Proteomics and Metabolomics studies in *Linum usitatissimum*

Thesis submitted towards the partial fulfilment of

BS-MS Dual degree programme

By

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Certificate

This is to certify that this dissertation entitled ***Proteomics and Metabolomics studies in Linum usitatissimum*** towards the partial fulfilment of the BS-MS dual degree programme at the Indian Institute of Science Education and Research (IISER), Pune represents original research carried out by *Pranita Somawar, Reg No. 20091056* at IISER Pune under the supervision of *Dr. Narendra Kadoo and Dr. Vidya Gupta*, Biochemical Sciences Division, CSIR-NCL Pune during the academic year 2013-2014.

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Declaration

I hereby declare that the matter embodied in the thesis entitled ***Proteomics and Metabolomics studies in Linum usitatissimum*** is the result of the investigations carried out by me at the Bio-chemical Division, IISER Pune under the supervision of Dr. Narendra Kadoo and the same has not been submitted elsewhere for any other degree.

Pranita Somawar

BS-MS Dual Degree Student

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Pranita

Abstract

Linum usitatissimum, commonly known as flaxseed, flax or linseed, is an economically important crop long known for its uses as a fibre crop and oilseed. Existing literature and recent researches have established its nutritional and health benefits. Earlier studies that focused on the nutritional and disease preventive benefits of flax seeds were mainly diet based, where any improvement in the ailment was linked up with the dietary intake of the flaxseed or linseed oil. But the extensive studies carried on the plant lignan, secoisolariciresinol diglucoside (SDG), found in flax complements the diet based studies and affirms the anti-aging, cardiovascular disease preventive and anti-cancerous properties of flax. Function of a cell is guided by its gene expression profile and the proteins that are expressed in the cell. Thus, a proteome essentially has the capability of briefing the observer about the metabolic scenario of the cell. Proteomics approaches that are often opted for protein profiling enable the screening of the proteins in a large-scale. This technique at its best, can resolve around a thousand proteins in a single separation procedure. During the course of the project, studies were carried out to understand various aspects of plant biochemistry in linseed.

Contents

Chapter 1

Introduction

Linum usitatissimum belongs to the genus *Linum* and family *Linaceae.* It is a commercial crop known for myriad of uses and many health benefits. When grown for its fibre or as a fodder for animals, the species is commonly called flax and when grown for oil production it is termed as linseed. Fiber flax is around three feet tall in height with fewer branches and less seeds whereas, linseed plants are short, less than two feet tall, with many branches and even more seeds. Out of the many linseed varieties available, NL-260 was chosen for the study. This variety is well-suited for rabi season and can grow up-till an average height of 50 centimetres. This variety belongs to early maturing group and its life-cycle spans over a period of about 105 days. NL-260 is believed to be mildly resistant to diseases and pests like, powdery mildew and budfly (Reddy et al. 2013). The oil obtained from linseed plants are used in the manufacturing of varnishes, paints, ink and serves as a potential biofuel (Dembris 2009). The fibres of the flax stem are strong and durable and are therefore used in the linen industry and the remaining plant serves as pulp for the paper industry. For our study, the NL-260 linseed variety was grown on fields and the area provided suitable environmental conditions for its growth.

Flaxseed displays a wide variety of plant lignans and exceptionally high alfa-linolenic acid content, which is an omega 3 fatty acid (Bickert et al. 1994). Among the various plant lignans in flax, the plant lignan secoisolariciresinol diglycoside (SDG) is of great research interest especially for its role as an antioxidant and has been widely studied (Kitts et al. 1999). In animal models secoisolariciresinol diglucoside (SDG) in flax has been shown to suppress as well as regress hypercholesterolemic atherosclerosis (Kailash Prasad 2008). Flax serves as one of the richest sources of plant based omega 3 fatty acids. Studies also suggest that the nutritional properties of flax can be useful in combating coronary artery diseases (Erkkilä et al. 2003, Lamotte et al. 2006, Leyva et al. 2011). The anti-cancerous role of dietary flax has also been established and it aids the recovery of the ailment in more than one ways. Its mechanism of action here includes suppression in the tumor growth and associated angiogenesis,

modulating the estrogen metabolism via estrogen receptor and signalling factor which results in apoptosis of the cancerous cells (Mason et al. 2013, Mense et al. 2008). Flax has been shown to exhibit anti-inflammatory property in mouse system (Dupasquier et al. 2006). The anti-oxidant property of flaxseed is also well established and same has been proven by conducting in-vitro studies (Kitts et al. 1999). A diet based study conducted in animal system, albino rats highlights the antioxidant property of flax (Rajesha et al. 2006).

The motivation of our study, on *Linum usitatissimum*, lied in the exploration and comprehension of the basic chemical pathway underlying the fatty acid biosynthesis in linseed and further exploit and manipulate the key role-player molecules to amplify the ALA production in the seeds.

To have an in-depth knowledge of the biological mechanisms in a cell and to crack their bio-chemical pathway, proteomics and metabolomics can serve as a very powerful tool for research. Proteomics encompasses the study and whereabouts of the proteins in a cell. The study of proteins, their structural folding and functional analysis is very resourceful but at times can prove to be little tricky because of the dynamic nature of the expression, activity and denaturation of the protein inside the cell. On one hand, where proteomics is mainly concerned with the study of proteins, metabolomics is another approach/branch of omics study that deals with the study of smaller molecules involved in the biosynthetic pathway. Proteins assist in understanding the genetic makeup and metabolites shed light on its phenotypic outcome. The two approaches, proteomics and metabolomics, are complimentary to each other and together have the capability of summing up a basic understanding of the biochemistry in an organism, in a broader fashion. So the above stated omics approaches were perceived as necessary and sufficient approaches to kick-start the initial phases of the study, in my project.

As remarked earlier the initial intensions of the study was to focus on the proteomic and metabolomics profiling in Linum usitatissimum, and so seed germination, tagging and tissue collection were the primary work that were undertaken. But in the due course of time as the work progressed the interest

shifted to the study of a vital plant metabolite acyl:acyl carrier protein thioesterase. The reason of the shift was rather technical which included persistent and perennial unavailability of the slots and bookings for the liquid chromatography mass spectrometry machine LC-MS that was required for the study. The efforts that were made for the processing of the samples and the standardization of the same have been described by me in the report.

In plants, presence of components like cellulose of the plant cell wall, lipids and storage polysaccharides like oils and fats interfere with the protein extraction. The solubility of the proteins is thus dependent on their localization inside the cell and correspondingly the extraction protocols are altered to enrich the protein of interest. In the course of extraction the protein pool is extracted in aqueous buffer, by detergents and then precipitated. One of the commonly employed methods for the extraction of plant protein that was adopted by us was the phenol extraction method. Phenol is partially miscible in water and interacts with the proteins forming a hydrogen bond. This results in the denaturation and consequent solubilisation of the protein in the organic phase. Phenol extraction method had been reported to yield maximum number of glycoprotein (Saravanan et al. 2004) and the efficiency had been attributed to the fact that the extraction procedure minimizes the molecular interaction between the protein and other material, thus reducing the degradation. One of the method that was tried as an alternative to the phenol protein extraction method was the Trichloroaetic acid/acetone method. The pellets that were obtained following the Trichloroacetic acid method had a pinkish tinge. In any protein extraction protocol the colour of the protein pellet serves as an affirmation of the purity of the pellet obtained which means that the pellet will be largely devoid of contamination of the secondary metabolites which might interfere during the fractionation of the protein in the subsequent steps. The step of protein extraction and its quantitation in terms of concentration was followed by the enrichment of the low abundant proteins in the pool. The task of enrichment was attempted by passing the protein dissolved in suitable buffer through the High throughput liquid chromatography (HPLC) column. The fractions that were obtained were vacuum-dried, reconstituted and trypsinized in order to prepare them for the LC-MS study.

The extraction procedures carried out by me for the metabolite extraction have been discussed in the material and method section. The metabolites were extracted dissolved in suitable buffer and kept in HPLC vials for the pre-fractionation procedure.

Fatty acid synthesis

Fatty acid synthesis is central to the biochemical mechanisms in plants. In plants there are known to be two major mechanism that govern the de-novo synthesis of fatty acids. The de-novo fatty acid synthesis takes place in the plastid, and the synthesis follows a pathway that is referred to as the 'prokaryotic pathway' (Ohlrogge et al. 1979). There is an alternate pathway for the fatty acid transport, called as the 'eukaryoric pathway' that is followed by the seed and other tissues that are non-photosynthetic in nature. This eukaryotic pathway also helps in the export of acyl-ACP thioesters from the stroma to the outer membrane of the chloroplast (Figure 1). The thioesters are then transported to the endoplasmic reticulum where they participate in further processes (Ohlrogge and Browse 1995).

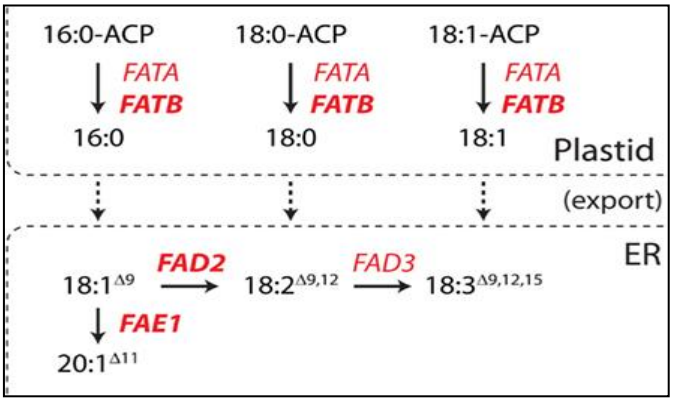


Figure 1. Transport of acyl ACP thioesterase following the eukaryotic pathway

During the fatty acid synthesis Thioesterases are enzymes that are involved in the termination of long fatty acids. For the termination of the fatty acid synthesis within the plastid, the plastidal acetyl transferases direct the transfer of acyl group from the acyl:acyl carrier protein (acyl-ACP) to produce glycerolipids. An alternate

mechanism of action that is followed for the termination of the fatty acyl chains is the hydrolysis of acyl:acyl carrier protein with the help of acyl ACP-thioesterase (FAT). The hydrolysis yields free fatty acid moiety and acyl carrier protein. It has been reported that in plants tissues around ninety percent of the plastidal fatty acid synthesis products are transported to the cytosol as free fatty acids (Browse et al, 1993). This reinforces the significance and role of acyl:acyl carrier protein thioesterases in the eukaryotic metabolic pathway. Since acyl-ACP thioesterases are involved in the hydrolysis of the acyl group, the chain length and saturation of the fatty acid exported from the plastid to the cytosol is guided by the substrate specificity of the thioesterases (Pollard et al., 1991).

Acyl:acyl carrier protein thiesterases (FAT) can be comfortably acknowledged as a class of enzymes that plays a key role in determining the fatty acid composition in oilseeds. These are enzymes that exhibit high specificity in terms of substrate binding and their expression pattern decides the length of the chain they terminate (Shine et al. 1976). The FAT gene that encodes these thioesterases have been classified into two types depending on their functionality. The enzymes belonging to the type A, FAT A, are involved in the termination of the long chain, unsaturated (18:1) $^{\Delta 9}$ fatty acid esters. The enzymes that fall in the type , FAT B, are specific to 16:0 and 18:0 saturated fatty acid esters (Figure 2). It is believed that FATB evolved previously than FATA (Jones et al. 1995).

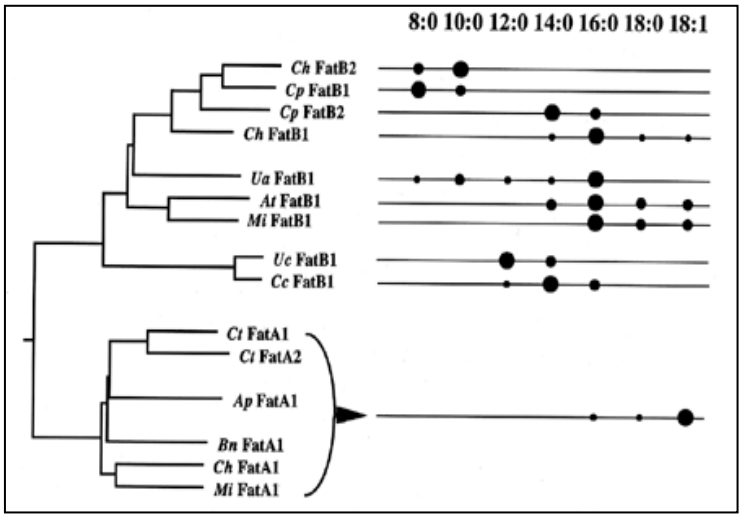


Figure 2. Phylogenetic tree depicting the fatty acid thioesterases and their corresponding specificity.(Jones et al. 1995)

To get a better understanding of the fatty acid synthesis pathway in the plastid and the role of fatty acid thioesterases in the whole picture, figure 3 has been put that depicts the fatty acid synthesis pathway.

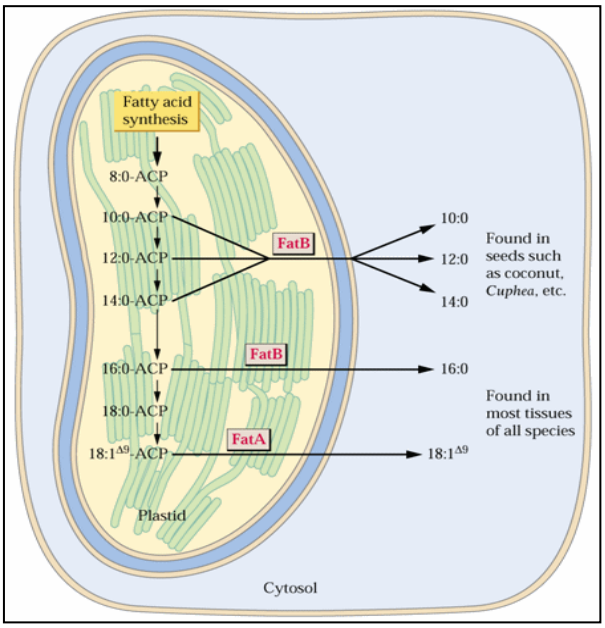


Figure 3. fatty acid synthesis in plasmid,and chain length specificity of the fatty acyl thioesterases. (BCH/PPA/PLS 609 plant biochemistry lecture 19 lipids).

Multiple sequence alignment studies carried out between various FAT A and FAT B cDNA sequence in plants showed that FAT A sequences were fairly shorter than FAT B sequences. In addition FAT B is also thought to exhibit a unique N-terminal sequence (Mayer K.M. et al, 2005).

Although the FAT genes have been studied in *Arabidopsis* and *Jatropha curcas,* their studies in flax have not quiet been established and hence, it posed as an interesting research topic for the project. Fatty acid desaturases which had been earlier studied in my lab was used in my project work to check for its amplification. The fatty acid desaturase (FAD 2) which was utilized had been already extracted earlier. The 18s RNA was used for the amplification in order to check the quality of the RNA that was extracted by me for the FAT gene related studies.

GUS assay had been standardized earlier in the lab and was also performed by me so that it could be used in the future for developing transgenics using the gene of interest like FAT A.

Chapter 2

Material and Method

1.1Tagging and collection

Approximately 500 seeds of the variety NL-260 of linseed were sown at CSIR-NCL, were watered twice in a week. Upon flowering the flowers were tagged and the bolls were collected after 4,8,12,16, 22, 30 and 48 days after anthesis (DAA). 16 DAA bolls were collected to a greater extent as they have higher metabolic activity for method standardization.

2. Protein extraction

2.1 Phenol extraction method

For the extraction, about 500 mg of the seed tissue was ground into a fine powder with the help of mortar and pestle. The tissue was constantly kept in liquid nitrogen during its grinding. Then 10 ml of extraction medium was added to the ground tissue and the slurry formed was again churned for some time. However before adding the extraction buffer it was ensured that there was no liquid nitrogen left in the mortar. If not taken care the tissue, liquid nitrogen and extraction buffer mixture turns into an unmanageable solid. The extraction medium had 0.7 Molar sucrose, 0.5 Molar tris ,30mM hydrochloric acid ,50 mM ethylenediaminetetraacetic acid, 0.1 Molar potassium chloride, 2 % betamercaptoethanol and 10% insoluble polyvinylpolypyrrolidone and a pH of 8.8.

The buffer and tissue mixture which can call as a crude extract was then centrifuged at 35000g for 30 minutes at 4 º C. The supernatant obtained after the centrifugation was transferred into a clean tube and again subjected to centrifugation at 35,000g for 30 minutes at 4º C. The supernatant thus obtained was again transferred to a fresh tube and extracted with equal volumes of water saturated phenol by vortexing the solution for 10 minutes at room temperature. This solution was then centrifuged at 35,00g for 20 minutes at 4ºC. The centrifugation in the above step resulted in a phenolic phase that was transferred to a fresh tube and re-extracted with equal volumes of cold extraction medium by vortexing it at room temperature for 5 to 10 minutes. But the extraction buffer utilized in the preceding step was without polyvinylpolypyrrolidone. The solution containing the phenolic phase and the extraction buffer was centrifuged at 35,000g for 10 minutes at 4ºC. The phenolic phase obtained after the centrifugation was once again re-extracted with the extraction buffer and subjected to centrifugation. The phenolic phase was then transferred to a clean tube and the dissolved proteins were precipitated by adding 3 to 5 volumes of cold methanol containing 0.1 M ammonium acetate and 10 mM β - mercaptoethanol. At this step, the onset of the precipitation was observed with the white particles settling at the bottom. This solution was allowed to stand overnight at – 20 º C. The following day, the solution mixture was centrifuged at 5,000g for 20 minutes at 4ºC. In order to wash the pellet obtained after the centrifugation, 1ml of cold methanol with 0.1M ammonium acetate and 10 mM

β - mercaptoethanol was added to the protein pellet and then it was transferred to a clean 1.5ml eppendorf tubes. The pellet with the wash buffer was centrifuged at 5,000 g for 5 minutes. The pellet was once again washed in the above stated manner and then stored in methanol at - 80 º C, until required. A major chunk of the entire protocol was carried out in the fume hood owing to the presence and use of ingredients like phenol and β-mercaptoethanol in the extraction procedure.

2.2 Trichloroacetic acid /acetone

The phenol extraction protocol can prove to be very tedious and extremely time consuming, especially while dealing with a large number of samples. So, one of the extraction procedures tried for the extraction as a less time consuming

alternative was TCA/acetone extraction method. The TCA/acetone extraction method had a fairly simple protocol where the powdered tissue were suspended in the trichloro-acetic acid buffer and was kept for overnight precipitation of the protein. The composition of the TCA extraction buffer consisted of 10% (volume/volume) TCA in acetone and 2% of β-mercaptoethanol. The amount of TCA buffer added was 1.5 ml per 100mg of tissue. The crushing of the tissue was tried by hand in the mortar and pestle as well in the machine, the bead-beater. In case of the machine crushed tissue, small beads were added to the tissue and then kept in the machine. The crushed tissues obtained from the machine were on the coarser side and were not very finely powdered. The crushed tissues were suspended in the extraction buffer and kept at -20 ºC for overnight precipitation. The following day the mixture was centrifuged at 5000g for 30 minutes at 4ºC. The supernatant was discarded and the pellet was washed thrice with the cold acetone and given a centrifugation of 10 minutes after each wash. The pellet was kept in the fume hood for air -drying following which 1ml of phenol was added to the protein pellet which was then vortexed for 10 minutes and centrifuged for 30 minutes. The reason behind air drying the pellet after the acetone washes was to remove any suspicion of degradation or denaturation of the protein in the pellet caused due acetone, that would adversely affect the resolubilization in the subsequent stages. The step of addition of phenol was introduced as a modification to the classical TCA/acetone extraction method, where the pellets are directly stored at - 80 ºC after the washes. The modified protocol then required the recovery of the phenolic phase and addition of cold methanol, ammonium acetate and β-mercaptoethanol for the precipitation of any protein that might have got dissolved in the phenolic phase. The solution was centrifuged at 5000g for 20 minutes at 4ºC following the overnight incubation at - 20ºC. The pellet was then washed with 1 ml of cold methanol containing 0.1M ammonium acetate and 10mM β-meraptoethanol, transferred to a fresh plastic tube of 1.5 ml and centrifuged at 5000g for 5 minutes at 4ºC. The pellet obtained was stored in methanol at - 80º C.A common practice that prevailed during the processing of the stored protein pellet (- 80ºC) was, to wash the protein pellet thrice with acetone before every use. While using the Trichloroacetic acid / acetone extraction protocol, great care was taken to remove any reminiscent trichloroacetic acid which would have otherwise interfered with the solubility of

the protein in isoelectric focussing (IEF) buffer during the successive steps. Any amount of trichloroacetic acid left in the protein pellet pose a risk of degradation or modification of the protein.

3. Sodium dodecyl sulphate, Polyacrylamide gel electrophoresis

12% resolving gel

	Per 10 mL
Water	3.3mL
30 % acryl mix	4 mL
1.5 M tris (pH 8.8)	2.5 mL
10% sodium dodecyl sulfhate	100µL
10% ammonium persulfate	100µL
Tetramethylethylenediamine	4µL

Table 1. Composition of 12% resolving gel

5% stacking gel

Water	6.8 ml
30% acryl mix	1.7 ml
1M tris (pH 6.8)	1.25 ml
10% sodium dodecyl sulfhate	100 µl
10% ammonium persulfate	100 µl
Tetramethylethylenediamine	10 µl

Table 2. composition of 5% stacking gel.

Tris - Glycine buffer

Every 500mL of buffer had 1.51 g of Tris, 9.40 g of glycine, 10% sodium dodecyl sulphate.

Sample preparation (proportion of dye added)

4. RAMA staining

RAMA stain is a modification of coomassie stain and its components are 0.05% coomassie brilliant blue R-250(CBB R-250), 10% acetic acid, 15% methanol and 3% ammonium sulplhate. For the preparation of the stain 0.05% of CBB R-250 was prepared from the stock solution of 0.25% CBB R-250 in 60% methanol and 3% ammonium sulphate was prepared from the stock solution of 40% ammonium sulphate. For one litre of stain preparation 300 ml of 60% methanol was prepared and 0.6 grams of CBB R-250 was added to it. This resulted in a 0.2% stock solution, of which 250 ml was taken in a beaker. This was followed by addition of 100 ml of 100 % acetic acid and 150 ml of methanol and finally the volume was made up by adding milli Q water. The solution was stirred and filtered and then stored.

5. In-gel digestion:

Protein samples (80 µg each) were loaded on 12% SDS polyacrylamide constant separation gel with a 4% stacking gel and electrophoresed at 20 °C using a vertical PROTEAN II xi Cell (BioRad, USA) at constant current of 50 A/gel. The gels were visualized with coomassie brilliant blue staining (CBB) and scanned at 300 dpi using high resolution image scanner GS 800 (Biorad, USA). Each lane (representing a seed developmental stage) of the gel was sliced with a scalpel into 13 pieces based on protein abundances. Each slice was transferred into a 1.5 mL tube, and in-gel digestion using trypsin was performed as described by Haynes et al. (2008) with slight modifications. In brief, protein bands were excised from the CBB-stained gels, washed twice with milli-Q water, destained with a 1:1 (v/v) solution of 50% acetonitrile and 50 mM NH_4HCO_3, and then dehydrated in 100% acetonitrile (ACN) until the gel pieces were shrunken. The dried gel pieces were reduced with 10 mM dithiothreitol for 45 min at 56 °C and alkylated with 55 mM iodoacetamide in dark at RT for 40 min. Gel pieces were dehydrated and then digested with trypsin (Sigma, USA) at 37 °C overnight. The resulting peptides were extracted using sonicator (Branson, USA) twice by adding 200 µL of a solution containing 0.1% formic acid and 5% acetonitrile for 15 min, respectively. The peptides were dried in a SpeedVac (Labconco, USA) and then reconstituted in 10 µL of 5% aqueous ACN containing 0.1% formic acid for subsequent analysis.

Liquid Chromatography Mass Spectrometry Analysis. All the samples were analyzed by LC-MSE using a NanoAcquity ultra performance liquid chromatography (UPLC) system (Waters, USA) coupled to a SYNAPT high definition mass spectrometer (Waters, USA). The nano-LC separation was performed using a bridged-ethyl hybrid (BEH) C18 reversed phase column (1.7 µm particle size) with an internal diameter of 75 µm and length of 150 mm (Waters, USA). The binary solvent system that was used, comprised 99.9% water and 0.1% formic acid (mobile phase A) and 99.9% acetonitrile and 0.1% formic acid (mobile phase B). The samples were initially preconcentrated and desalted online at a flow rate of 5 µL/min using a Symmetry C18 trapping column (internal diameter 180 µm, length 20 mm) (Waters, USA) with a 0.1% B mobile phase. Each sample (total digested protein) was applied to the trapping column and flushed with 0.1% solvent B for 3 min at a flow rate of 15 µL/min. After each injection, peptides were eluted into the NanoLockSpray ion source at a flow rate of 300 nL/min using a gradient of 2−40% B over 50 min. The lockmass calibrant peptide standard, 600 fmol/µL Glu-fibrinopeptide B (Sigma-Aldrich, USA), was infused into the NanoLockSpray ion source at a flow rate of 300 nL/min and was sampled during the acquisition at 30 s intervals. The mass spectrometer was operated in V-mode at a resolution of at least 9000 full width at half height(fwhh). For LC-MSE, full scan (m/z 50−2000) data were collected using the “expression” mode of acquisition, which acquires alternating 1 s scans of normal and elevated collision energy. Data were collected at a constant collision energy setting of low (4 V) and high (ramp from 20 to 40 V) energy mode MSE.

6. Plant tissue culture and GUS assay

6.1 Germination media

For every 500 ml of the germination media ,2.2 g of Murashige and skoog basalt,15 g of sucrose and 4g of plant tissue culture grade agar was added to the distilled water and the pH was adjusted to 5.8. The agar was added to the later stage, once the pH was set. The media was then heated and poured in to glass jars up-to a height of about 1.5 cm. The jars were then autoclaved and later kept at room temperature for the media in it to set.

6.2 Steps for seed inoculation

The NL-260 linseeds were placed in 70% ethanol for a couple of minutes. The seeds were then removed and placed in sterilization solution which is 4% sodium hypochlorite and a drop of tween 20, for about 10 to 12 minutes until total discoloration of the seeds. Upon discoloration of the seeds were washed in distilled water thrice and dried on whatmann filter paper and then inoculated in the glass jars. Around 15 seeds were inoculated in a circular locus near the periphery of the bottom of the jars.

6.3 Callus induction media

Callus induction media was prepared by adding 0.44g MS salt, 3 g sucrose and 0.8g agar to 100 ml of distilled water. The pH of the solution was adjusted to 5.8 before the addition of agar. The solution was transferred to a conical flask and was covered with cotton plugs and paper and then autoclaved. The media was allowed to cool and growth hormones benzylaminopurine (BAP) and 1-naphthaleneacetic acid (NAA) were added to it, the media was dispensed into petridishes and allowed to set. The amount of BAP added was 1mg per litre and NAA was added 0.02mg per litre.

6.4 Callus culture

The plantlets obtained after a growth period of 10 to 12 days were washed in distilled water, dried on whatman filter paper. The tender cotyledon that appear on the top and the hypocotyl region that follow were cut using a sterilized scalpel. The tissues were then gently placed on the callus induction media.

6.5 Agrobacterium mediated transformation

Engineered binary vector pRESC200 was introduced into modified Agrobacterium tumefacien , strain GV3101. The competent cells were prepared using the calcium chloride method (Sambrook et al. 1989), and were transformed following the Freeze Thaw method (Hofgen and Willmitzer 1988). The transformed cells were then plated on the agar media containing the antibodies and then incubated for 24 to 35 hours at 28ºC. The positive colonies obtained after the screening and consequent colony polymerase chain reaction were used for making agro-bacterium suspension for the transformation of the linseed tissue. The agro bacterium suspension was centrifuged at 3300 rpm at 4°C,

20min and the pellet was suspended in MS-basal medium. The ex plants were dipped for 5 minutes into the suspension thus formed and then dried and placed back into their petridishes. The explants were then incubated overnight at room temperature in a dark room.(details in supp. data)

6.6 GUS assay

The selected tissues after the transformation were immersed in the 5-bromo-4-chloro-3-indolyl β-glucoronide solution (X-Gluc) for 6 hours at 37º C. The composition of the X-Gluc solution had 100mM sodium phosphate (pH-7),0.5 mM potassium ferrocyanide,0.5mM potassium ferricyanide,10mM Ethylene diamine tetra acetic acid and 0.1%(w/v) X-Gluc.

7. RNA extraction

Total RNA was extracted from the tissues of the leaf, the bolls and seeds. RNA extraction was carried out using the Spectrum *plant total RNA kit, sigma.* Firstly the plant tissues of interest were finely ground in liquid nitrogen and then lysis buffer was immediately added to the fine powder thus obtained/The tissue samples required for the extraction were hand ground in liquid nitrogen using mortar and pestle until they turned into a fine powder. 1ml of lysis solution containing β-mercaptoethanol was then added per 50 mg of ground tissue. The motivation was to extract the intra-cellular material into the solution and separate it from the cellular debris. The cell lysate thus obtained was mixed with the binding solution and passed through the binding column. The column was then washed by the wash solution and the RNA bound to the column was eluted into a fresh tube with the help of elution buffer. Primary quantitation of the yield was done in nanodrop machine.

DNase treatment for the RNA samples was carried out using RQDnase kit (Promega)

The reaction for DNase digestion was set as follows

RNA	For every 3 µg
RQ1 reaction buffer 10x	1µl
RQ1 DNase	1µl

Total volume	10µl

Table 3 Composition of the reaction mixture for DNase treatment.

The reaction mixture was incubated at 37º C for 30 minutes and then 1µl of DNase stop solution was added per 3 µg of RNA. This was followed by an incubation of 10 minutes at 65ºC.

8. Complimentary DNA strand synthesis

The RNA sample obtained after the DNase treatment given DNase was added into 10 µl of 2x RT master mix containing 10x RT buffer, dNTP mix, oligo - dT primers, reverse transcriptase and RNase inhibitor and nuclease free water. The reaction mixture was then passed through a thermal cycle of 25º C for 10 minutes; 37º C for 120 minutes; 85º C for 5 minutes and then kept on hold at 4º C.

All the reactions were carried out in 0.1% diethylpyrocarbonate treated water which was autoclaved twice before use.

2 x master mix components	Volume (µL)
10 x RT buffer	2.0
25 x dNTP mix	0.8
Oligo dT primer	1.0
Reverse transcriptase	1.0
RNase inhibitor	1.0
Nuclease free water	4.2

applied bio-systems Table 4. Composition of PCR

9. Polymerase chain reaction amplification of Fat gene

Primers for the 14 acyl ACP thioesterase from flax were designed using the 5' and 3' regions of the genes (cDNA sequences) with an additional restriction site at the 5' end of the primer. 100pmol stocks were made for the primers and 1:10 dilution of these stocks were used for the PCR.

Table 5

10 x buffer	1 µL
$Mgcl_2$	0.5 µL
Forward primer	0.5 µL
Reverse primer	0.5 µL
DNTPs	2.5 µL
Template	1 µL
Taq Polymerase	0.2 µL
Water	3.8 µL

Table 6. Composition of PCR reaction

PFX Buffer	2.5 µL
$Mgcl_2$	0.5 µL
DNTPs	2.5 µL
Forward primer	0.5 µL
Reverse primer	0.5 µL
Template	1 µL
PFX enzyme	0.5 µL
Water	1.5 µL

10. Metabolite extraction

Metabolites were extracted from the seed, the boll and leaf

10.1 Acetone – MeoH

For the metabolite extraction by this method, 400µl of cold acetone (kept at -20ºC) was added per 50 mg of crushed tissue. The mixture was vortexed for 30 seconds and incubated in liquid nitrogen for one minute. The sample was then thawed and the process of vortexing and incubation was repeated twice. This was followed by an hour incubation at -20ºC and centrifugation at 13,000 rpm for 15 minutes. The resultant supernatant was transferred to a separate vial, whereas the precipitate was mixed with 200µl of cold methanol, water and formic

acid solution for re-extraction of the metabolite from the precipitate. The methanol, water and formic acid was mixed following the ratio of 86.5:12.5:1. The solutions were similarly treated and were vortexed for 30 seconds and sonicated for 10 minutes at 4º C. The sample was then kept at -20ºC for an hour for incubation. The sample solution was then subjected to centrifugation of 15 minutes at 13,000 rpm at 4º C and the supernatant thus obtained was pooled with the supernatant previously obtained. The pool of supernatant was dried with the help of a vacuum concentrator, speed vac, at room temperature and was re-dissolved in 300µl of 95% acetonitrile. The final solution was then centrifuged for 10 minutes at 13,000 rpm and the supernatant was transferred to the high performance liquid chromatography vials (HPLC) vial.

For the standardization purposes three more metabolite extraction procedures were selected/ pinned down.

10.2 Chloroform method

Methanol, chloroform and water was taken in the ratio of 5:2:1, this was added to 50 mg of tissue and was subjected to centrifugation at 12,500 g at 4º C. The polar phase obtained after the centrifugation was recovered and kept in ice. The pellet or the apolar phase was treated with 70% methanol vortexed and then kept at room temperature for 10 minutes.

10.3 Consecutive extraction protocol (CE)

To 50 mg of crushed and ground tissue, 1.5 ml of 50 %methanol was added and vortexed. The supernatant was collected and dried in the vacuum concentrator. The remaining pellet that was left after the recovery of the supernatant was homogenised in 1.5 ml of dichloromethane and methanol in the ratio of 3:1. This was centrifuged and the supernatant was dried overnight in fume cupboard.

10.4 Two layer extraction method

Here for every 50 mg of the tissue 400 µl of methanol and 125µl of water, followed by 200µl water and 400 µl of dichloromethane.

10.5 Cold acetone

To 50 mg of finely ground tissue 2 ml of cold acetone was added and vortexed for 30 seconds. The solution was incubated in liquid nitrogen for one minute, after which the mixture was centrifuged at 13,000 rpm for 15 minutes and the supernatant was transferred to a separate vial. The precipitate was mixed with 200µl of cold methanol, water and formic acid solution in the ratio (865:125:10). This solution was vortexed for 30 seconds then sonicated for 10 minutes at 4ºC and then kept at -20º C for an hour incubation. After the incubation the sample was centrifuged at 13,000rpm for 15 minutes at 4ºC and the supernatant obtained was pooled with the earlier one. The supernatant solution was dries in the vacuum concentrator at room temperature and then re-dissolved in 500µl of 95%acetonitrile. This was finally centrifuged for 10 minutes at 13,000rpm and the supernatant was transferred to HPLC vials.

Chapter 3

Results and Discussion

1. Protein extraction and estimation

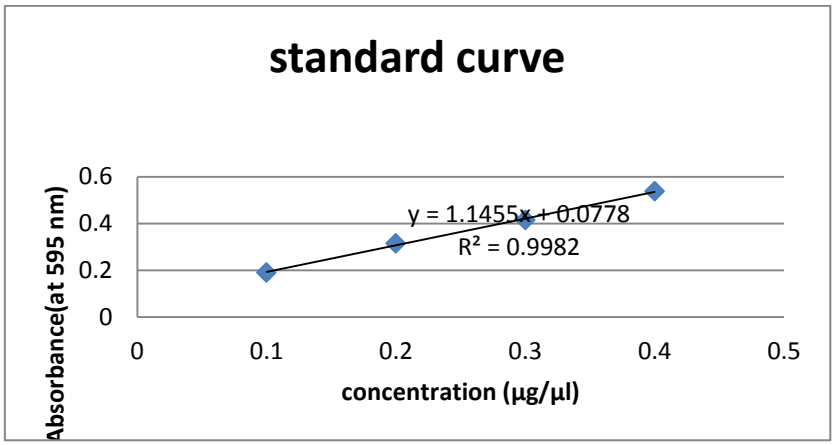
The step of precipitation in the phenol extraction protocol helps in the extraction of the proteins even the low abundant ones in the sample, removal of the impurities that might be interacting with the proteins and interfering with the protein extraction procedure and hampering the purity of the sample .For instance it necessary to inhibit protease activity during the extraction procedure.

For estimating the protein concentration of the protein extracted Bradford assay was performed. A fairly reliable standard curve was obtained from the assay for the quantitation. Bradford is a light sensitive colorimetric assay and so the standardization of the assay within the acceptable range of absorption, i.e optical density was important. Proteins were diluted into the ratio of 1:10; 1:100 and 1:1000, but only the dilution of protein in the ratio of 1:100 appeared to showcase acceptable and reasonable absorption values. For more reliable and reproducible results the proteins were dissolved in iso-electric focussing(IEF) buffer. The standard curve thus obtained was used for the estimation of the extracted protein. The estimated concentrations of protein were cross-checked by running sodium dodecyl sulphate polyacrylamide gel and later staining them in RAMA stain. The results provided for the qualitative confirmation of the protein concentration estimated by the Bradford assay.

Graph 1 is an illustration of the type of calibration curve that was obtained and was required for the protein quantitation. Similar graphs were used for the protein concentration estimation during course of the project.

Upon estimation of the protein, the protein pool obtained were enriched with low abundance protein by pre-fractionation by using high performance liquid chromatography mass spectrometry. The fractions collected from this step were dehydrated and reconstituted for trypsinization it was at this step that different buffers were again tried and there occurred a need to look for another method for protein estimation .The Bradford assay used earlier could not deliver reliable

results at this stage. The alternative that was chosen for the estimation of the protein concentration was bicinchoninic acid assay (BCA).I was amidst the above mentioned step, and was checking for robust reproducibility results of the assay when we had to halt and discontinue the progression of the work due to technical issues.



Graph 1. Standard curve obtained by keeping bovine serum albumin (BSA) protein as standard.

2. In-solution digestion

The sample preparation for the proteomic study, following the in-solution digestion protocol was carried by extracting the proteins from the seed tissues, estimating and reconstituting it in three different buffers to test it for trypsinization of the proteins into peptides. Urea was thought to be an excellent choice for protein re-solubilisation because exhibits a tendency to cancel the secondary and tertiary structures thereby bringing the proteins into a single conformation. Urea helps in keeping the hydrophobic protein in solution and also keeps the protein-protein interaction in check. Apart from this sodium dodecyl sulphate, ammonium bicarbonate and iso-electric focusing buffer were also tried for solubilisation. Re-solubilisation was tried for RB , 2% SDS ,ABC ,Methanol and acetone (image,1)

Trysinization

Protein digestion by trypsin was tested by dissolving 100 µg of proteins in various buffers. The amount of trypsin added was 2µg for every 100µg of protein followed by an incubation of 16 hours at 37ºC.(prior to this, the samples were kept at -

80,treated with dithiothreitol (DTT) and indole 3 acetic acid (IAA). Methanol, acetone and ACN dissolved samples did not settle in the well indicating that it required certain modification Methanol, acetone and ACN did not settle in the well indicating that it required certain modification. The protein samples used in the above experiment were obtained from mature seeds

Pre-fractionation, Re-solubilisation and trypsinization for proteomics

C1 a1 C2 a2 C3 a3

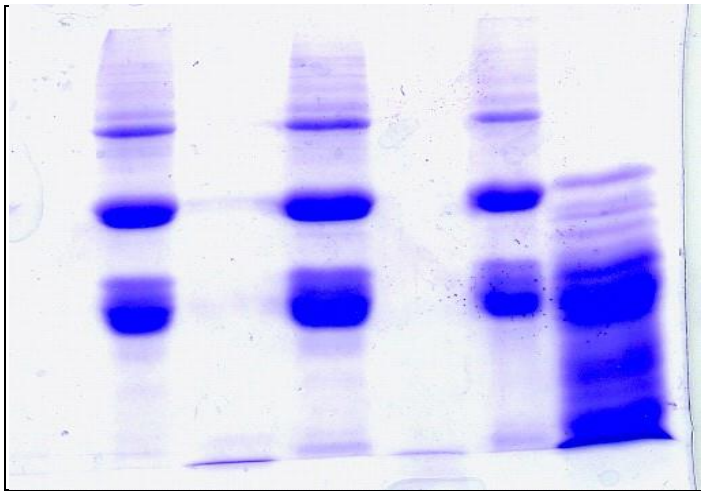


Image 1. Coomassie stained gel of trypsin digested protein

C 1- protein dissolved in ammonium bicarbonate. C 2 – protein dissolved in sodiumdodecylsulfate. C 3 – protein dissolved in urea a1 – after trypsinization of C1. a 2 – after trypsinization of C2. a3 – after trypsinization of C

trypsin added in the ratio 1:50 #each well contains 20µg protein

The in-solution digestion was attempted in order to acquire a global protein profile. The in- gel digestion is carried out for the targeted protein profiling.

3. In-gel digestion

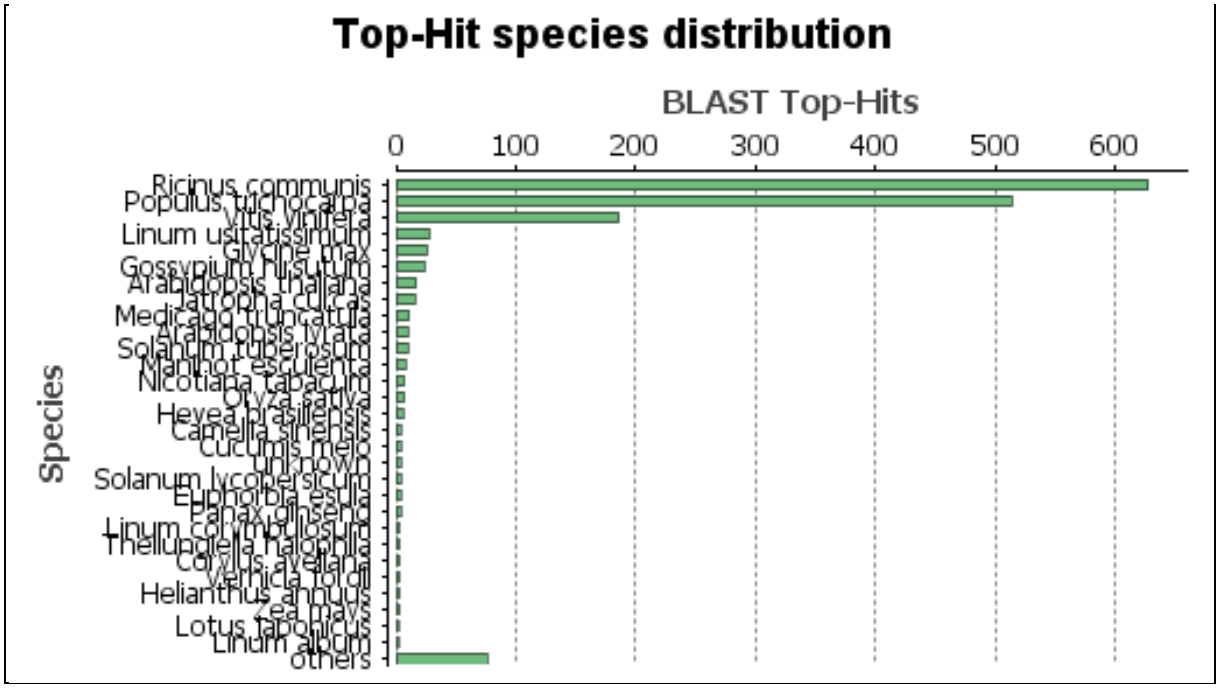
The in-gel digestion was performed by extracting the proteins by phenol extraction protocol. The protein samples were given SDS-PAGE runs. The destained gel pieces with the protein bands were ,excised ,dehydrated and

alkylated and trypsinized. The samples were then injected in the LCMS machine. The liquid chromatography mass spectrometry (LCMS) analysis of the extracted proteins from the mature seeds yielded 1751 proteins. These proteins were analysed with the help of Blast2go which is a bio-informatics tool, since the flax gene sequences were not previously annotated.

3.1 Blast2go Blast Statistics:

The chart below represents the statistics of the top hits of the blast of the obtained proteins against that of the redundant protein sequences of species in the database. The species in the database have annotated gene sequences. Here *Ricinus communis* exhibited maximum hits and hence similarity (graph 2).

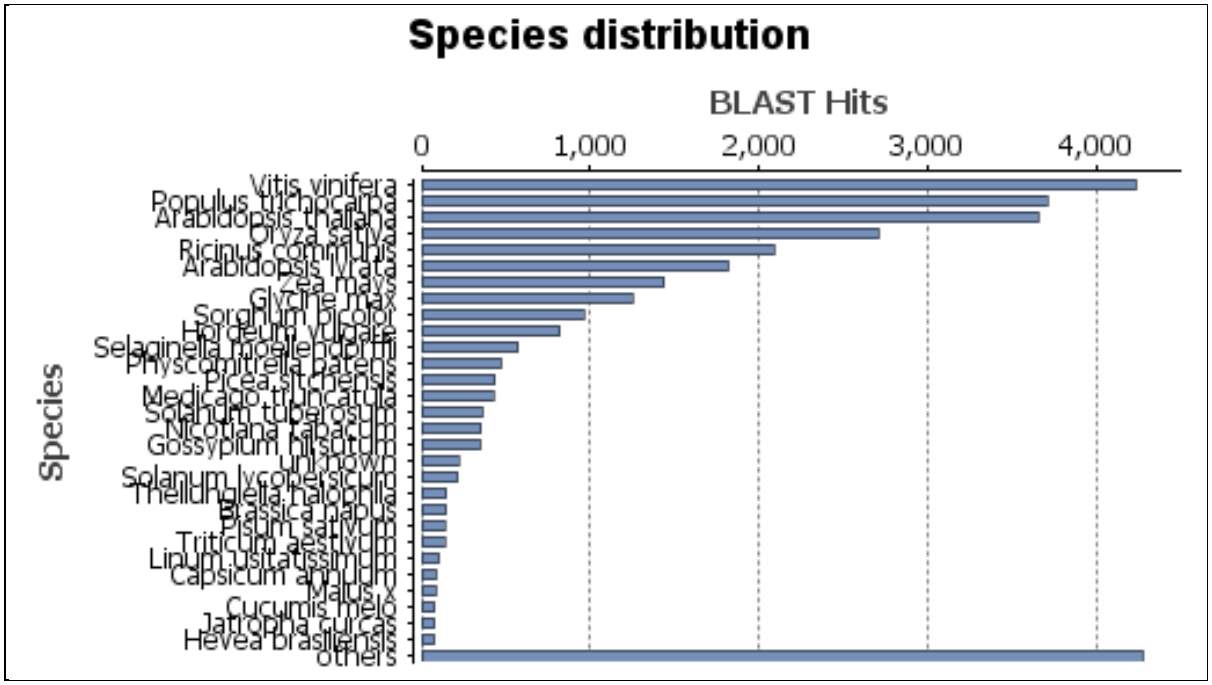
3.1. Top-Hit Species:



3.2. Species Distribution:

The data obtained are required to be fed to the tool, which then produces statistics that can be easily to interpreted. When similar blast functions, as mentioned above (section 3.1), were carried out with all the sequences of

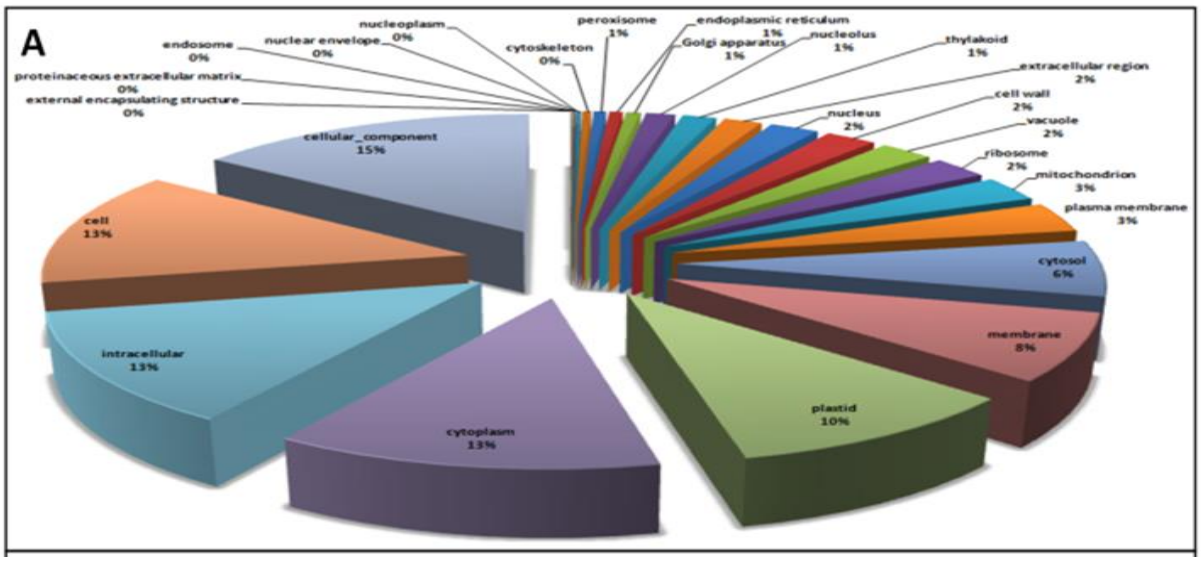
particular specie in the database the following statistics were obtained. In this case *vitis vinifera* maximum hits and hence most similarity in terms of amino acid sequence



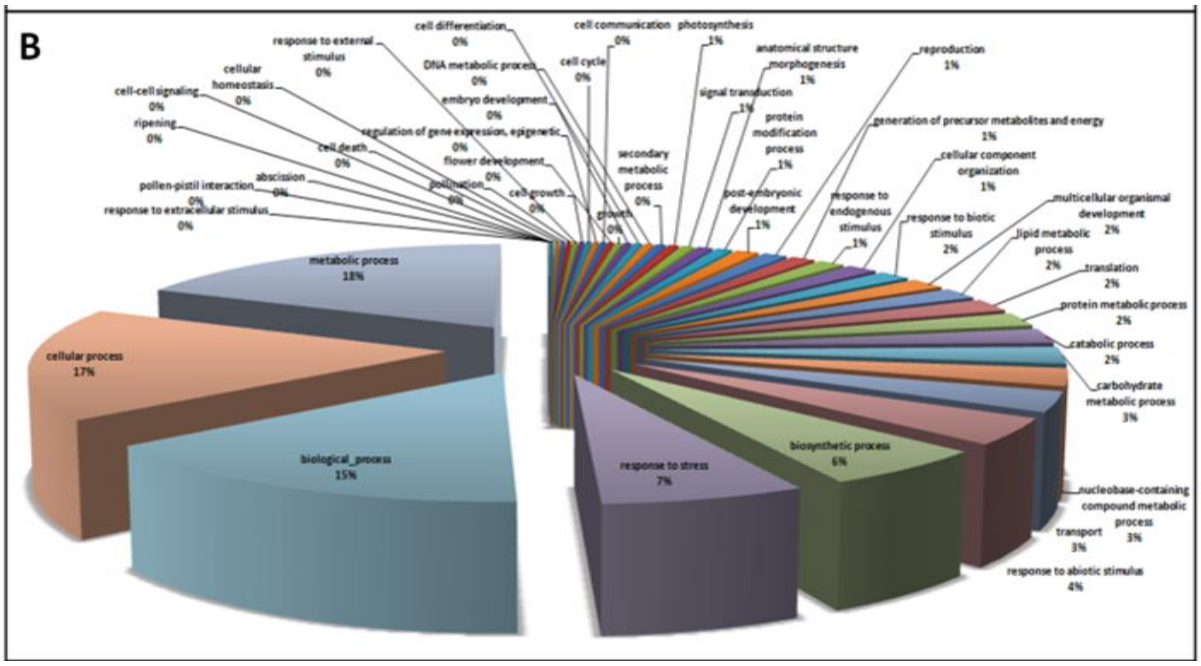
3.3 Blast2go identification and characterization of identified proteins:

A .Cellular Components:

The pie-chart represents the percentage of protein population found in a particular organelle in a cell. Again these predictions are made on the basis of the information of specie in the database that had maximum similarity with proteins in our study. (figure A ,below).As is evident from the illustration maximum percentage of the protein pool detected from mass spectrometry came from the cytoplasmic pool followed by the plastidal protein population



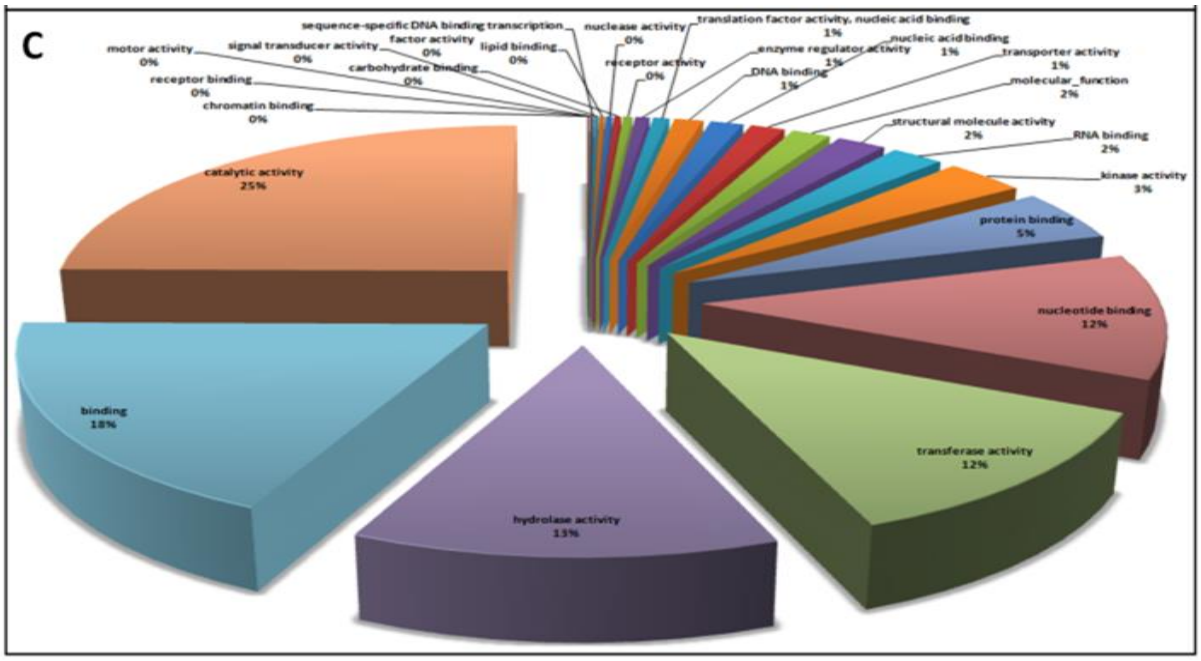
2. Biological Process:



The KEGG maps (bio-chemical pathways) that were formulated during the analysis with the help of Blast2go, helped in assigning the biological process that the proteins would have been involved in.(figure B above).A dominant percentage of proteins seem to be participating in metabolic processes , cellular processes and biological processes.

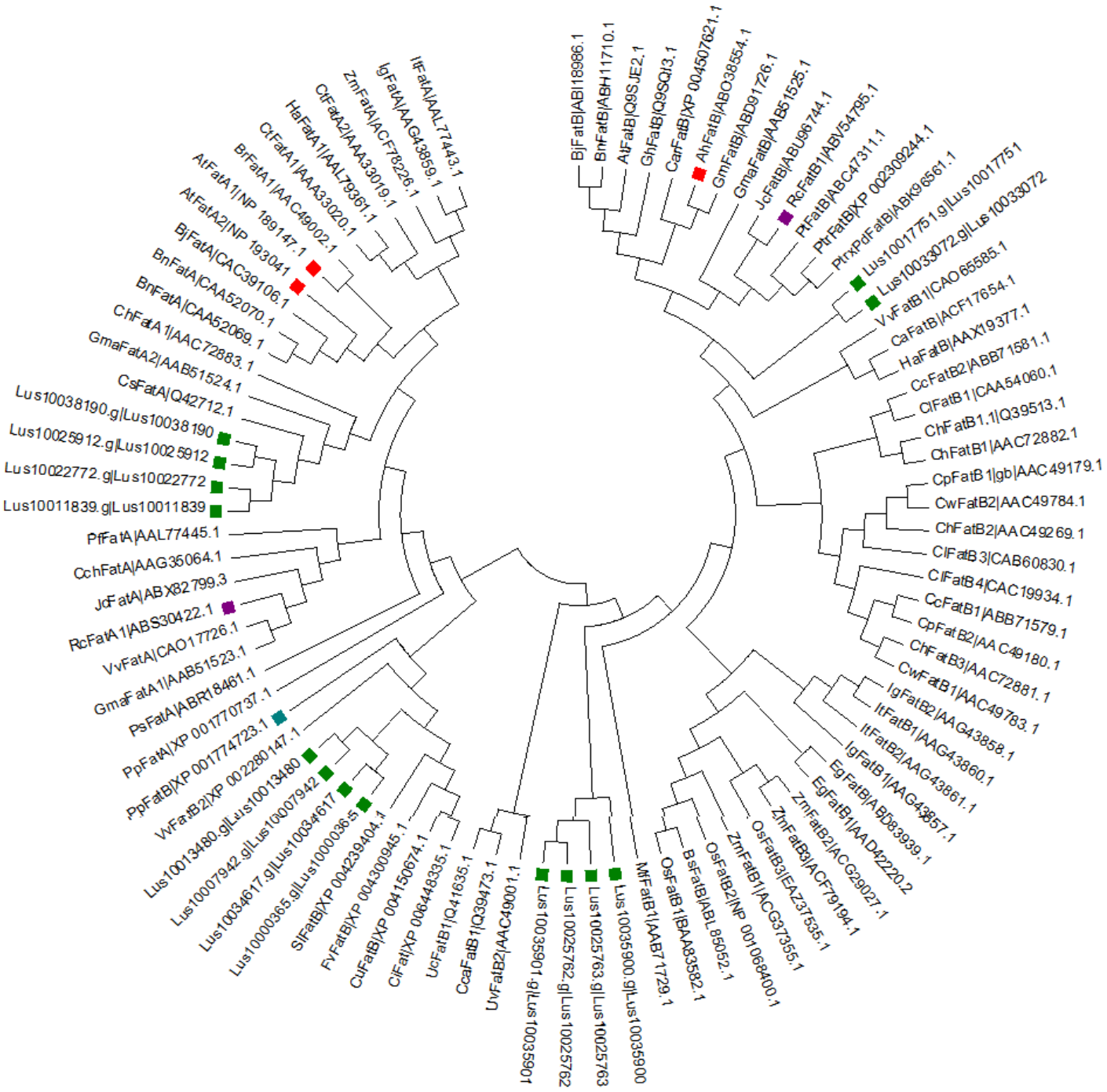
3. Molecular Functions:

One of the other aspects is that this tool also aids in acquiring a fair idea of the molecular functions that these proteins are involved in. (figure C below) For example the chart depicts the percentage of proteins involved in binding activity i.e 15%, 18%of protein are involved in hydrolase activity and 12% each in transferase and nucleotide binding activity in the cell. The information of protein percentage involved in transferase activity was useful data as later half of my project concerned with the molecules involved in transferase activity.



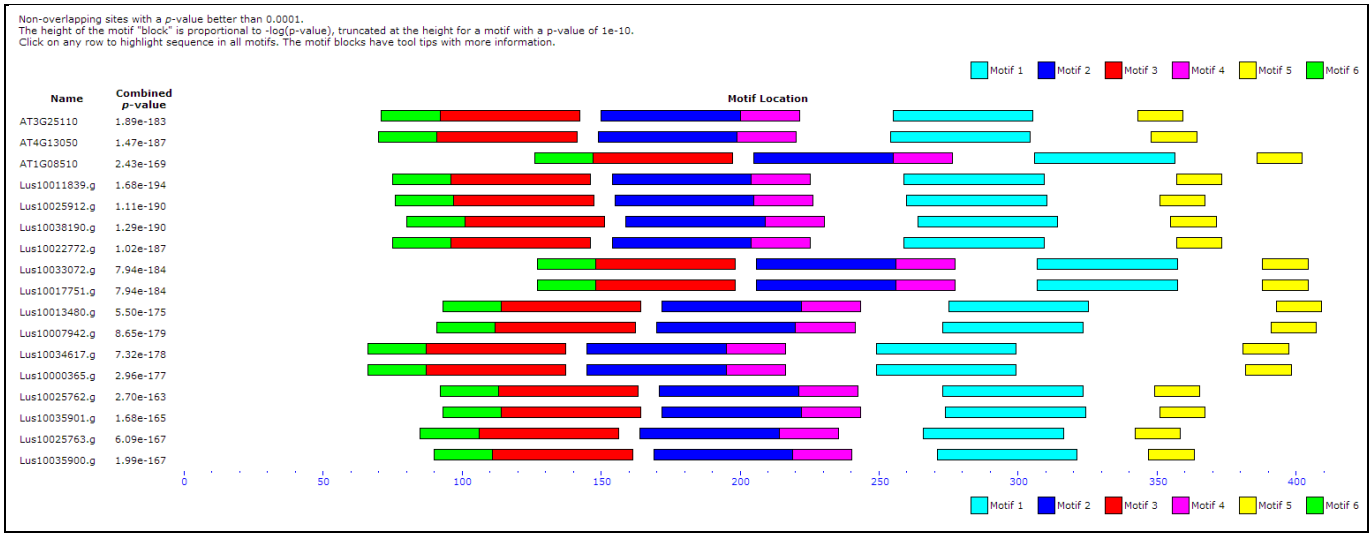
4. Phylogenetic tree construction of acyl:acyl carrier protein thioesterase from Flax:

The sequences for FAT A and FAT B genes from various sources were downloaded from NCBI and Phytozome, which were aligned and a phylogenetic tree was constructed in MEGA6.0.The tree depicted the species and the sequences with maximum similarity, it along with shedding light on the evolutionary history of the FAT gene. The primers were accordingly designed and ordered and the task of standardizing their amplification was taken up. In order to study the impact of the FAT gene in the *Linum Usitatissimum* system this was a necessary step.


BjFatB|ABI18986.1
BnFatB|ABH11710.1
AtFatB|Q9SJE2.1
GhFatB|Q9SQI3.1
CarFatB|XP 004507621.1
AhFatB|ABO38554.1
GmFatB|ABD91726.1
GmaFatB|AAB51525.1
JcFatB|ABU96744.1
RcFatB1|ABV54795.1
PtFatB|ABC47311.1
PtrxPdFatB|XP 002309244.1
Lus10017751.g|Lus10017751
Lus10033072.g|Lus10033072
Lus10033072.g|Lus10033072
VvFatB1|CAO65585.1
CaFatB|ACF17654.1
HaFatB|AAX19377.1
CcFatB2|ABB71581.1
ClFatB1|CAA54060.1
ChFatB1.1|Q39513.1
ChFatB1|AAC72882.1
CpFatB1|gb|AAC49179.1
CwFatB2|AAC49784.1
ChFatB2|AAC49269.1
ClFatB3|CAB60830.1
ClFatB4|CAC19934.1
CcFatB1|ABB71579.1
CpFatB2|AAC49180.1
ChFatB3|AAC72881.1
CwFatB1|AAC49783.1
IgFatB2|AAG43858.1
ItFatB1|AAG43860.1
IgFatB1|AAG43861.1
EgFatB1|AAG43857.1
EgFatB1|ABB83939.1
ZmFatB2|AAD42220.2
ZmFatB3|ACG29027.1
OsFatB3|ACF79194.1
ZmFatB1|EAZ37535.1
OsFatB2|ACG37355.1
BsFatB|ABL85052.1
OsFatB1|NP 001068400.1
OsFatB1|BAA83582.1
MtFatB1|AAB71729.1
Lus10035900.g|Lus10035900
Lus10025763.g|Lus10025763
Lus10025762.g|Lus10025762
Lus10035901.g|Lus10035901
UvFatB2|AAC49001.1
CcaFatB1|Q39473.1
UcFatB1|Q41635.1
ClFat|XP 006448335.1
CuFatB|XP 004150674.1
FvFatB|XP 004300945.1
SlFatB|XP 004239404.1
Lus10000365.g|Lus10000365
Lus10034617.g|Lus10034617
Lus10007942.g|Lus10007942
Lus10013480.g|Lus10013480
VvFatB2|XP 002280147.1
PpFatB|XP 001774723.1
PpFatA|XP 001770737.1
PsFatA|ABR18461.1
GmaFatA1|AAB51523.1
VvFatA|CAO17726.1
RcFatA1|ABS30422.1
JcFatA|ABX82799.3
CchFatA|AAG35064.1
PfFatA|AAL77445.1
Lus10011839.g|Lus10011839
Lus10022772.g|Lus10022772
Lus10025912.g|Lus10025912
Lus10038190.g|Lus10038190
CsFatA|Q42712.1
GmaFatA2|AAB51524.1
ChFatA1|AAC72883.1
BnFatA|CAA52069.1
BnFatA|CAA52070.1
BjFatA|CAC39106.1
AtFatA2|NP 193041.1
AtFatA1|NP 189147.1
BrFatA1|AAC49002.1
CtFatA1|AAA33020.1
HaFatA1|AAL79361.1
CtFatA2|AAA33019.1
ZmFatA|ACF78226.1
IgFatA|AAG43859.1
ItFatA|AAL77443.1

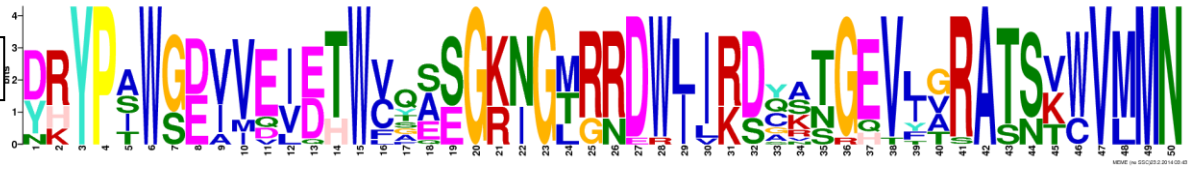
5. Motif analysis using MEME

MEME was used to determine the conserved motifs in FAT A and FAT B genes from *Linum* and were matched with that of *Arabidopsis thaliana.* The sequences of the conserved motifs have been described below. The function of a protein is extensively dependent on the conformation it acquires and its tertiary structure so a study of the conserved motif is an absolute necessity.

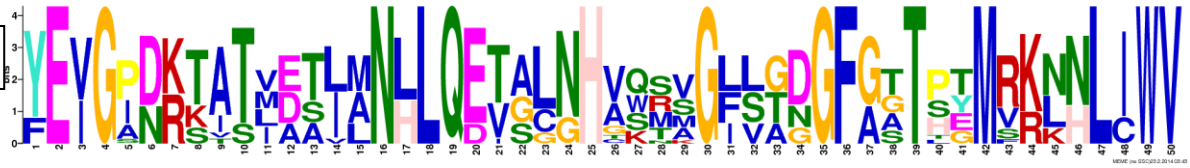


Motif 1

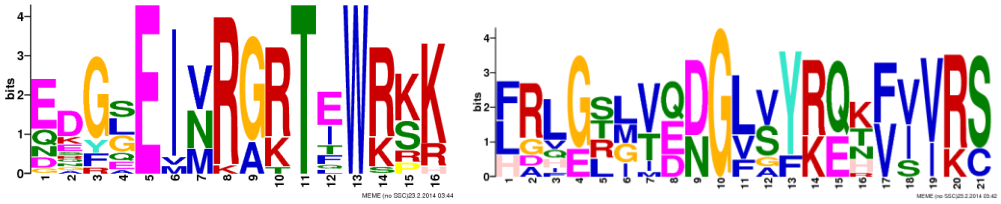
Motif 2



Motif 3



Motif 4



Motif 6

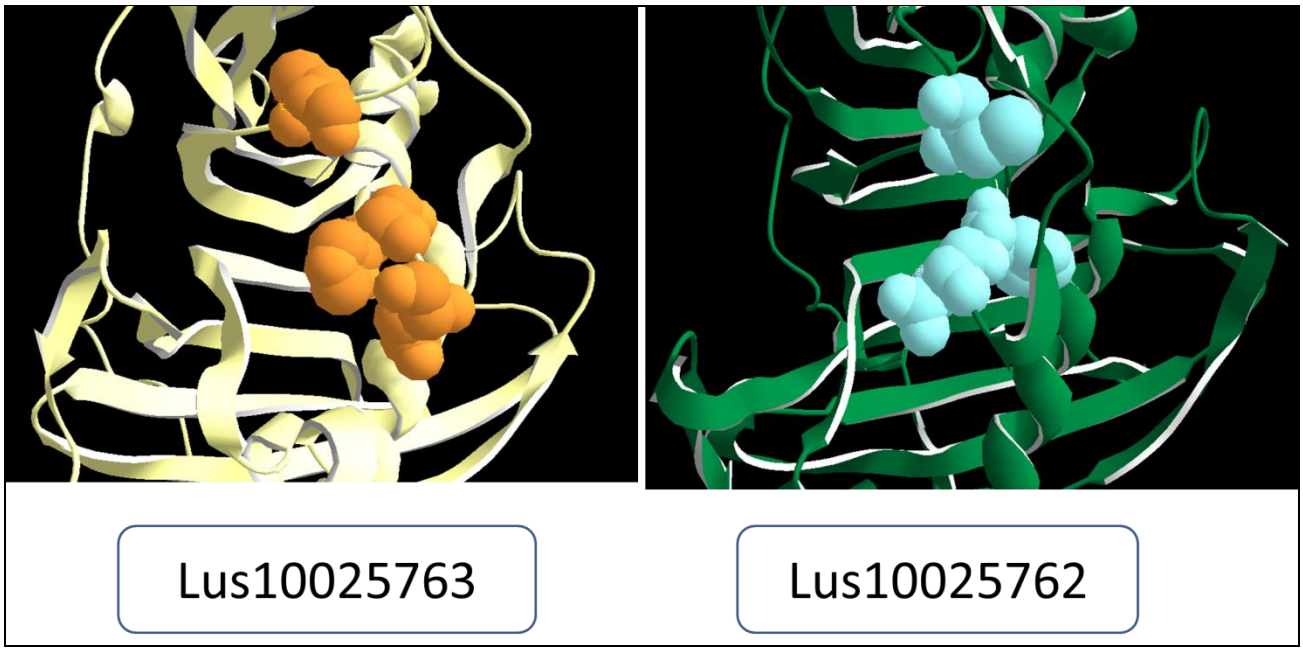
6. Structure Prediction using SWISS-MODEL workspace and expression analysis using flax EST database:

Linum fat genes (putative)	4GAK *Spirosoma linguale*	2OWN *Lactobacillus plantarum*	SEF3 Position *	Total No. of ESTs	Tissue Distribution
Lus10033072	266 aa mapped	-	Absent	17	Stem Peel, Mature Embryo
Lus10017751	266 aa mapped	-	Absent	32	Mature Embryo, Torpedo Embryo
Lus10007942	305 aa mapped	-	-218	-	
Lus10013480	305 aa mapped	-	Absent	50	Flower
Lus10000365	321 aa mapped	-	+986	02	Torpedo Embryo
Lus10034617	-	330 aa mapped	+982	-	
Lus10025762	262 aa mapped	-	+555, -625, +783	02	Globular Seed Coat
Lus10035900	-	266 aa mapped	+473	31	Globular Seed Coat
Lus10035901	263 aa mapped	-	-677, +797	02	Globular Seed Coat
Lus10025763	-	256 aa mapped	Absent	-	
Lus10011839		294 aa mapped		13	Torpedo Embryo
Lus10022772	295 aa mapped	-	+508	11	Torpedo Embryo
Lus10025912	280 aa mapped	-	Absent	09	Heart Embryo
Lus10038190	-	279 aa mapped	Absent	12	Torpedo Embryo

Overlay Models on 4gak and 2own

The homology model was created using the default settings, and the structures were overlaid on 4gak and 2own.The three dimensional structures help in prediction of the nature of interaction that protein will have with other proteins and other substrates.

6.1. Active Site determination in the homology model

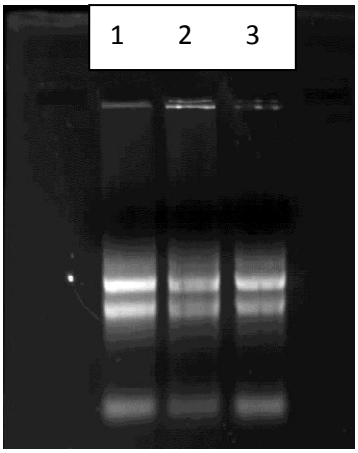


The image shows the binding site and the interaction of the model with the documented ligand in the database.

7. Genomic study

7.1. RNA extraction

RNA extracted from the leaf were run on the first lane and, second and third lanes had RNA extracted from bolls and seeds. The results forced us to take drastic steps to eliminate the degradation of the RNA during the extraction protocol.



7.2. 18 s RNA amplification

Polymerase chain reaction was carried out for the 18 s RNA using templates that had been made from the seed tissue. The template were synthesised earlier and were stored at - 20º C. The DNase treated and untreated samples were subjected to polymerase chain reaction. Positive amplification was obtained in case of DNase treated samples. The reaction mixture was prepared in the under stated manner.

10 x buffer	1 µL
$Mgcl_2$	0.5 µL
Forward primer	0.5 µL
Reverse primer	0.5 µL
DNTPs	2.5 µL
Template	1 µL
Taq Polymerase	0.2 µL
Water	3.8 µL

+C 1 2 3 4 L

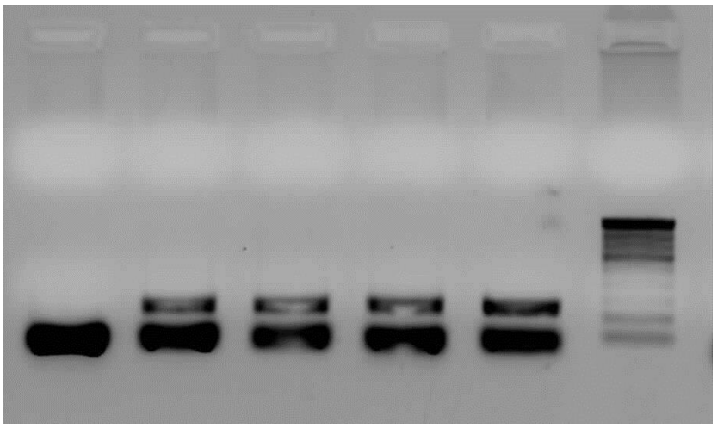


Image 2. Polymerase chain reaction of 18s RNA.

L -100 bp ladder ; NC- negative control ;1,2,3- Dnase untreated template

4,5,6- Dnase treated template

7.3. Fatty acid Desaturase 2

Lanes 1 2 3 4 L

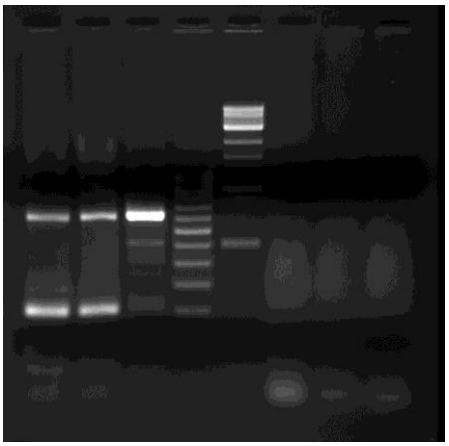


Image 3. PCR amplification of FAD 2 gene from the template extracted from leaf, bolls and seeds

The desaturase had already been extracted ,amplified and studied in the lab and was used for the amplification to affirm the functioning of the reaction mixture ingredients that was being used .Following are series of gel images that were obtained in the process of standardization of FAT gene amplification.

Image FATA_B

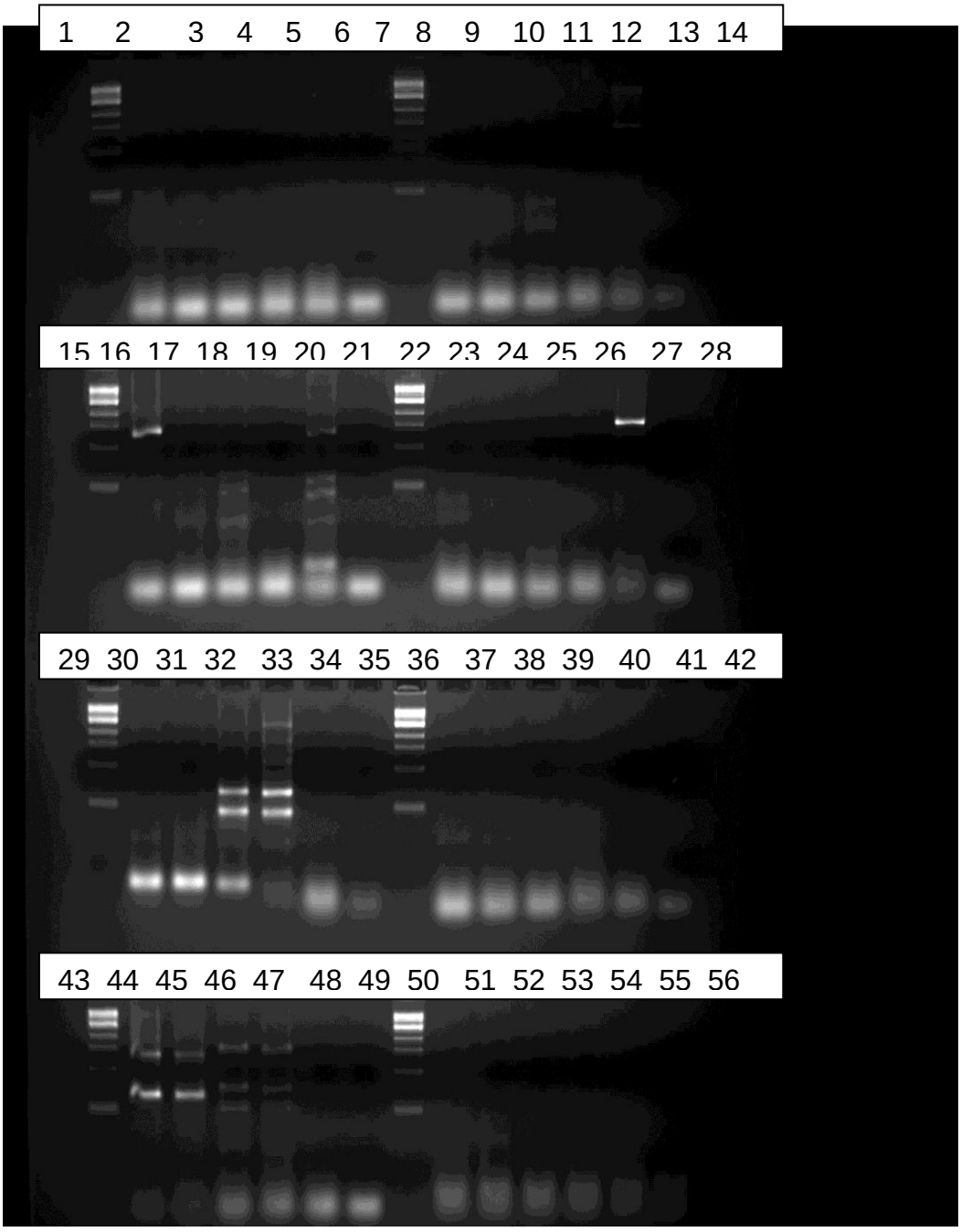


Image 4 lane [2 to 7] has FAT A1 gene amplification, lane [9 to 14] has FAT A2 gene amplification, lane [16 to 21] has FAT A3 gene amplification , lane [23 to28] has FAT B1 gene amplification , lane [30 to 35] has FAT B2 gene amplification lane [37 to 42] has FAT B3 gene amplification, lane [44 to 49] has FAT B4 gene amplification lane [51 to 58] has FAT B5 gene amplification. All were flanked with 1KB ladder. Gradient used 45, 47, 50, 52, 55 and 57ºC

5. FAT B

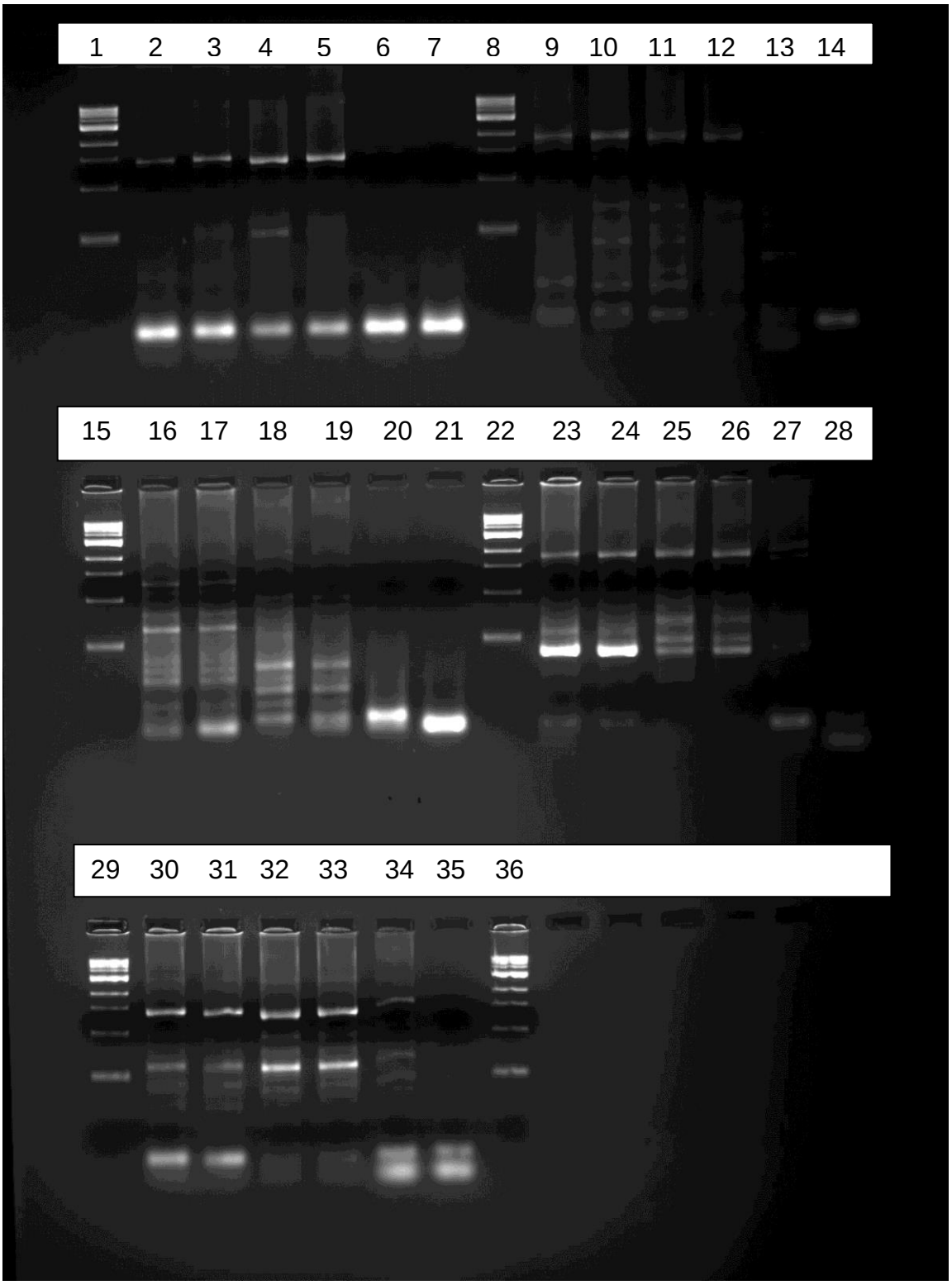


Image 5 lane [2 to 7,9 to 14] has FAT B6 gene amplification, lane [16 to 21,23 to 28] has FAT B7 gene amplification, lane [30 to 35, 37to 42] has FAT B8 gene

amplification. Gradient of temperature followed were 45, 47, 50, 52, 55, 57; 48, 51, 53, 58, 60, 63ºC.

6. In order to standardize the polymease chain reaction and find out a suitable annealing condition, gradient polymerase chain reactions were set at twelve different temperature. The temperature range was then narrowed down to 58º C to 60º C ,and the primers of the FAT B genes,eight of them were checked for their optimim annealing temperature.

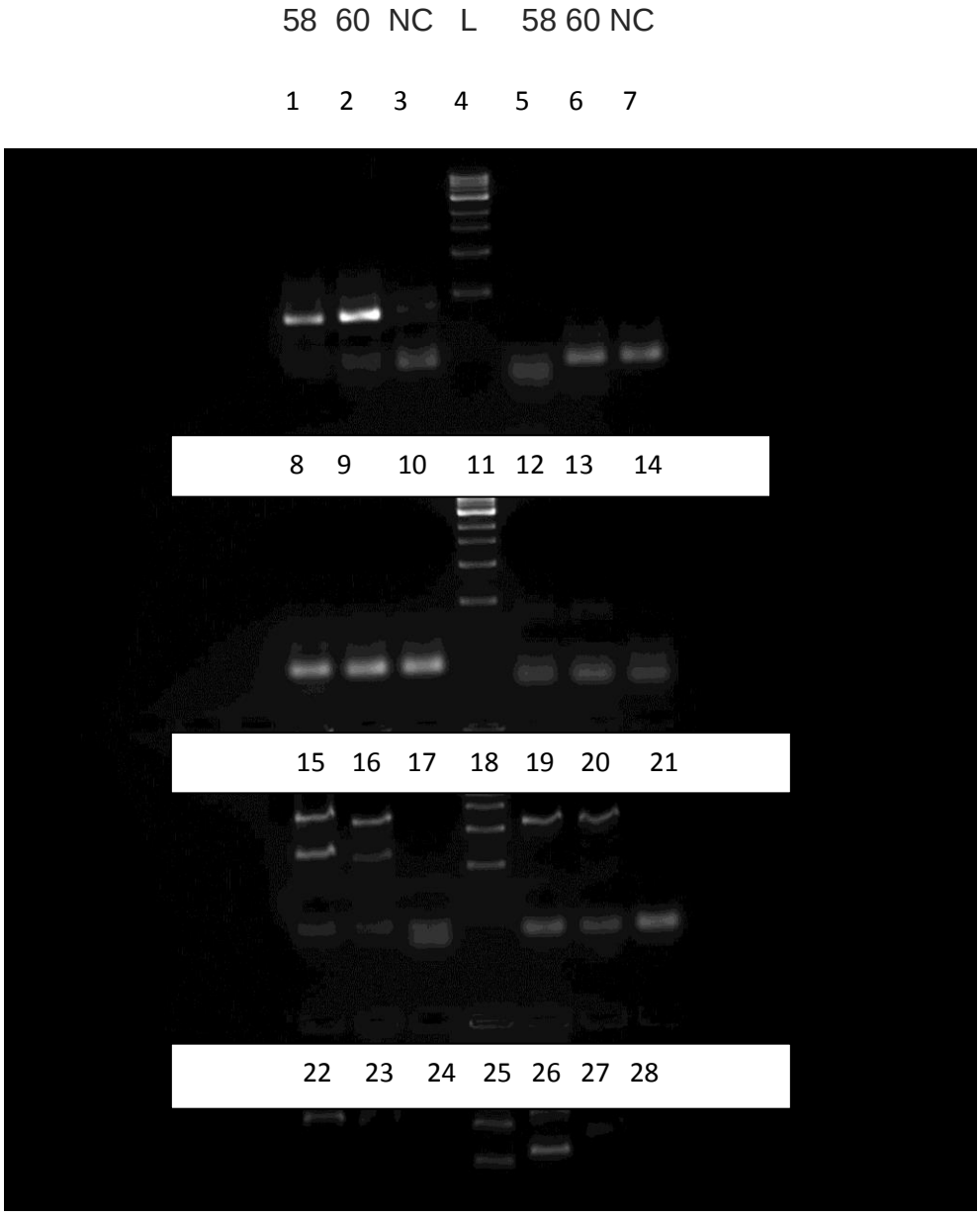


Image 6. Primer tested -[480],[365],[617],[762],[072],[763],[900],[751]

Primer [480]- 1 at 58ºC ,2 at 60 ºC,3 negative control at 60 ºC. Primer [365]- 5 at 58ºC ,6 at 60 ºC,7 negative control at 60 ºC. Primer [617]- 8 at 58ºC ,9 at 60 ºC,10 negative control at 60 ºC. Primer [762]- 12 at 58ºC ,13 at 60 ºC,14 negative control at 60 ºC. Primer [072]- 15 at 58ºC ,16 at 60 ºC,17 negative control at 60 ºC. Primer [763]- 19 at 58ºC ,20 at 60 ºC,21 negative control at 60 ºC. Primer [900]- 22 at 58ºC ,23 at 60 ºC,24 negative control at 60 ºC. Primer [751]- 26 at 58ºC ,27 at 60 ºC,28 negative control at 60 ºC.

The above images were that obtained during the proceedings of gradient PCR, carried out to pin down the denaturation temperature. The experiment is in process and in the last image the gene was addressed by their code. The range of the danaturation temperature to be tested for is constricting but the task of specifically enlisting them can be done after repeated replication of result on which the work is ongoing.

8. GUS assay

β-glucuronidase assay is one of the widely performed assays to detect gene transformation and promoter activity in plants. GUS gene acts as a reporter gene and assists in the monitoring the activity of gene of interest by forming a reporter gene product which is blue in colour and can be easily visualised. The T DNA region of the vector that was introduced into the bacterial genome had T DNA at both the ends, to mark the boundary for specifying the gene of interest. The selectable marker helps in locating the cell exhibiting the T DNA region. The fusion gene is made of the promoter of interest and a polyadenylation signal sequence to create the translational fusion.

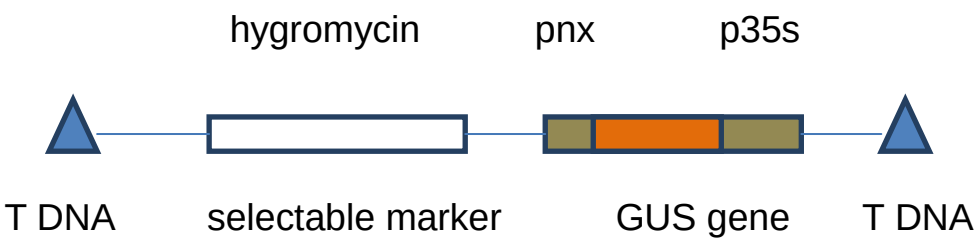


Figure 4 TDNA promoter GUS gene fusion in Agrobacterium Tumifacien. The transformed tissue exhibited blue coloration confirming the transformation.

Image 7 GUS assay in linseed

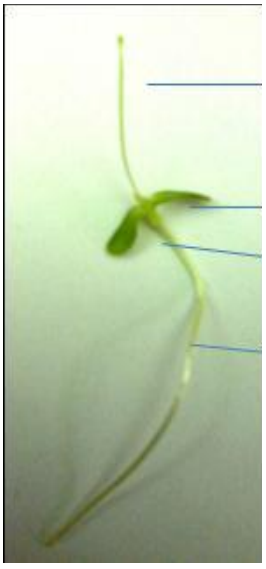


Image 8. Plantlet of the linseed

The results for the initial phase were positive and in the above picture the blue coloration shows successful transformation of the bacterial enzyme β-glucuronidase into the linseed genome.

Plan of the project under execution: The aim is to analyse the seed and boll tissues belonging to the seven different stages of growth i.e. fourth, eighth, twelfth, sixteenth, twenty-second, thirtieth and fortieth day after anthesis. The idea is to give the samples high-throughput liquid chromatography runs (HPLC) in three biological replicates and two technical replicates, which will serve as a pre-fractionation step for the sample preparation. And the samples will be studied by liquid chromatography mass spectroscopy analysis.

A parallel study that is being carried out is the metabolome analysis of linseed. The extraction method and various modifications are being tried for the metabolite extraction from the linseed. Some of the extraction protocols that were carried out have been mentioned in the materials and method section. The GUS assay could be used in future as a reporter gene to the gene of interest.

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