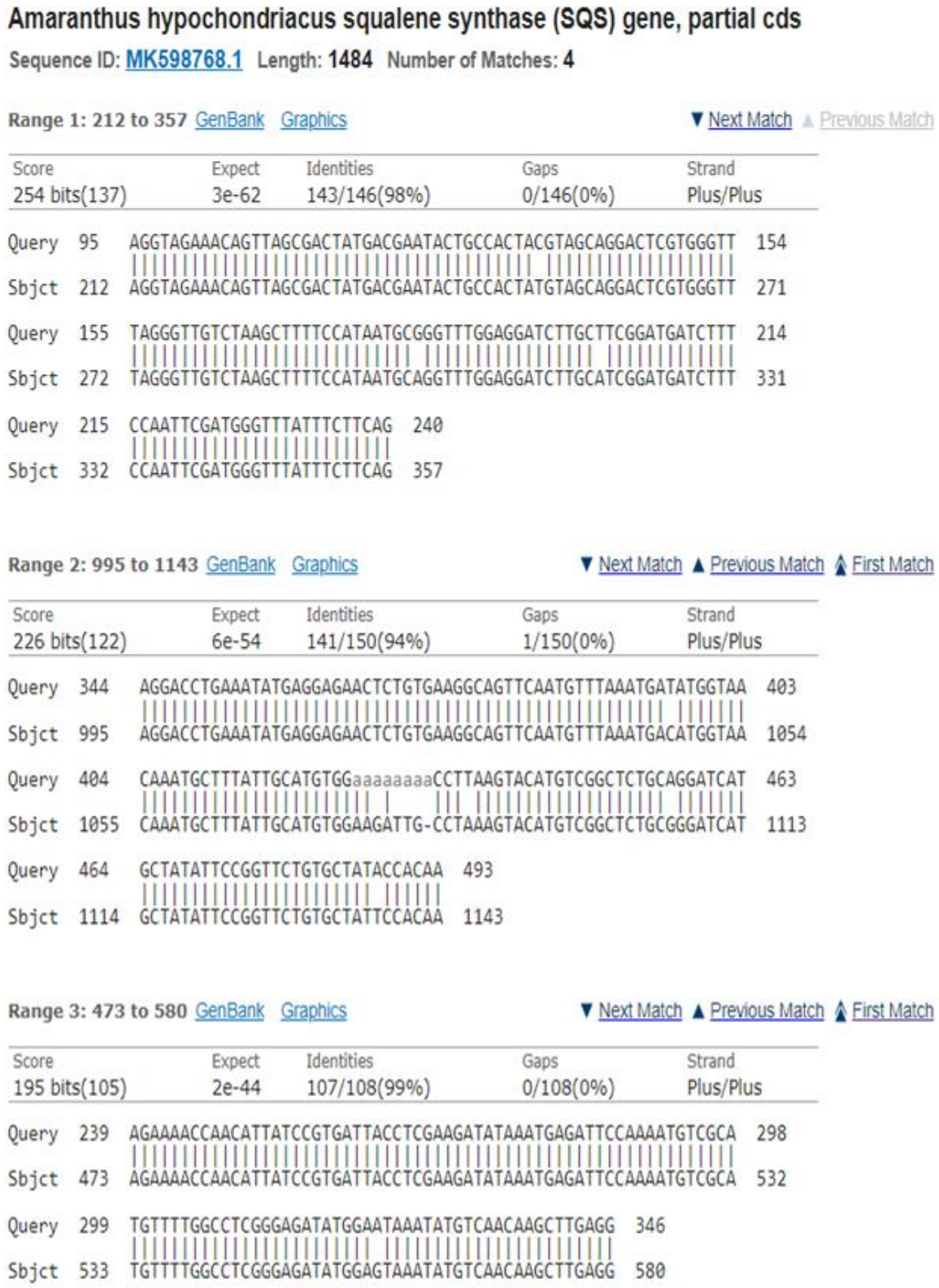


Figure 13: *squalene synthase* sequence using reverse primers and BLAST

3.9 Amplification:

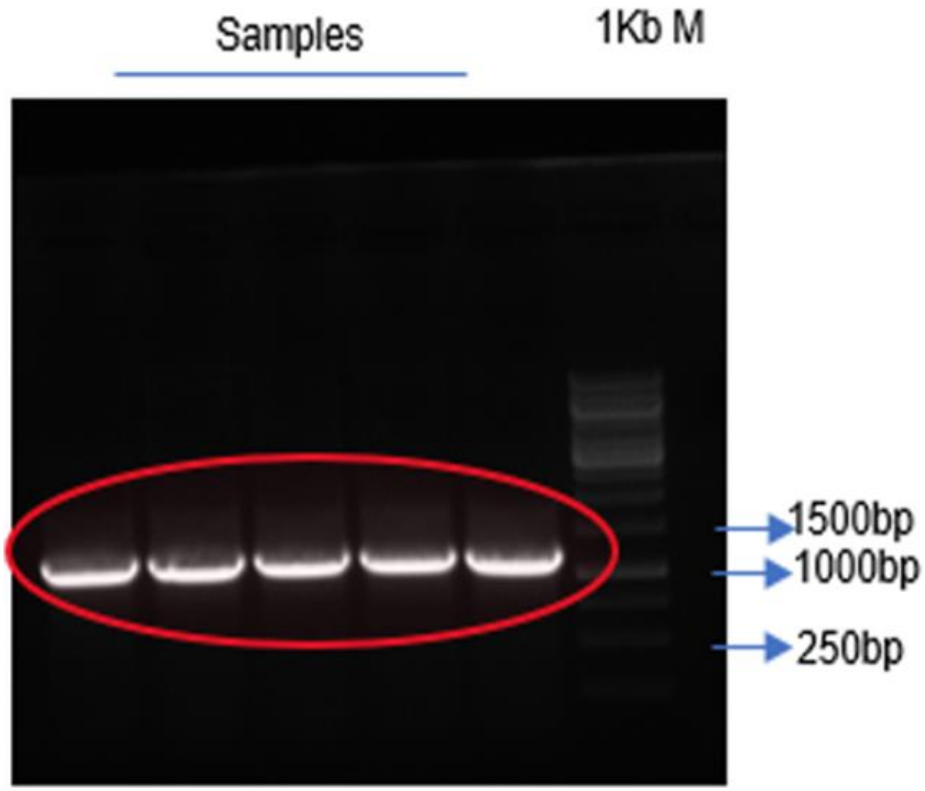


Figure 14: Red circle represents the amplification of the O.E SQS gene(1300bp) from plasmid.

3.10 Gel elution:

Figure 15: Red circle represents the gel elution Nanodrop reading.

3.11 Restriction digestion:

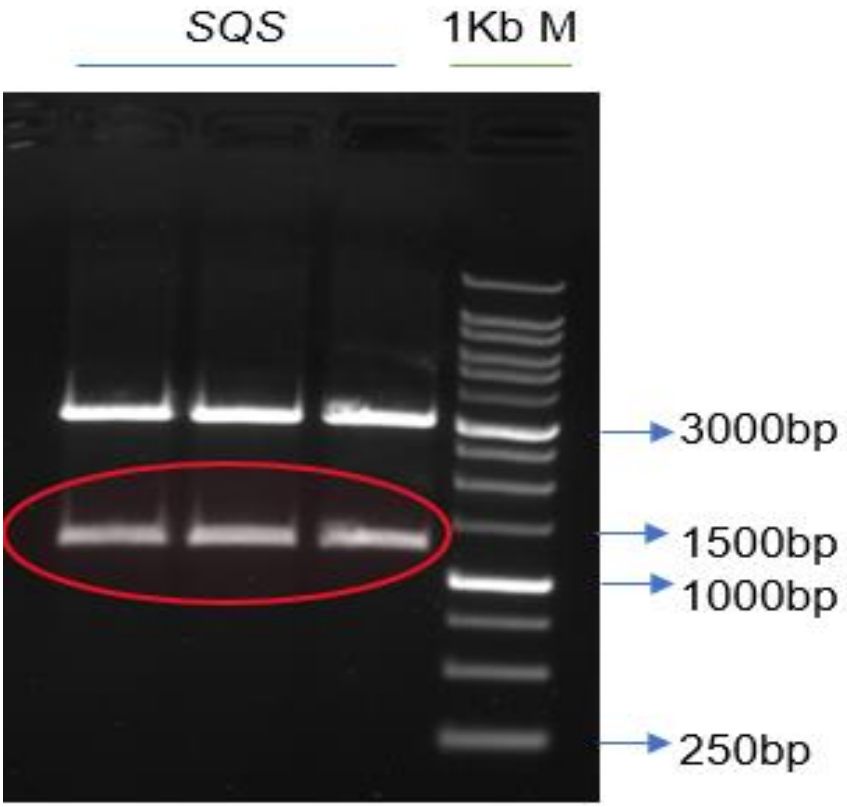


Figure 16: Restriction digestion of SQS red circle represent *SQS* gene .

3.12 Purification:

Figure 17: PCR purification Nanodrop reading.

3.13 Colony PCR:

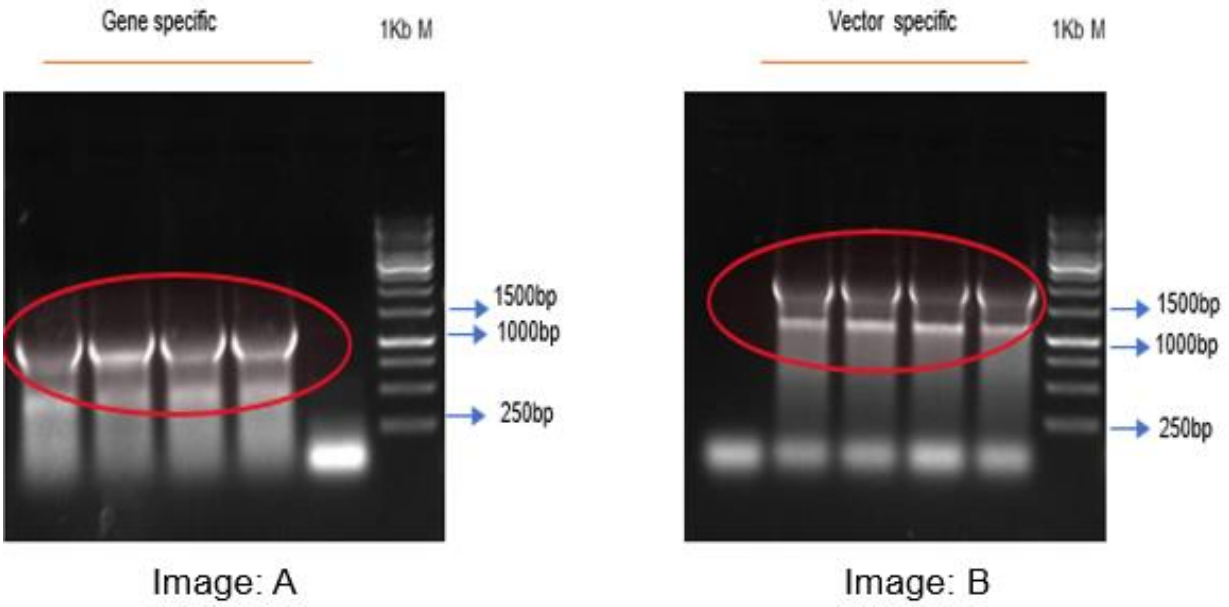


Figure 18: Colony PCR of *SQS* gene(1300bp) in Cloning vector, Image (A) Gene-specific and Image (B) Vector specific

3.14 PCR from plasmid:

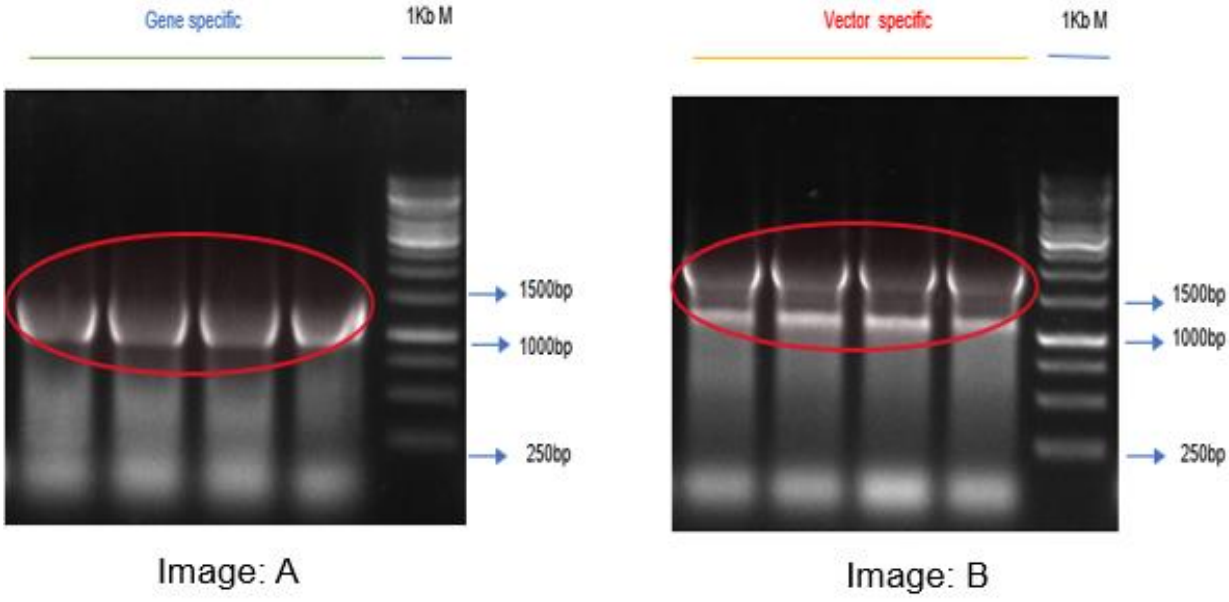


Figure 19: Colony PCR of *SQS* gene(1300bp) in Cloning vector, Image (A) Gene-specific and Image (B) Vector specific

3.15 Colony PCR (*Agrobacterium* Transformant's):

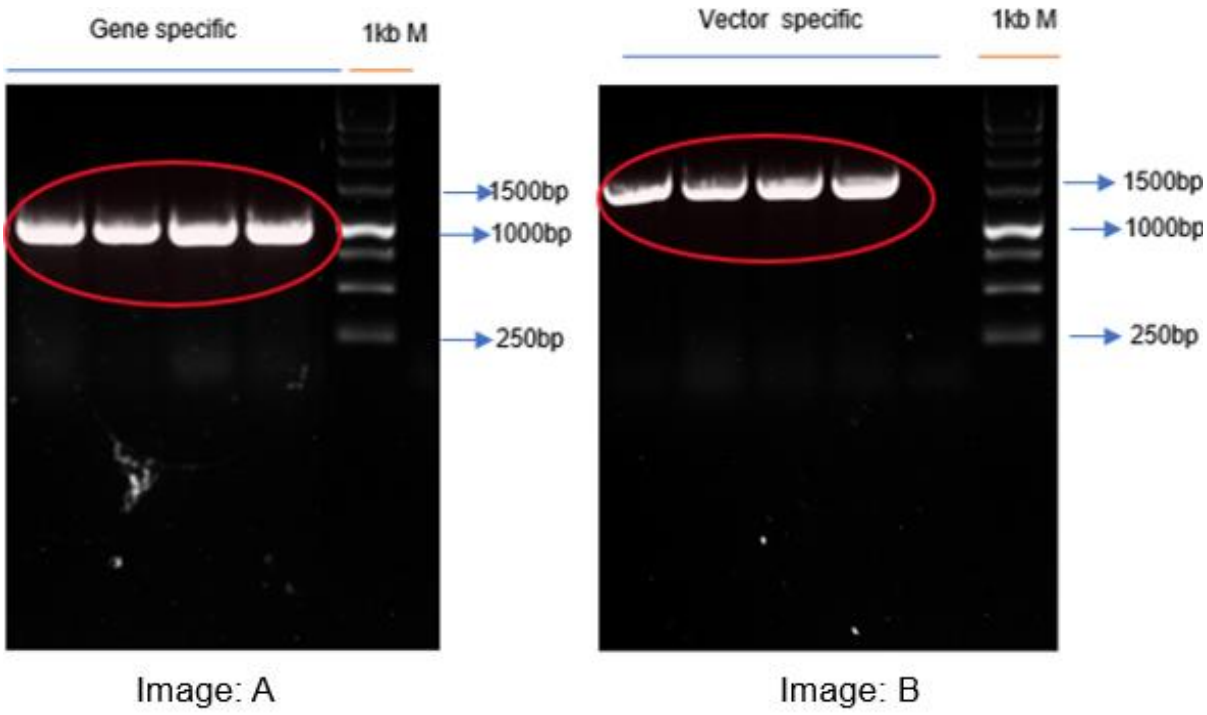


Figure 20: Colony PCR of *Agrobacterium tumefaciens SQS* gene(1300bp) in expression vector, Image (A) Gene-specific and Image (B) Vector specific.

3.16 Tissue cultured *Agrobacterium* Transformant's:

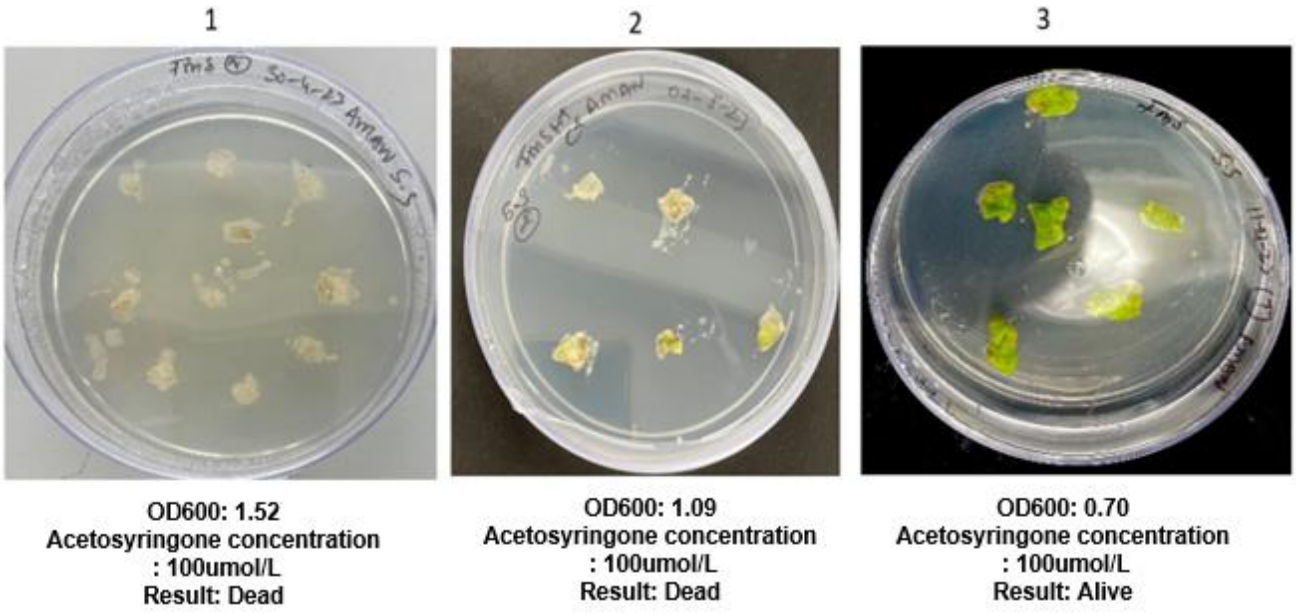


Figure 21: *Nicotiana benthamiana Agrobacterium* Transformant's grown under tissue cultured medium at different growth parameters.

3.17 Floral dip:

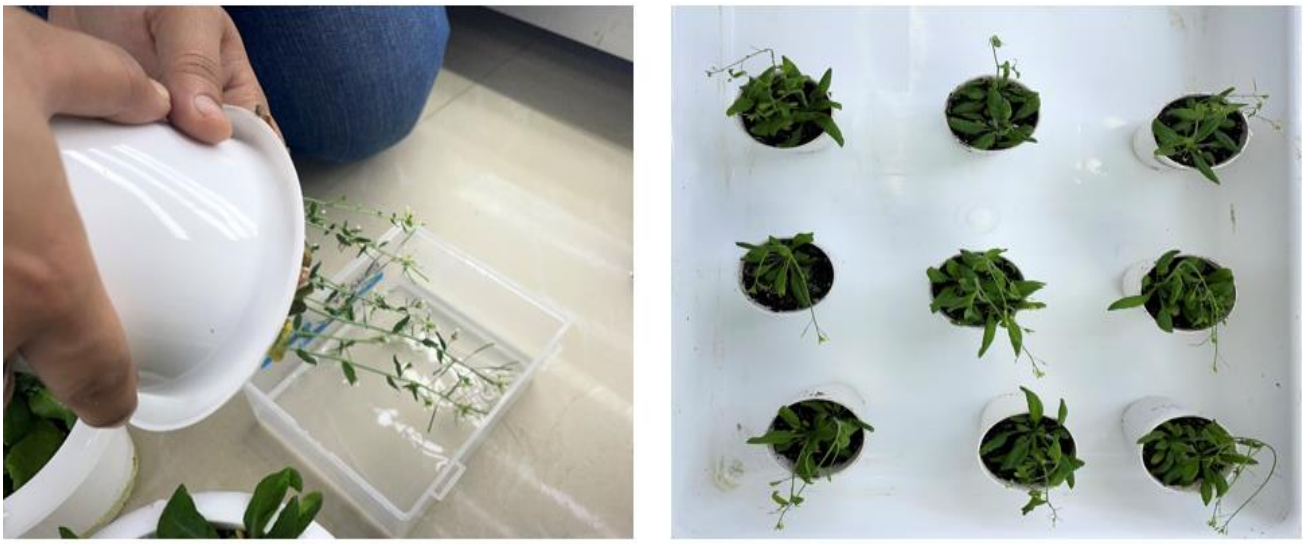


Image: A Image: B

Figure 22: Image (A) is floral dip method and Image (B) plants after floral dip.

3.18 Amplification:

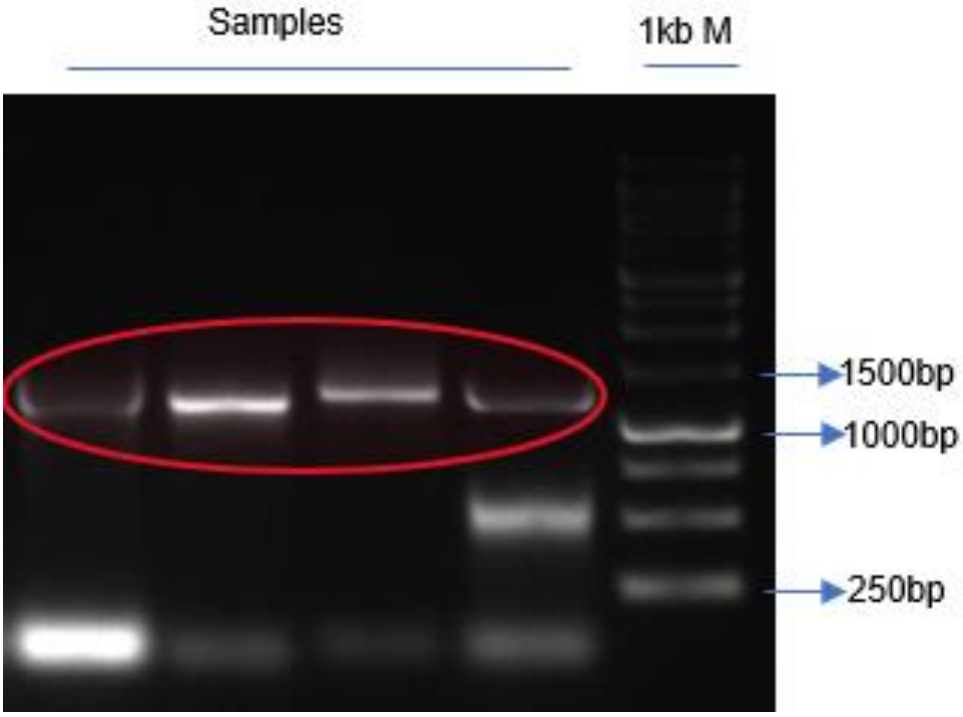


Figure 23: Red circle represents the amplification of the SQS gene for pET cloning (1300bp) from cDNA.

3.19 Colony PCR:

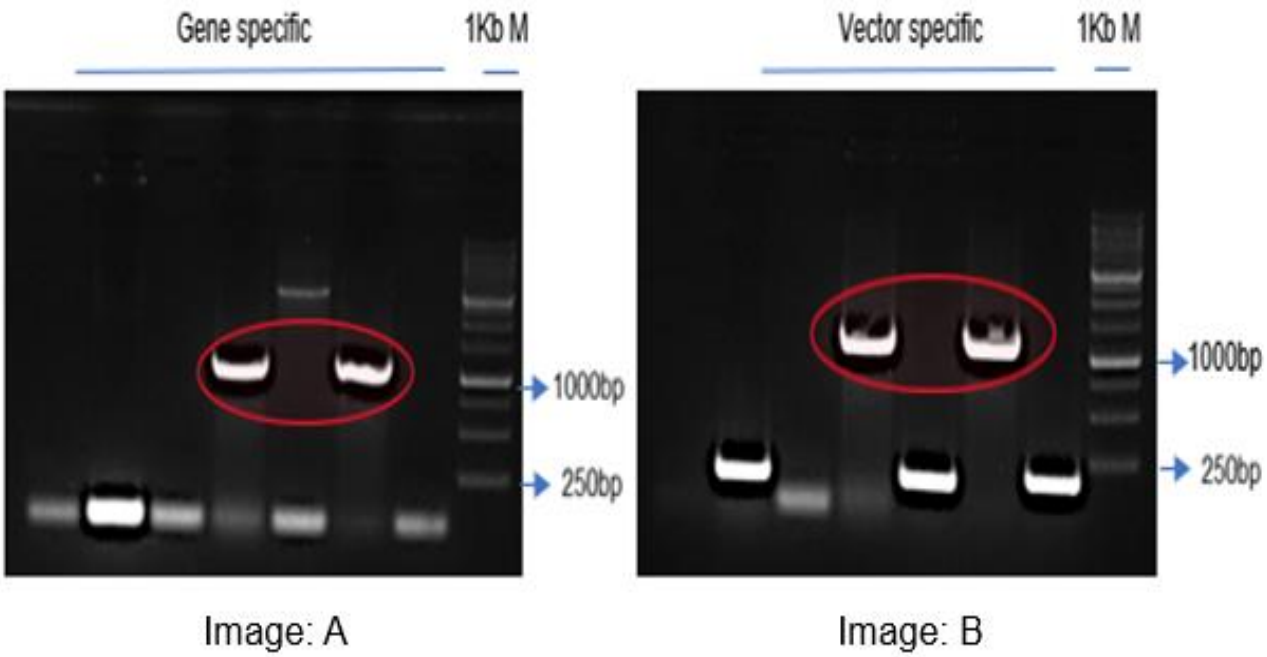


Figure 24: Colony PCR of SQS gene (1300bp) in cloning vector, Image(A) gene-specific and Image(B) Vector specific.

3.20 PCR from Plasmid:

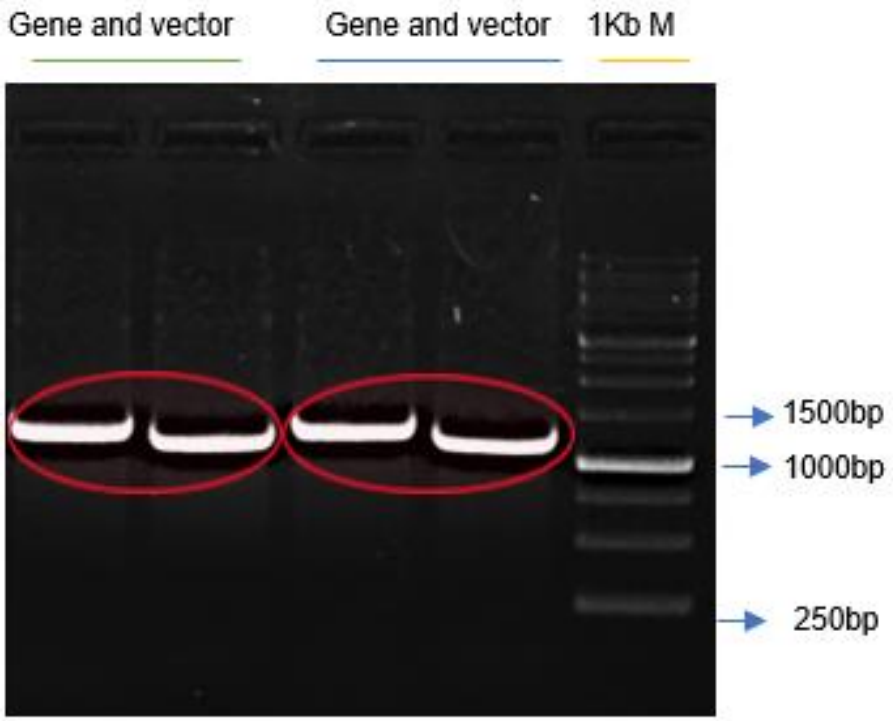


Figure 25: Plasmid PCR of SQS gene (1300bp) in cloning vector, gene-specific primers, vector-specific primers.

3.21 SQS(*squalene synthase*) sequence using forward primers:

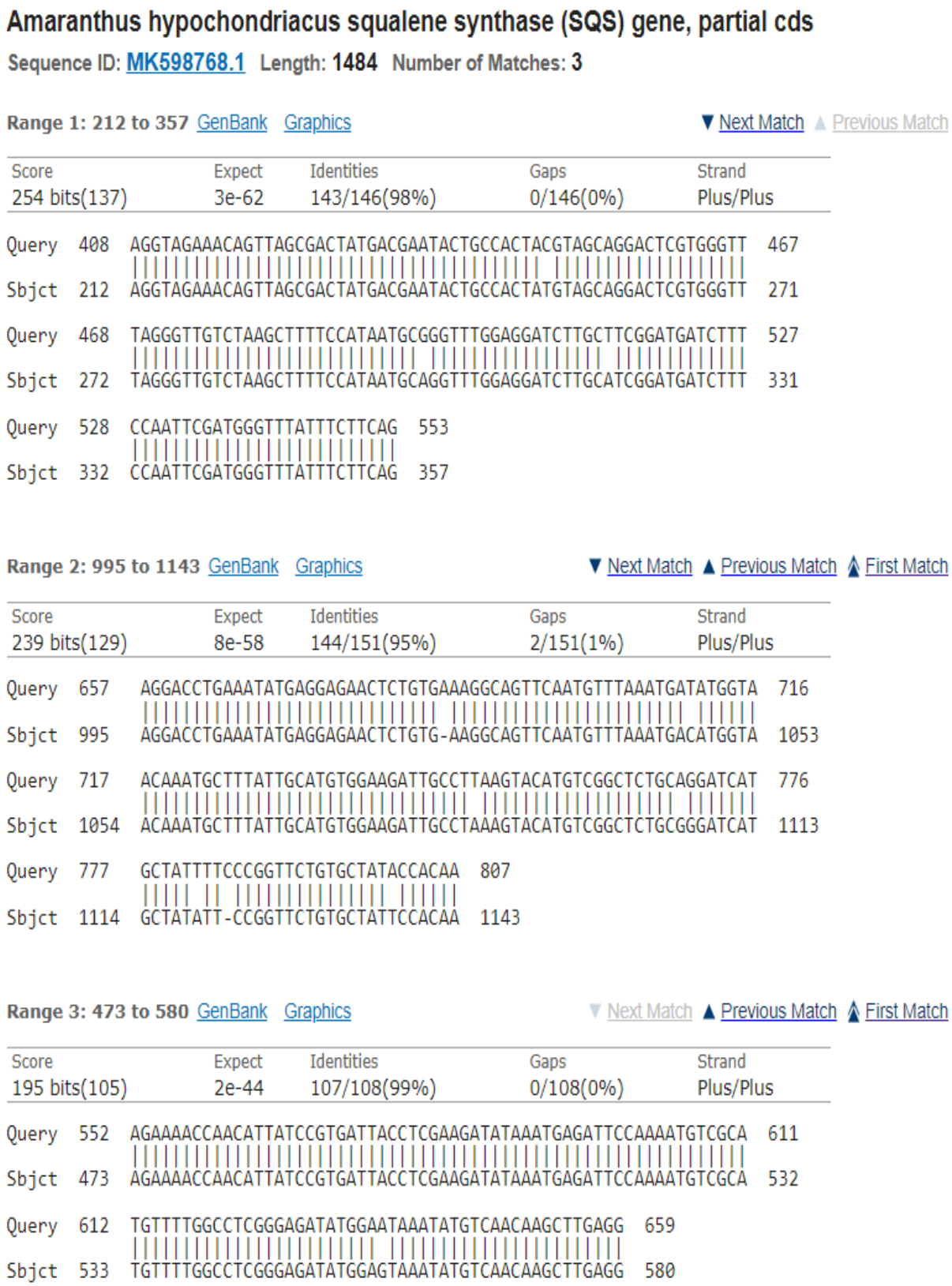


Figure 26: *squalene synthase* sequence using forward primers and BLAST

3.22 SQS(*squalene synthase*) sequence using reverse primers:

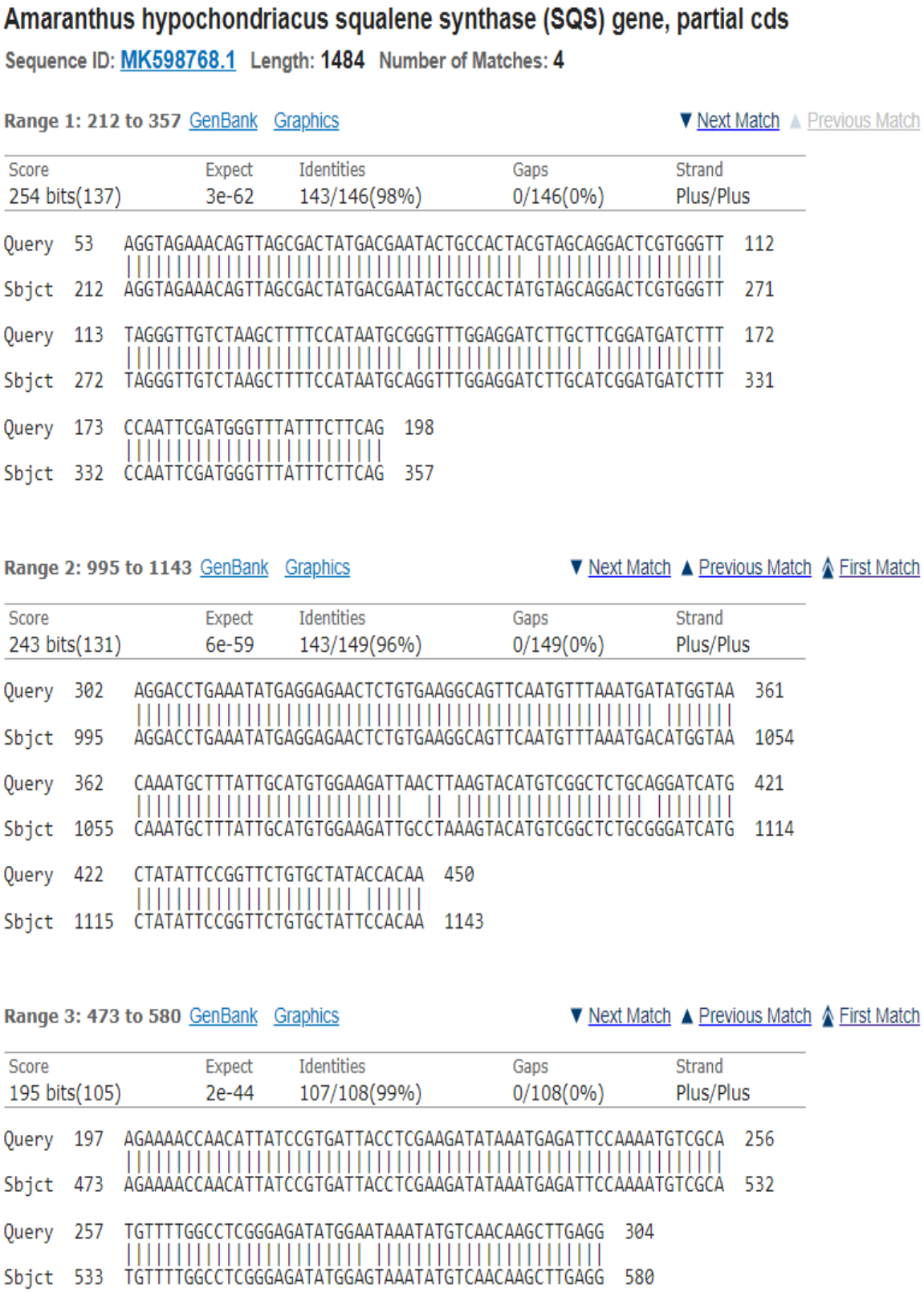


Figure 27: *squalene synthase* sequence using forward primers and BLAST

3.23 Restriction digestion:

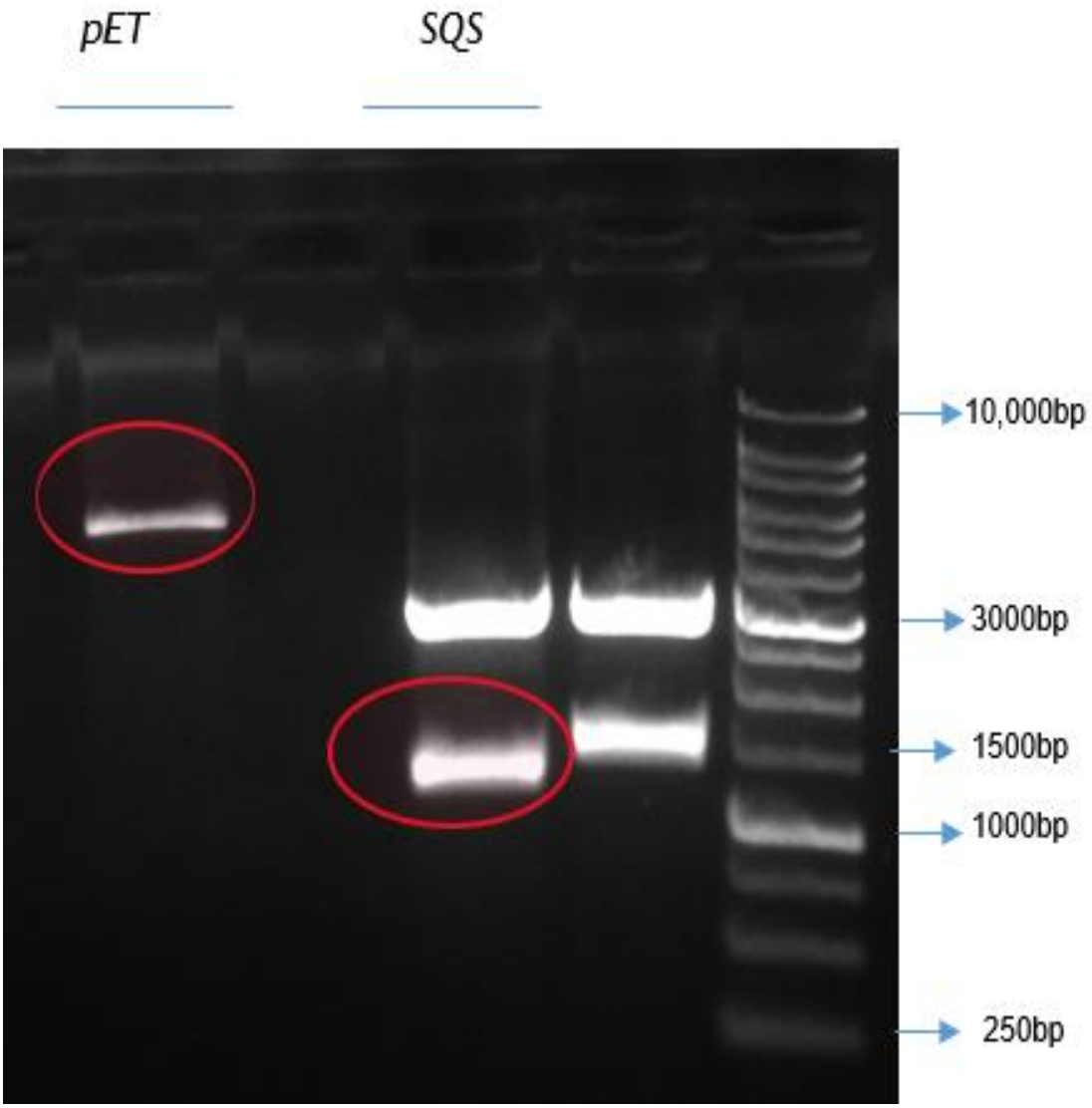


Figure 28: Restriction digestion of SQS gene and pET-28(a)+ vector

4. Discussion:

Molecular cloning and characterization of *Squalene synthase* from grain amaranth is an area of research that can provide valuable insights into the biosynthesis of squalene and other important lipids. Amaranth is a highly nutritious and versatile crop that has been used for centuries in traditional diets and medicinal practices. However, little is known about the molecular mechanisms underlying its beneficial properties.

The cloning and characterization of *Squalene synthase* from grain amaranth can help to fill this knowledge gap. This process involves isolating and amplifying the DNA sequence that codes for squalene synthase, as well as characterizing the enzyme's biochemical properties, such as its substrate specificity, enzyme kinetics, and protein structure.

One potential application of this research is the development of new strategies for improving the nutritional value of amaranth and other crops. Squalene is a precursor to many important lipids, including cholesterol, vitamin D, and steroid hormones. Understanding the biosynthesis of squalene and other lipids in amaranth can help to identify new targets for genetic engineering and breeding programs aimed at improving crop yields, nutrient content, and overall plant health.

Another potential application of this research is the development of new drugs and therapies for human diseases. *Squalene synthase* is a target for several drugs used to treat hypercholesterolemia and other lipid-related disorders. Understanding the structure and function of squalene synthase from grain amaranth can help to identify new drug targets and improve the efficacy and safety of existing therapies.

The molecular cloning and characterization of *Squalene synthase* from grain amaranth is an important area of research with numerous potential applications in agriculture, medicine, and biotechnology. By understanding the biosynthesis of squalene and other important lipids in amaranth, researchers can develop new strategies for improving crop yields, nutrient content, and human health.

Amaranthus, a potentially rich source of squalene, is becoming a model plant for research in the oilseed crop area. Isolation and characterization of a *SQS* gene from grain amaranth is the first step toward understanding the regulation of the squalene biosynthetic pathway. Genes encoding SQS have been isolated from many sources, such as fungi (Moriyoshi et al., 1991), bacteria (Lee & Poulter, 2008), animals (McKenzie et al., 1992), and plants (Park & Nishikawa, 2012).

Amaranth was a sister to the subgroup consisting of other dicots, and the amaranth *SQS* was most closely related to the *A. thaliana SQS.* Differences in the sequences of genes between amaranth and other angiosperms are a general characteristic of this crop, reflecting differences in their genetic background.

Next, we investigated the tissue-specific expression of *SQS* in grain amaranth by qRT-PCR. Previous studies showed that SQS transcripts and protein in the higher plant were mainly localized in storage organs (Akamine et al., 2003), what we get is that mature leaf expressed more than other tissues.

This study provides useful information about the molecular characterization of the *SQS* clone isolated from grain amaranth. A basic understanding of these characteristics will contribute to further studies on the amaranth SQS.

5. Conclusion:

The molecular cloning and characterization of *Squalene synthase* from grain amaranth is a promising area of research that has the potential to advance our understanding of the biosynthesis of squalene and other important lipids in plants. By isolating and characterizing the DNA sequence that codes for squalene synthase, researchers can gain insights into the enzyme's biochemical properties, such as its substrate specificity and enzyme kinetics. This information can be used to develop new strategies for improving the nutritional value of amaranth and other crops, as well as for developing new drugs and therapies for human diseases.

Furthermore, this research can contribute to the wider scientific community's understanding of plant biochemistry and genetics, which can have implications for sustainable agriculture and food security. The cloning and characterization of genes involved in lipid metabolism can also provide insights into lipid-related disorders in humans and animals and offer potential targets for the development of new drugs and therapies.

Overall, the molecular cloning and characterization of *Squalene synthase* from grain amaranth is a valuable and promising area of research that can contribute to a wide range of fields, from agriculture and nutrition to medicine and biotechnology.

5.1 Future prospects:

i. Screening of Squalene synthase transformants in tissue culture media containing hygromycin antibiotic up to T1, T2, T3 generations.

ii. Screening of Squalene synthase in *Nicotiana benthamiana* plant in tissue culture medium and at the molecular level.

iii. Transformation in E. coli. *BL21DE3* cells, SDS-PAGE, transactivation assay.

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