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GENE EXPRESSION IN A MICROSPORE-DERIVED CELL SUSPENSION CULTURE OF *BRASSICA NAPUS* EXHIBITING ENHANCED OIL PRODUCTION

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LETHBRIDGE, ALBERTA, CANADA

TO MY PARENTS,
DONALD AND PENNY DAVOREN

ABSTRACT

Triacylglycerol (TAG) production in the microspore derived (MD) cell suspension culture of *Brassica napus* L. cv Jet Neuf was enhanced when the sucrose concentration in the growth medium was increased from 2 to 14 % (w/v). mRNA differential display by polymerase chain reaction was used to examine gene expression in cells grown at different sucrose concentrations in order to identify mRNAs which could be associated with oil formation. The anchored primer, $T_{12}AA$, was used to screen one subset, representing approximately one twelfth of the transcript population, isolated from cultures grown in media supplemented to 2, 6 and 14 % (w/v) sucrose. Analysis of this mRNA subset revealed thirteen cDNAs which appeared to be upregulated as the sucrose concentration was increased. Cloning and sequencing revealed multiple cDNA fragments for each signal detected by differential display. RT-PCR analysis of sixteen different cDNAs revealed that eight encoded mRNAs which were upregulated in parallel to the increase in media sucrose. Comparison of the eight upregulated cDNAs to other sequences in GenBank revealed the following: (1) BSS8A had a 100% identity with the last 25 amino acids of an acyl carrier protein from *Arabidopsis thaliana*, (2) BSS1A displayed homology to a number of sequences of unknown function, (3) BSS11B displayed weak but significant homology to a number of sequences of unknown function, (4) BSS13A displayed homology to four members of the thioredoxin family from *A. thaliana* and (5) four had no significant homology to previously reported sequences which makes them potential candidates to encode lipogenic enzymes. These results indicate that differential display of mRNA may be a simple and rapid method for the identification of sucrose-modulated gene expression changes in this system and for the characterization of novel sequences potentially encoding lipogenic proteins.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

ACP	acyl carrier protein
A. thaliana	*Arabidopsis thaliana*
B. napus	*Brassica napus*
BLAST	basic local alignment search tool
BSC	*Brassica napus* sucrose control
BSS	*Brassica napus* sucrose stimulated
cDNA	complementary deoxyribonucleic acid
CoA	coenzyme A
CPT	CDP-choline: 1,2-diacylglycerol cholinephosphotransferase
CRTC	Controlled ribonucleotide tailing of cDNA ends
DAG	*sn*-1,2-diacylglycerol
DD	differential display
DGAT	diacylglycerol acyltransferase
DGDG	digalactosyldiacylglycerol
DTT	dithiothreitol
DW	dry weight
EF-TU	the nuclear encoded mitochondrial translation elongation factor - TU
ER	endoplasmic reticulum
FAD	fatty acid desaturase
FAMES	fatty acid methyl esters

FAS	fatty acid synthesis
FW	fresh weight
G3P	*sn*-glycerol-3-phosphate
GPAAT	glycerol-3-phosphate acyltransferase
HPTLC	high performance thin layer chromatography
HSP	high scoring segment pair
KAS	β-ketoacyl-ACP synthase
LPA	lysophosphatidate
LPAAT	lysophosphatidate acyltransferase
LPCAT	acyl-CoA: lysophosphatidylcholine acyltransferase
MD	microspore derived
MGDG	monogalactosyldiacylglycerol
MoMULV	Moloney Murine Leukemia Virus
mRNA	messenger ribonucleic acid
NADH	nicotiamide-adenine dinucleotide
NADPH	nicotiamide-adenine dinucleotide phosphate
PA	phosphatidate
PAP	phosphatidate phosphatase
PC	phosphatidyl choline
PCR	polymerase chain reaction
PL	phospholipid
RFLP	restriction fragment length polymorphism

RT	reverse transcription
SDS	sodium dodecyl sulfate
sn	stereochemical numbering
SQDG	sulfoquinovosyldiacylglycerol
SSC	sodium chloride sodium citrate
TAG	triacylglycerol
TL	total lipid
UDP	uridine diphosphate

INTRODUCTION

Many plant species store large amounts of lipids in seeds. Currently, world trade in vegetable oils derived from such seeds is about 65 million tonnes per year (Murphy, 1994). In Canada, oilseed sales represent approximately one quarter of crop production (Scarth *et al.*, 1992). Canola represents approximately 63 % of the vegetable oil produced in Canada (McDonald, 1990). Canola refers to oilseed rape species, such as *Brassica napus* and *Brassica compestris*, which have been bred to have low levels of erucic acid in the oil component and low levels of glucosinolates in the meal (Anonymous, 1990). On average seven million acres of canola are planted annually in Canada (Anonymous, 1994). Canola seeds typically contain over 40 % oil (Stymne and Stobart, 1987). The oil is useful for both edible and industrial applications. Following extraction of the oil, the remaining meal can be further processed for use as a livestock feed because it contains approximately 38 % protein in addition to minerals, vitamins and fibre (Bell, 1993).

Triacylglycerol (TAG) is the chemical form of storage lipid in almost all plants. Triacylglycerol biosynthesis has been proposed to take place via the reactions of the Kennedy pathway (Kennedy, 1961; Stobart and Stymne, 1987). The enzymes of this pathway are associated with the endoplasmic reticulum (ER) membrane and catalyze the sequential acylation of the *sn*-1, 2 and 3 positions of *sn*-glycerol-3-phosphate (G3P), with the removal of the phosphate group occurring just prior to the final acylation. In mature seeds, the TAG molecules are stored in densely packed and spherically shaped lipid bodies approximately 1 μm in diameter (Huang, 1992; Murphy, 1993; Herman, 1994). In oilseed rape, the cotyledons of the embryo are the major site of TAG accumulation

(Murphy, 1993).

Despite the obvious importance of vegetable oils, little is known about the biochemical regulation of TAG biosynthesis. This lack of knowledge can be partially attributed to the difficulties associated with studying seed tissues, such as the long growth period to obtain developing seeds, the discontinuous accumulation of lipids (Harwood, 1989) and the difficulties in obtaining sufficient material synchronized to well defined developmental stages (Gurr, 1980). In order to facilitate the study of seed oil biosynthesis, a number of *in vitro* systems with well-defined developmental stages have been developed (Murphy, 1993). One of the best characterized systems is the microspore derived (MD) embryo system of *B. napus* (Taylor *et al.*, 1990; Pomeroy *et al.*, 1991). Microspore derived embryos have been shown to accumulate TAG in a manner similar to that of zygotic embryos (Taylor *et al.*, 1990; Pomeroy *et al.*, 1991). Furthermore the TAG which accumulates in MD embryos is similar in composition to the seed storage lipid (Taylor *et al.*, 1991). Cell suspension cultures have also been shown to contain TAG and manipulation of the culture conditions have been shown to have a strong influence on the lipid content and composition (Radwan and Mangold, 1976; Theimer *et al.*, 1991). A non-differentiating MD cell-suspension culture of *B. napus* L. cv Jet Neuf, developed by Wilf Keller and colleagues has been shown to accumulate some TAG (Weselake *et al.*, 1993).

In the past few years, the understanding of the basic metabolic pathways that lead to the synthesis of the major plant glycerolipids has advanced rapidly. Many of the advances can be attributed to the use of molecular biological approaches to isolate

cDNAs encoding enzymes of these pathways. Furthermore, the potential economic value of genetically engineered oil crops has stimulated research in industrial laboratories. Recent attempts at genetic engineering of oil seed crops have been successful in the production of new and improved vegetable oils (reviewed by Kinney, 1994; Ohlrogge, 1994; Topfer *et al.*, 1995). Almost all of the soluble enzymes involved in the production of fatty acids have been cloned (Topfer and Martini, 1994). Many of the membrane bound enzymes of fatty acid biosynthesis have been isolated by map based cloning (Arondel *et al.*, 1992), T-DNA or transposon tagging (Okuley *et al.*, 1994; James *et al.*, 1995) and complementation of mutants (Brown *et al.*, 1994; Dewey *et al.*, 1994).

Compared to the large amount of work being done on fatty acid biosynthesis, little has been directed specifically at the enzymes of the Kennedy pathway. Molecular genetic studies of these enzymes has been impeded by difficulties in their solubilization and purification. Diacylglycerol acyltransferase (DGAT, EC 2.3.1.20) catalyzes the final step in TAG biosynthesis. Studies with developing seeds and MD embryos have shown that the activity of DGAT increases markedly during the rapid phase of oil accumulation (Tzen *et al.*, 1993; Weselake *et al.*, 1993). As well, a substantial accumulation of *sn*-1,2-diacylglycerol (DAG) during TAG formation suggests that DGAT may exert a significant flux control over oil accumulation (Perry and Harwood, 1993a). Despite the central role of DGAT in TAG biosynthesis, it has not been purified from any source. Thus at present there is no direct method of identifying a cDNA encoding DGAT because there is no information about the amino acid sequence of DGAT. It is possible, however, that a cDNA for DGAT has been sequenced by the large sequencing projects presently

underway for *Arabidopsis thaliana* (Hofte *et al.*, 1993; Newman *et al.*, 1994). Recently a mutant of *A. thaliana* has been identified which has been found to have reduced DGAT activity (Katavic *et al.*, 1995). It may be possible to identify a gene encoding DGAT or genes which affect the activity of this enzyme from the mutant. The identification of such genes, however, may be difficult as the mutant was produced by chemical mutagenesis which alters a few nucleotides at random sites throughout the genome.

As a unique approach to the identification of TAG biosynthetic enzymes, this thesis has examined gene expression associated with increased oil accumulation in the cell suspension culture of *B. napus* L. cv Jet Neuf. The technique of differential display (DD) of mRNA developed by Liang and Pardee (1992) was found to be very sensitive for the detection of genes which were differentially expressed and as such proved to be useful for the detection of mRNAs which may encode lipid biosynthetic enzymes. The cell suspension cultures were an easily maintained source of cells that were active in TAG biosynthesis. The culture produced more DGAT activity and TAG when the media sucrose concentration was increased. Furthermore, these non-differentiating cells facilitated studies of gene expression associated with TAG biosynthesis by avoiding gene expression associated with the other complex processes that normally occur in developing seeds such as embryogenesis, preparation for dessication and other developmental specific gene expression.

LITERATURE REVIEW

Global Importance of Seed Oil

Triacylglycerols are stored predominantly in oleaceous seeds and some fruits such as avocado but minor accumulation also occurs in leaves, stems and roots (Gurr, 1980). Most higher plants form TAGs in the cotyledons of the embryo or endosperm tissue during maturation and store them in subcellular organelles called oil bodies (Murphy, 1993). The TAG molecule is a glycerolipid which consists of three fatty acyl groups esterified to the three carbon atoms on a glycerol "backbone" (Gurr, 1980). The fatty acyl groups of TAG represent an efficient energy store since they contain fully reduced carbon which yields maximal energy upon oxidation.

Currently, vegetable oils represent an important economic commodity with world trade figures of about 65 million tonnes per year (Murphy, 1994). Approximately 84 % of this oil is produced by the following six crops: soybean, palm, oilseed rape, sunflower, cotton seed and ground nut. The demand for vegetable oils has grown with the discovery that they are more suitable, than animal fats, for human consumption as well as being useful for various industrial applications such as high temperature lubricants and foaming agents. A number of environmentally friendly commodities, such as cosmetics and biodiesels, are now being produced from the more natural vegetable oils rather than petrochemical oils (Topfer *et al.*, 1995). Approximately 90 % of the vegetable oils produced are used in the manufacture of margarines, shortenings, salad oils and frying agents (Robbelen, 1988).

Because of their economic importance, a significant amount of work has been dedicated to the determination of fatty acid compositions of TAGs from the seeds of many species (Weiss, 1983). A large variety of fatty acid structures have been identified in nature (Gunstone, 1986), but only five account for approximately 90 % of the commercial vegetable oils produced (Browse and Somerville, 1991). These five fatty acids are 16:0, 18:0, 18:1, 18:2 and 18:3. They are commonly found in membranes and TAG. In addition, certain plant species produce specialized fatty acids that are excluded from the membrane lipid fraction, but are used for TAG biosynthesis (Battey and Ohlrogge, 1989; Vogel and Browse, 1996). Triacylglycerols of oilseed rape contain the long-chain, monounsaturated fatty acids, 20:1 and 22:1. Palm kernel oil, coconut oil and the oils of several *Cuphea* species contain high levels of the medium chain saturated fatty acids which include 10:0, 12:0 and 14:0. Fatty acids containing hydroxyl groups, triple bonds and conjugated double bonds have also been identified in the TAG fraction of a few plant species (Weiss, 1983). The utility and quality of vegetable oil, for most purposes, depends upon the relative amounts of the five major fatty acids. Oils consisting primarily of oleic acid are oxidatively stable, display superior flavor characteristics and, potentially, provide health benefits while oils rich in polyunsaturated fatty acids have poor flavor and low stability (Neff *et al.*, 1992).

Oilseed rape encompasses the *Brassica* species which include *Brassica napus* L. and *Brassica rapa* L.(*B. campestris* L.). Oilseed rape was initially cultivated in Canada during the Second World War as a source of oil for the lubrication of marine engines.

This oil, however, was not suitable for consumption because it contained approximately 50 % erucic acid. High erucic acid oils have been shown to cause heart lesions in pigs (Sauer *et al.*, 1989). As well, the early rapeseed meal left over after oil was extracted was not useful as an animal feed because it contained minor, but toxic, constituents known as glucosinolates. Early plant breeding efforts led to the release of Oro, in 1968, the first low erucic acid oilseed rape cultivar. By 1974, the first "double low" cultivar, Tower, was released having both low erucic acid levels and low glucosinolate content. Double low cultivars are classified as canola quality if they have less than 2 % erucic acid in the oil and less than 30 μmoles of glucosinolates per gram of the meal component (Anonymous, 1990). More recent breeding efforts have resulted in the production of low linoleic acid (18:2 Δ9,12) varieties such as the cultivar Stellar which has both canola quality and less than 3 % linoleic acid. The reduction in linoleic acid has been shown to improve the stability of the oil.

The increasing utility of oilseeds has led to the application of genetic engineering to the improvement of plants. The use of genetic engineering, in combination with classical breeding techniques, has already led to some major advances in vegetable oil production (Topfer *et al.*, 1995). By altering the position and number of double bonds, fatty acid chain length and the introduction of functional groups to fatty acids in model systems it has become possible to customize the oil production of a plant to meet different needs. The technology used to modify the lipid composition of these model systems is currently being applied to crop plants such as oilseed rape and soybean in

order to enable the efficient production of customized oils. Recently, the cloning of a number of important genes encoding fatty acid biosynthetic enzymes has enabled their manipulation in transgenic plants to produce oil with modified fatty acid composition (Topfer *et al.*, 1995).

Triacylglycerol Biosynthesis in Developing Oilseeds

Triacylglycerol biosynthesis generally can be categorized into three major parts: 1) the production of the glycerol backbone, 2) the assembly of fatty acids and 3) the esterification of the fatty acids to the glycerol backbone (Stymne and Stobart, 1987). Initially, sucrose transported through phloem tissue to developing seeds, is converted into hexose phosphates and triose phosphates by cytosolic glycolysis. The triose phosphate, G3P, is later acylated to form the TAG molecule. Hexose phosphates are transported into the plastids by the action of specific translocators located in the membrane of this organelle.

De novo fatty acid biosynthesis (FAS) has been reviewed quite extensively (Gurr, 1980, Gurr and Harwood, 1991, Sparace and Kleppinger-Sparace, 1993; Ohlrogge and Browse, 1995). The process of *de novo* fatty acid biosynthesis has been shown to occur exclusively in the stroma of plastids. Fatty acid assembly is catalyzed by a series of enzymes and requires acyl carrier protein (ACP). Fatty acids can be used for the production of membrane lipids in the plastid, or transported into the cytoplasm for the synthesis of membrane lipids or storage TAGs.

The first committed step in FAS is the reaction of acetyl-CoA and HCO_3^-,

catalyzed by acetyl-CoA carboxylase (ACCase, E.C. 6.4.1.2), in the presence of adenosine triphosphate (ATP), to form malonyl-CoA (Post-Beittenmiller *et al.*, 1992). In plants and animals, ACCase has been studied extensively because it has been shown to be the rate limiting step for fatty acid biosynthesis (Post-Beittenmiller *et al.*, 1992). It has been established that at least one of the two types of plant ACCase is a large multi functional enzyme of over 200 kDa per subunit each having a biotin binding site. Genes encoding ACCase have been cloned from a number of plants including alfalfa (*Medicago sativa* L.) (Shorrosh *et al.*, 1993), *Cyclotella cryptica* (Roessler and Ohlorogge, 1993), wheat (*Triticum aestivum*) (Gornicki *et al.*, 1993) and *B. napus* (Schulte *et al.*, 1993). Characterization of the *B. napus* ACCase clone showed that the enzyme consists of 32 exons placed over 12 kbp with similar functional domains to the sequences isolated from yeast and rat tissues.

Acyl carrier protein is a non enzymic fatty acid synthase protein which covalently binds to growing acyl chains by its phosphopantetheine group (Ohlrogge, 1987). All ACPs identified have a molecular weight of approximately 10,000 and are acidic proteins (Ohlrogge, 1987). Acyl carrier proteins initially enter the fatty acid synthetic pathway by the action of malonyl-CoA:[ACP] transacylase E.C. 2.3.1.39 which catalyzes the transfer of malonyl from CoA to ACP. Following the production of malonyl-ACP, a series of enzymes known as the type II fatty acid synthase (FAS) consecutively add two carbon units derived from malonyl-CoA to a growing acyl chain bound to ACP. The first condensation of acetyl-CoA and malonyl ACP is catalyzed by β-ketoacyl-ACP synthase

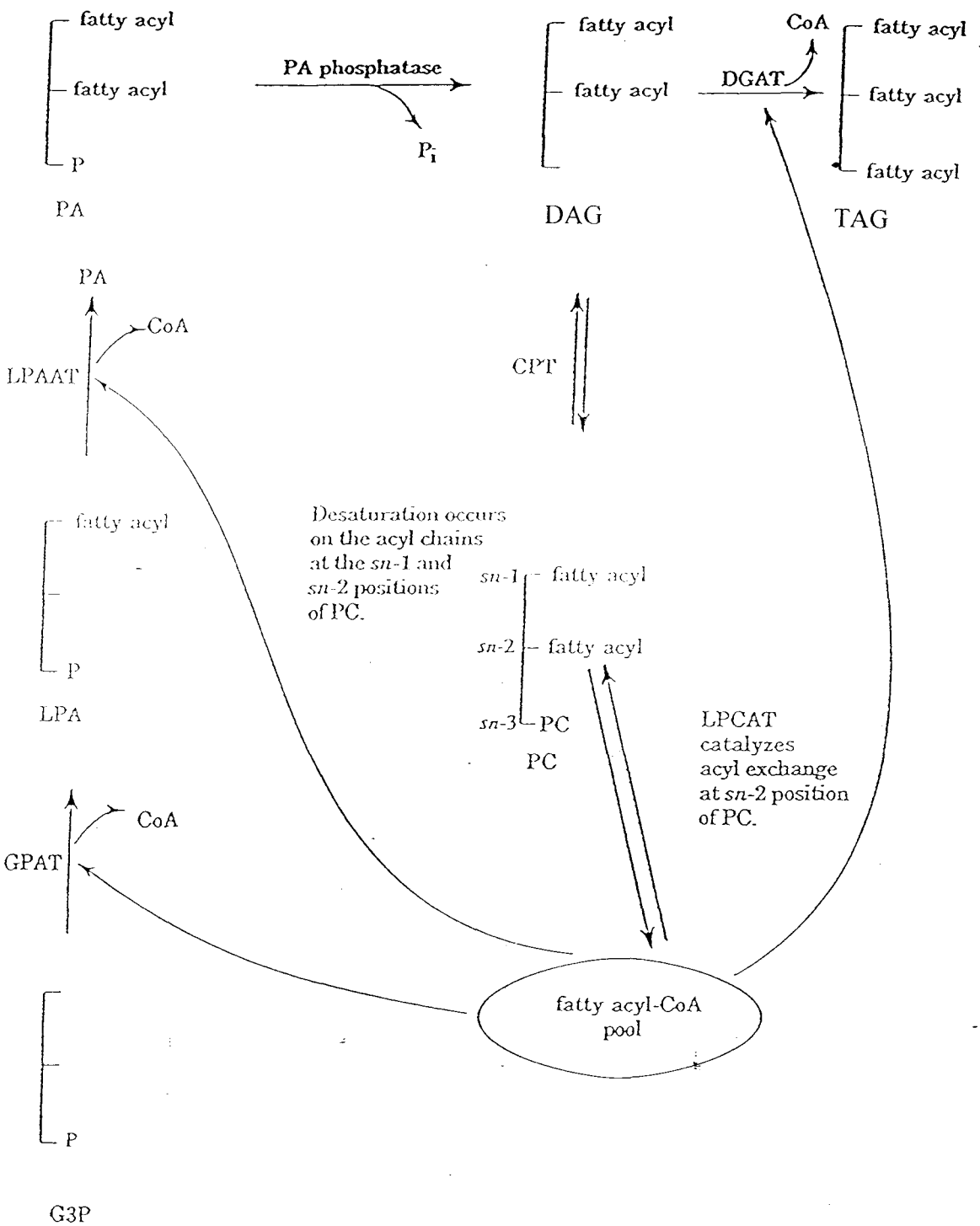
III (KASIII) (Jaworski *et al.*, 1989). Following this initial condensation, a second condensing enzyme (KASI) catalyzes seven condensations to produce palmitoyl-ACP, a C_{16} acyl thioester (Shimakata and Stumpf, 1983). In most tissues, the palmitoyl-ACP may then be elongated to stearoyl-ACP C_{18} by the action of a specific KASII enzyme (Shimakata and Stumpf, 1982). After the addition of the two carbon units by the condensing enzyme in each cycle of fatty acid synthesis, the chain is reduced by β-ketoacyl-[ACP] reductase E.C. 1.1.1.100 with NADPH as a cosubstrate to form β-hydroxyacyl-ACP (Caughey and Kenwick, 1982). Following reduction, the chain is dehydrated by β-hydroxyacyl-ACP dehydratase to form enoyl-ACP. A second reduction is catalyzed by enoyl-ACP-reductase E.C. 1.3.1.9 in the presence of NADH as a cofactor.

Fatty acyl moieties are released from ACP by the action of specific thioesterases such as oleoyl-ACP thioesterase (E.C. 3.1.2.14) (Shine *et al.*, 1976). Thioesterases are especially important in plants such as elm, coconut and camphor where they have been shown to control the chain length of fatty acids shorter than 16 carbon atoms (Davies, 1993). Fatty acids may either be used directly for plastid lipid synthesis or released from ACP and exported to the cytoplasm. Once released from ACP, the acyl residues are exported to the cytoplasm and converted to acyl-CoA esters by an acyl-CoA synthetase located in the outer envelope of the plastid (Stymne and Stobart, 1987; Browse and Somerville, 1991). Once in the cytoplasm, fatty acyl- CoAs enter an acyl-CoA pool and serve as substrates in glycerolipid assembly.

TAGs are formed in the ER (Stobart *et al.*, 1986) by the stepwise acylation of

G3P according to the Kennedy pathway (Kennedy, 1961) (Figure 1). The G3P backbone is supplied primarily through the reduction of dihydroxyacetone phosphate catalyzed by G3P dehydrogenase. Dihydroxyacetone phosphate is derived from either the glycolytic or pentose phosphate pathways (Stymne and Stobart, 1987). Alternatively, glycerol kinase also catalyzes the production of G3P but at a much slower rate than G3P dehydrogenase (Gurr, 1980). The first enzyme of the Kennedy pathway, glycerolphosphate acyltransferase (GPAT, E.C. 2.3.1.15), catalyzes the acylation of G3P at the *sn*-1 position to form lysophosphatidate (LPA). Recently GPAT was solubilized and partially purified from avocado mesocarp (Eccleston and Harwood, 1995). The second enzyme, lysophosphatidic acid acyltransferase (LPAAT, E.C. 2.3.1.51), catalyzes the acylation of LPA at the *sn*-2 position to form phosphatidate (PA). Coleman (1992) reported identification of a cDNA encoding an LPAAT from *Escherichia coli*. The LPAAT was identified by complementation with the *E. coli* mutant. Recently cDNAs encoding putative LPAAT enzymes have been identified from maize (Brown *et al.*, 1994), meadowfoam (Hanke *et al.*, 1995) and yeast (Nagiec *et al.*, 1993), by complementation with the *E. coli* mutant. Davies *et al.* (1995) reported on the solubilization of LPAAT from immature coconut endosperm which led to the partial purification and cloning of a cDNA that encoded LPAAT (Knutzon *et al.*, 1995). PA is dephosphorylated by PA phosphatase (E.C. 3.1.3.4) to form DAG. Recently PA phosphatase was purified from Avocado (Pearce and Slabas, 1997). Diacylglycerol acyltransferase catalyzes the final and committed step of TAG biosynthesis, the acylation of DAG at the sn-3 position. Recent findings, however, have indicated that DGAT is not the only enzyme catalyzing theformation of TAG. Lehner and Kuksis (1993)

Figure 1. Schematic representation of TAG biosynthesis in relation to phosphatidylcholine (PC) synthesis and modification in developing oilseeds. Adapted from Stymne and Stobart (1987) and Stobart and Stymne (1990).


fatty acyl
fatty acyl
P
PA
PA phosphatase
P_i
fatty acyl
fatty acyl
DAG
CoA
DGAT
fatty acyl
fatty acyl
fatty acyl
TAG
PA
CoA
LPAAT
CPT
fatty acyl
P
LPA
Desaturation occurs on the acyl chains at the *sn*-1 and *sn*-2 positions of PC.
sn-1
fatty acyl
sn-2
fatty acyl
sn-3
PC
PC
LPCAT catalyzes acyl exchange at *sn*-2 position of PC.
CoA
GPAT
fatty acyl-CoA pool
P
G3P

demonstrated that diacylglycerol transacylase catalyzes a transesterification reaction using two molecules of DAG to form TAG and a monoacylglycerol. The enzymes GPAT, LPAAT and DGAT have specific acyl preferences which govern the non-random distribution of fatty acids at the *sn*-2 and 3 positions of PA, DAG, TAG and other glycerolipids (Kocsis and Weselake, 1996).

It is difficult to discuss the assembly of TAG without consideration of the biosynthesis of membrane lipids because both processes share the same steps up to and including, the formation of DAG (Gardiner *et al.*, 1983; Frentzen, 1993). Two major forms of glycerolipids, phosphoglycerols and glycosylglycerols, are known to exist. The phosphoglycerols have a phosphorus molecule as a head group whereas glycosylglycerols have a sugar molecule as a headgroup (Harwood, 1989). The fatty acid composition at the *sn*-1 and *sn*-2 positions of the glycerol backbone is used to further classify the glycerolipids (Frentzen, 1993). "Prokaryotic" glycerolipids have a 16 carbon fatty acid at the *sn*-2 position and predominantly 18 carbon fatty acids at the *sn*-1 position while "eukaryotic" glycerolipids have an 18 or 16 carbon fatty acid at the *sn*-1 position and exclusively an 18 carbon fatty acid at the *sn*-2 position (Harwood, 1989; Frentzen, 1993). The "prokaryotic" glycerolipids are found exclusively in the plastid of higher plants while eukaryotic forms occur both within and outside the plastid. PA has been shown to be an important intermediate in the formation of membrane lipids as it can be used as a substrate for the production of cytidine diphosphate diacylglycerol which can be further manipulated to form phosphatidylglycerol and phosphatidylinositol. PA can also be hydrolyzed by phospholipase A to yield G3P (Harwood, 1989). DAG is also an

important intermediate in the formation of membrane phospholipids such as phosphatidylcholine (PC) and phosphatidylethanolamine (Harwood, 1989).

Glycosylglycerol biosynthesis, within the chloroplast, has been shown to yield the following major lipid forms: monogalactosyldiacylglycerol (MGDG), digalactosyldiacylglycerol (DGDG), and sulphoquinovosyldiacylglycerol (SQDG) (Harwood, 1989). Monoglactosyldiacylglycerol is formed by the transfer of galactose from uridine diphosphate (UDP)-galactose to diacylglycerol. MGDG lipids characteristically contain the fatty acid 16:3 at the *sn*-2 position of the glycerol backbone and have been shown to have both prokaryotic and eukaryotic patterns of fatty acid structure. DGDG is synthesized by either the transfer of a second galactose from UDP-galactose to MGDG or by a interlipid transfer between two MGDG molecules (Harwood, 1989). In eukaryotes the MGDG and DGDG lipids typically contain polyunsaturated fatty acids (Harwood, 1980). The exact route for production of SQDG is still unknown, but Gurr and Harwood (1991) suggested that it may occur in the plastid because all the enzymes necessary for sulfolipid synthesis have been detected there.

In developing seeds, the majority of fatty acids produced in the plastid are exported into the cytoplasm where they become part of the acyl CoA pool. Once in this pool, fatty acids are subject to a variety of modifications which contribute to the vast complexity of lipids. It has been shown that DAG produced by the dephosphorylation of PA may be converted into PC through a freely reversible reaction catalyzed by cholinephosphotransferase (CPT, EC 2.7.8.2) (Harwood, 1989). The PC may then be rapidly desaturated by either the Δ12 or Δ15 cytoplasmic desaturases (Figure 1) (Stymne

et al., 1983; Stymne and Stobart, 1987). This desaturation reaction is responsible for the variations of common 16 and 18 carbon fatty acids found in membrane lipids. Other fatty acyl CoA molecules can be transferred to the *sn*-2 position of PC by the action of acyl-CoA:Lysophosphatidylcholine acyltransferase (LPCAT, EC 2.3.1.23) (Figure 1) (Stobart and Stymne, 1990). This exchange reaction is also freely reversible and allows for the incorporation of polyunsaturated fatty acids back into the acyl CoA pool for acylation of other positions of the glycerol backbone. This exchange of polyunsaturated fatty acids from PC to the free fatty acid pool has been shown to be responsible for the enrichment of TAG with polyunsaturates (Stymne *et al.*, 1983).

Certain products of *de novo* fatty acid biosynthesis in some species are called unusual fatty acids because they do not accumulate to significant quantities in all species. These lipids include medium chain fatty acids of 8 to 14 carbon atoms in length, very long chain fatty acids of twenty to twenty-four carbon atoms in length or fatty acids with substitutions such as hydroxyl, epoxy and cyclopropane groups (Battey and Ohlrogge, 1989; Vogel and Browse, 1996). The very long chain fatty acids are produced by an elongation of fatty acids, with malonyl-CoA, in the ER (Browse and Somerville, 1991). Erucic acid (22:1) has a number of industrial applications. Presently there are over 1000 different patent applications submitted for C_{22} oleochemicals and their derivatives (Sonntag, 1991). Unusual fatty acids, characterized to date, are excluded from the membrane lipid fraction and only accumulate in TAG. The targeting of unusual fatty acids to TAG may be due to a spacial separation of two DAG pools, one for membrane lipid synthesis and the other for TAG biosynthesis (Vogel and Browse, 1996).

In developing oilseeds, TAG accumulates in specialized organelles known as lipid or oil bodies. It has been suggested that oil bodies are synthesized in the ER by vesiculation (Huang, 1992). Others have proposed that the formation occurs on the outside of the ER membrane as "naked" oil droplets accumulate and the oleosins are added later (Napier *et al.*, 1996). The vesiculation theory proposes that TAGs are produced in the ER and sequestered, due to their hydrophobicity, between the two phospholipid (PL) layers of the ER membrane (Huang, 1996). Oil accumulates in a tiny "bud" and PLs synthesized in the ER diffuse to the surface of this structure. As oil is being added to this structure proteins termed oleosins are simultaneously added. Oleosins are synthesized on polyribosomes bound to the ER and are guided, presumably by their hydrophobic central domain, to the budding oil body. The budding particle consists of TAGs surrounded by a half unit layer of PLs and oleosins. The second theory suggests that oil is produced "naked" on the outer membrane of the ER and that oleosins are added later in development. Once the budding particle reaches a certain size, dictated by the species, it then buds off the ER and forms a mature oil body. Mature oil bodies, on average, range from about 0.6-2.0 μm in diameter (Tzen *et al.*, 1993). This size has been suggested to be partially controlled by the ratio of TAG to oleosin (Ting *et al.*, 1996).

Oleosins are found in a number of plant seeds as well as in pollen (Huang, 1996). In mature seeds of *B. napus,* oleosins are fairly abundant, representing up to 8 % of the total cellular protein (Huang, 1996). Every oleosin has a highly conserved central hydrophobic stretch of 72 amino acids. The N-terminal sequences have few similarities in length or sequence, which contribute to the majority of differences observed between

oleosins. The carboxylic side is conserved in the form of an amphipathic alpha helical stretch. This amphipathicity has been suggested to allow the protein to interact with the PLs on the surface of the oil body. The combination of N terminal, central and carboxy terminal portions serve to anchor the protein to the surface of the oil body (Huang, 1992). The secondary structure of the oleosin serves to achieve maximal stability in the unique environment of the oil body surface. It has been further postulated that the oleosins interact among themselves to acquire extra stability (Huang, 1996). Oleosins create a barrier of negative charges which prevents the coalescing of oil bodies, due to repulsion. In addition to their structural role, oleosins may also serve as receptors for the attachment of lipases facilitating the degradation of TAG during seed germination (Huang, 1992).

Triacylglycerol Deposition In Developing Seeds

and Microspore Derived Cultures of Oilseed Rape

A number of studies have examined the accumulation of storage lipid (Appelqvist, 1972; Appelqvist, 1975; Fowler and Downey, 1970; Norton and Harris, 1975) and protein (Crouch and Sussex, 1981) in developing seeds of oilseed rape. For example Murphy and Cummins (1989) have examined the timing of both TAG and protein accumulation. For approximately 2 weeks after anthesis, embryos were shown to grow by cell division until they reached about 1 mm in length. During this time TAG accumulated, but at a very slow rate. At approximately 2 to 3 weeks after anthesis, cell division stopped and cell expansion began. The deposition of TAG occurred rapidly between 4 and 6 weeks post anthesis with most of the oil accumulating in oil-bodies, 2 to

4 μm in diameter. Storage protein synthesis begins at about 5 weeks after anthesis. Storage proteins such as napin and cruciferin accumulated at maximal rates between 5 and 7 weeks after anthesis followed by an accumulation of oleosin protein which occurred about 7 to 10 weeks after anthesis. The accumulation of oleosins was shown to coincide with a reduction in the average size of the oil body to 0.3 - 1 μm. From these results, Murphy and Cummins (1989) concluded that storage product accumulation was tightly regulated and shown to occur in four distinct phases: (i) a cell division phase, (ii) a phase during which storage oil accumulated rapidly, (iii) a phase dominated by polar protein synthesis and (iv) a phase where oleosin synthesis was predominant.

A number of important TAG biosynthetic enzymes have been shown to be very active during the rapid oil accumulation phase of seed development (Stymne and Stobart, 1987). In developing seeds of *B. napus,* ACCase (Turnham and Northcote, 1983), β-ketoacyl-ACP synthase (MacKintosh *et al.*, 1989), ACP (Hannapel and Ohlrogge, 1988) and enoyl-ACP reductase (Slabas *et al.*, 1986) have been shown to have maximum activity during the time of TAG accumulation. In the final dessication stage, however, the activity of these proteins has been found to be very low or undetectable. The rapid accumulation of lipids has been shown to be due to an increased production of TAG biosynthetic enzymes caused by an increased level of gene expression (Hannapel and Ohlrogge, 1988). Furthermore, in studies of the *B. napus* enoyl-ACP reductase, both transcript levels and protein levels were maximal during the rapid phase of TAG accumulation (Kater *et al.*, 1991). Thus, enoyl-ACP reductase appears to be regulated via gene expression.

Maturing seeds are not a very convenient source of tissue for the study of TAG biosynthesis because it is difficult to obtain sufficient quantities of seed at the correct stage of development. To facilitate the study of plant TAG biosynthesis, a number of plant culture systems have been developed (Murphy *et al.*, 1993; Weber *et al.*, 1992). Plant cells in culture generally contain low levels of both unusual fatty acids and TAGs, indicating that these cells are quite different from oil storage tissues (Weber *et al.*, 1992). Recent studies of both somatic and gametophytic embryos from oil crops, however, have revealed that embryo formation in these cultures parallels many aspects of embryogenesis, in developing seeds, such as storage lipid assembly and fatty acid elongation (Taylor *et al.*, 1991; Weber *et al.*, 1992). Microspore derived embryos of *B. napus* have been used quite extensively for biochemical studies of plant TAG biosynthesis (Taylor *et al.*, 1990; Pomeroy *et al.*, 1991). The utility of the MD embryo system is attributable to the ability to obtain specific developmental stages which possess high capacities for storage lipid biosynthesis and accumulation *in vitro* (Weber *et al.*, 1992). MD embryos have been shown to accumulate TAG in a similar manner to that of zygotic embryos (Taylor *et al.*, 1990; Pomeroy *et al.*, 1991). Furthermore, the TAG produced by MD embryos has been shown to be of similar fatty acid composition to that of zygotic embryos.

Cell suspension cultures developed from various oilseeds contain TAG (Radwan and Mangold, 1976), as examplified by the MD cell suspension culture of *B. napus* L. cv Jet Neuf, originally described by Orr *et al.*, (1986). Both MD embryos and MD cell suspension cultures have been used in the characterization, solubilization and partial

purification of TAG biosynthetic enzymes (Weselake etal., 1993; Taylor *et al.*, 1993; Little *et al.*, 1994; Kocsis *et al.*, 1996). Zygotic and MD embryos of *B. napus* go through a sequence of developmental stages as they accumulate TAG (Pomeroy *et al.*, 1991; Taylor and Weber, 1994), whereas, in the cell suspension system, the cells remain in a non-differentiated state (Orr *et al.*, 1986; Simmonds *et al.*, 1991).

Alterations in the sucrose concentration of plant cell cultures have a profound impact on lipid biosynthesis. The relationship between media sucrose concentration and lipid biosynthesis is not fully understood, but this effect has been reported in numerous studies involving cell cultures, MD and zygotic embryos. It has proven difficult, however to discern the relative contributions of sucrose as a carbon source versus sucrose as an osmoticum in influencing both lipid accumulation and fatty acid composition (Khuri and Moorby, 1995). In developing seeds of *B. napus* it has been shown that exogenous ABA treatment has an influence on the accumulation of transcripts and gene products of the major seed storage proteins (Finkelstein *et al.*, 1985), lipid profiles (Finkelstein and Somerville, 1989) and on the levels of oil-body associated proteins (Taylor *et al.*, 1990). Many of the responses stimulated by ABA are also affected by water stress or elevated osmotica (Finkelstein *et al.*, 1985; Finkelstein and Somerville, 1989). From studies of soybean embryos cultured in high osmotica, Bray and Beachy (1985) suggested that increased protein accumulation was due to the increased ABA content from the osmotic stress. Alternatively, Finkelstein and Crouch (1986) found that storage protein accumulation could be affected by osmoticum and ABA independent of

one another. The effects of osmoticum on TAG accumulation have been noted for several tissues, including somatic cultures of *Daucus carota* L. (Dutta and Appelqvist, 1989; Ross and Murphy, 1993), developing embryos of *Triticum aestivum* L. (Rodriguez-Sotres and Black, 1994), cultures of *Pimpinella anisum* L. (Radetzky and Langheinrich, 1994) and cultured somatic embryos of *Picea glauca* (Moench) Voss (Attree *et al.*, 1992). Sucrose concentration and high osmotic potential in growth media have also been shown to influence the fatty acid composition of plant cultures. Increasing the sucrose concentration in the media of asexual embryos of *Theobroma cacao* L. cultured *in vitro* resulted in a shift from mostly polyunsaturated fatty acids to saturated and monounsaturated fatty acids (Pence *et al.*, 1981). High osmoticum, produced by the addition of 0.69 M sorbitol, to the media of zygotic embryos of *Brassica napus* L. cv Nugget, resulted in a larger accumulation of eicosenoic acid (20:1) and erucic acid (22:1). Since ABA and osmoticum are a normal part of the developing seed it is possible that embryogenesis is coordinated through these effectors. In general, the effects of osmoticum in promoting increased TAG and protein accumulation were enhanced by the addition of ABA.

Modification Of Seed Oil Composition and Content

The fatty acid composition of a seed oil determines its physical and chemical properties and therefore its potential applications (Mancha *et al.*, 1994). Plant breeders have been modifying the lipid composition of oilseed crops for a number of years to produce improved oils for both human consumption and industrial applications.

Conventional plant breeding has brought about a number of beneficial changes to oilseeds, such as the development of canola quality rapeseed. Furthermore, plants such as oilseed rape have been shown to tolerate relatively large alterations in fatty acid composition without any obvious deleterious effects on growth and development of the plant. Despite advances made by conventional plant breeding, seeds of the major oil crops still have fatty acid compositions that are considered to be less than optimal to meet the requirements for industrial applications and the food industry.

Modification of oil quality in crop plants has been reviewed recently (Topfer *et al.*, 1995, Kishore and Somerville, 1993; Knauf, 1987). Presently, twelve fatty acids are of particular importance for food and non-food applications (Topfer *et al.*, 1995). The other thousand fatty acids found naturally and others not found naturally, may prove to be useful, but they are not yet available because they occur in non-crop plants or other organisms. A number of research projects have begun addressing the possibility of engineering crop plants to produce fatty acids which are not normally found in plants. Extensive work has been done attempting to improve the oil quality for industrial applications and for human consumption. There appears to be great potential for the use of vegetable oils to reduce the demand for non-renewable fossil fuels. The use of vegetable oils would be considerably better for the environment and could potentially be cheaper where extensive processing of petrochemical oils is required. As well, the production of homogeneous or near homogenous oils in plants would be of considerable value as the cost of processing a mixture of TAGs to separate out only those with a specific fatty acid composition is quite expensive and time consuming (Topfer *et al.*,

1995). If the oleic acid content of rapeseed could be increased to over 90 %, it would be more cost effective than using petroleum oils for the production of a number of commodities. This modification would also be beneficial in the production of margarine because there would be less cholesterogenic polyunsaturated fats present that partially hydrogenate to produce unhealthy trans-fatty acids. Nine carbon fatty acids are used as plasticizers and in the manufacture of polymers, fibers, films and adhesives (Henkel, 1994). Presently oleic acid is oxidized by ozone to produce two C_9 straight chain saturated dibasic acids (Topfer *et al.*, 1995). To simplify the production of C_9 fatty acids, work is presently underway to increase the oleic acid content of oilseed rape.

Mutant lines of oilseed rape have been developed that accumulate up to 79 % oleic acid. Attempts to increase the oleic acid content over 80 %, by conventional plant breeding, however, resulted in reduced cold tolerance (Miquel and Browse, 1994). This reduced cold tolerance was probably the result of a reduction in unsaturated fatty acids in the membranes of these plants. Hausemann *et al.* (1995) produced a transgenic line of oilseed rape that expressed an antisense transcript to the Δ12 desaturase to avoid the undesired agronomic traits produced by breeding for further increases in oleic acid content. The antisense repression in this line increased the oleic acid content of the TAG fraction from 62 % to 83 %. A further increase of oleic acid was obtained by crossing the transgenic line with the mutant line that had 79 % oleic acid in the TAG fraction to produce a line containing 88 % oleic acid in the TAG fraction (Hitz *et al.*, 1995).

Interest in LPAAT stems from its strong selectivity for particular fatty acids thereby dictating the fatty acid composition of TAG at the *sn*-2 position. In a recent

study, *sn*-2 acyltransferase from yeast was expressed in a rapeseed line (Zou *et al.*, 1997) with the hope of producing trierucin. The native *Brassica* LPAAT will not insert erucic acid at the *sn*-2 position of the TAG molecule (Bernerth and Frentzen, 1990; Cao et al., 1990). The expression of yeast *sn*-2 acyltransferase in the oilseed rape line resulted in a substantial increase in TAG accumulation. In transgenic lines, TAG accounted for 33 - 41.2 % of the dry weight of the seed as opposed to 34 % in the control line (Zou et al., 1997). The effect of expressing *sn*-2 acyltransferase in oilseed rape suggests that regulation of carbon flow through the Kennedy pathway is under a complex set of controls. This increased oil accumulation may suggest that the yeast *sn*-2 acyltransferase is under different control in this system and possibly not subject to the same regulation as the native enzyme was. It should be noted that these results are from a preliminary study in the greenhouse. It will be interesting to see if the plants maintain the increased oil accumulation in field trials.

Modification of DGAT activity in oilseed rape appears to hold promise for the future. Biochemical and molecular evidence suggests that this may also be a key enzyme to target for altering seed oil content. The activity of DGAT has been found to be closely correlated to the rate of TAG accumulation, but it was much lower than that of the other Kennedy pathway enzymes (Ichihara *et al.*, 1988; Ichihara and Noda, 1982). Furthermore, developing safflower seeds fed ^{14}C labeled acetate were found to accumulate a significant amount of labeled DAG during TAG accumulation (Ichihara *et al.*, 1988). From these results, it was proposed that the acylation of DAG was the rate limiting step in the production of TAG from G3P. Similar results were also obtained

from studies examining the lipid composition of oilseed rape during development (Perry and Harwood, 1993a, 1993b). The build up of DAG in oilseed rape was found to occur during the rapid accumulation of TAG when DGAT activity was maximal. Perry and Harwood (1993a; 1993b) also concluded that DGAT activity was limiting the production of TAG and that it may exert a flux control on the pathway. The relatively low activity of DGAT and the accumulation of DAG, both imply that overexpression of DGAT, in developing seeds, may lead to the production of seeds with increased levels of TAG.

Recently, DGAT was partially characterized in developing MD embryos of *B. napus* L. cv Topas (Weselake *et al.*, 1991; Taylor *et al.*, 1991; Taylor *et al.*, 1992; Weselake *et al.*, 1993). The specific activity of DGAT has been shown to vary as the MD embryo develops. The highest specific activities observed were found in the early torpedo to late cotyledonary stages when rapid TAG accumulation occurred. The greatest specific activity occurred in the mid cotyledonary stage where it was approximately three fold higher than that of the heart to early torpedo stages (Weselake *et al.*, 1993).

Recently, a mutant of *A. thaliana* was identified which had reduced DGAT activity. The mutant, named AS11, was produced by treatments with ethylmethanesulfonate. It displayed reduced accumulation of 20:1 and 18:1 fatty acids with an increased storage of 18:3 (Katavic *et al.*, 1995). Examination of the intermediates involved in TAG accumulation resulted in the detection of increased DAG accumulation to approximately 8 - 12 % of the total lipid, whereas in the wild type, DAG characteristically accumulated to only about 1 % or less. Analysis of the fatty acid profiles of AS11 indicated that the mutation affected DGAT activity and consequently

altered the plants ability to perform extraplastidic fatty acid modifications (Katavic *et al.*, 1995). The reduced DGAT activity in AS11 was associated with delayed seed development, reduced TAG content and an increased production of 18:3 fatty acids at the expense of very long chain fatty acids. It was not certain whether the mutation directly altered the gene for DGAT or whether the gene affected was a regulatory factor that controlled DGAT. The study, however, indicated that DGAT was very important for effective accumulation of TAG and seed development.

Molecular Approaches Used in the Identification

of Genes Involved in Lipid Metabolism

The development of effective strategies in seed oil modification is dependent on accessing the genes encoding the lipid biosynthetic enzymes. The identification of genes involved in lipid production will only be given brief mention here because the topic has been reviewed rather extensively in the past few years. For a more detailed examination of this topic, the reader is refered to: Mackenzie, (1995), Murphy *et al.* (1994), Ohlrogge (1994), Kinney (1994) and Gibson et al. (1994). Presently, there are a number of methods which can be used to successfully isolate genes from plants. The conventional method of isolating a gene is to purify a protein which corresponds to the desired enzymatic activity. Following purification, antibodies are raised against the protein and used to screen a cDNA expression library. The antibody should recognize the expressed protein and the encoding cDNA can then be cloned. This approach was successful for the isolation of a few genes involved in lipid metabolism such as: the plastidial GPAT

(Ishizaki *et al.*, 1988; Voelker *et al.*, 1992), the stearoyl-ACP desaturase (Shanklin and Somerville, 1991) and enoyl-ACP reductase (Kater *et al.*, 1991). Alternatively, once a protein is purified to homogeneity, it can be partially sequenced and from the sequence, a degenerate oligonucleotide probe can be designed. The probe can then be used to screen a gene library because its sequence will hybridize to cDNA clones containing an insert which encodes the protein of interest. This method has been used to isolate β-ketoacyl-ACP reductase from *B. napus* and *A. thaliana* (Slabas *et al.*, 1992) and for ACP from spinach (Scherer and Knauf, 1987). Oligonucleotide primers can also be designed, based on sequence, so that they may be used in a PCR reaction to generate a probe which can be used to screen a library. This approach has been used to isolate cDNAs encoding the stearoyl-ACP desaturase from safflower (Thompson *et al.*, 1991), β-ketoacyl-ACP synthase I from barley (Siggaard-Andersen *et al.*, 1991), acyl-ACP thioesterase from *Umbellularia* (Voelker *et al.*, 1992) and β-ketoacyl-ACP reductase from *Cuphea* (Klein *et al.*, 1992).

A number of lipid biosynthetic enzymes, including DGAT, are membrane bound and it has proven difficult to isolate them using conventional procedures (Little *et al.*, 1994; Eccleston and Harwood, 1995; Kocsis *et al.*, 1996). Subsequently, molecular genetic approaches for gene isolation have been developed. Gene isolation, without protein purification, can usually be performed rather quickly if the gene has been isolated from another organism. In this case, heterologous gene probes may be used to screen a cDNA library from the organism of interest. This approach was used to isolate an LPAAT clone from *Limnanthes douglasii* because cDNAs were already isolated from

LPAAT clone from *Limnanthes douglasii* because cDNAs were already isolated from maize and *Escherichia coli* (Brown *et al.*, 1995).

Gene tagging also allows isolation of genes, without prior protein purification. This procedure can be performed with either transposable elements or T-DNA (Walbot, 1992). These two procedures involve the incorporation of DNA of known sequence into the genome of an organism. When the DNA is inserted into the middle of a gene, it will be inactivated. With this technique it is easy to identify a inactivated gene as the DNA causing the inactivation is of known sequence and therefore easily identified.

Another method commonly used is called "chromosome walking" (Arondel *et al.*, 1992). With this method a large number of plant seeds are mutated by physical or chemical means until the desired gene is turned off. Following identification of a plant that is mutated in the desired gene, the mutation is mapped to identify the closest restriction fragment length polymorphism (RFLP) marker. The closest RFLP marker is then used to identify a genomic clone from a yeast artificial chromosome or bacterial artificial chromosome library. Following identification, a genomic clone can be transformed into the mutant plant and if the desired gene is encoded, the mutation will be complemented. Genomic clones are typically 120 - 600 kb in length so they must be subcloned before being used in complementation studies. The smaller subclone that complements the mutation can be sequenced to identify the gene of interest. This process is particularly useful for the analysis of *A. thaliana* because many mutants have been identified and a detailed RFLP map has already been worked out (Chang *et al.*, 1988; Nam *et al.*, 1989; Lemieux *et al.*, 1990). By using this method, a gene encoding a Δ15

fatty acid desaturase (FAD3) gene was isolated (Arondel *et al.*, 1992).

Microbial mutants have been used in situations where plant mutants for the gene of interest were not available. Typically one prepares a cDNA expression library using plant mRNA and a vector designed to work in the system having the mutant. A large number of mutants can be grown and transformed with cDNAs from the library. The cDNA that functionally complements the mutant can be isolated and examined. This procedure has been used to isolate cDNAs encoding putative LPAATs from maize (Brown *et al.*, 1994) *and Limnanthes douglasii* (Brown *et al.*, 1995).

Analysis of Gene Expression in Plants

Analysis of gene expression in tissues or cells differing in TAG accumulation may also be helpful in identification of genes encoding TAG biosynthetic enzymes. Upregulated transcripts that correlated with increased TAG could represent TAG biosynthetic enzymes. Current methods for analyzing gene expression in closely related systems usually involve differential hybridization, subtractive hybridization or two dimensional protein gels. These methods have been used for a number of years and have become routine techniques in many molecular biology labs. Therefore, they will only be given brief mention here. For the purpose of explaining these methods, reference will be made to induced and non-induced tissues. Differential hybridization involves the hybridization of labeled cDNA fragments from induced and non-induced tissues to a cDNA library from the induced tissues. Messages present in one tissue, but not the other, will be identified by the lack of signal from the cDNA clones that are unique to the tissue

of interest (Hedrick *et al.*, 1984). Subtractive hybridization (Lee *et al.*, 1991) involves the production of cDNA from an induced tissue, hybridization of the cDNA to mRNA from a control tissue followed by the removal of the double stranded products. When the double stranded products are removed, the remaining cDNA fragments are those unique to the induced tissue. Two dimensional protein gels involve the separation of proteins in one dimension based on their isoelectric point followed by separation in a second dimension by their subunit molecular weight (O'Farrell, 1975; Croy and Pardee, 1983). Differential and subtractive hybridization have been used to isolate a number of genes implicated in tumorigenesis (Steeg *et al.*, 1988). Differential hybridization has shown to give a two directional comparison of gene expression, but the sensitivity is not as high as that of subtractive hybridization. Differential hybridization was used to isolate a 21.2 kDa oleosin isoform from *A. thaliana* by comparison with a mutant (Zou *et al.*, 1996). Since oleosins are known to be expressed rather abundantly, this technique was ideal for this purpose. Subtractive hybridization has been shown to recover genes that are expressed at low levels, but with this technique genes are recovered incompletely and the comparison of expression is only unidirectional (Liang and Pardee, 1994; Lee *et al.*, 1991). Further complications with the hybridization techniques stem from the need for relatively large amounts of RNA and the laborious and time consuming nature of the procedures. Protein gels have proven valuable in the analysis of protein synthesis, but often difficulties arise due to proteins which are very close to one another and in recovering sufficient protein to sequence (Croy and Pardee, 1983).

More recently, the method of differential display (DD) of mRNA (Liang and

Pardee, 1992) has been frequently used for comparisons of gene expression patterns in different tissues. Prior to the advent of DD, differential hybridization was the only technique that could be used to identify genes active in either of two situations. With the development of DD, the identification of genes active in either of two or more situations as well as the identification of the total number of genes subject to up- or down-regulation between multiple groups of cells has become routine for many molecular biologists.

The DD procedure has been used for the identification of differentially expressed genes in a number of mammalian systems (Liang *et al.*, 1994; Nishio *et al.*, 1994; Zimmerman and Schultz, 1994), plant systems (Sharma and Davis, 1995; Wilkinson *et al.*, 1995; Suty *et al.*, 1996; Torelli *et al.*, 1996), fungal systems (Appleyard *et al.*, 1995) and in plant pathogen interactions (Benito *et al.*, 1996). The DD procedure involves PCR amplification of subpopulations of first strand cDNAs using a specific set of short oligonucleotide primers. A 3' primer, which consists of 11 or 12 deoxythymidine residues plus two additional 3' bases to provide specificity, is used for reverse transcription (RT) of mRNA subsets and also for subsequent PCR amplifications. The 5' primer is a decamer of arbitrarily-defined sequence that, in practice, yields approximately 50 - 100 products of between 50 and 600 nucleotides in length upon PCR amplification of first-strand cDNAs in conjunction with the 3' primer. These PCR amplified cDNA tags are adjacent to the poly (A) tail and represent particular mRNAs from each cell type being analyzed. Radiolabelled amplification products derived from the same 5' and 3' primers, but different tissues or treatments, are displayed side-by-side on a

polyacrylamide gel in order to identify tissue, developmental or treatment specific expression. Different combinations of 5' and 3' primers are used to generate a panel of PCR products for two or more RNA samples that can be compared to theoretically identify all expression differences between cells.

When DD was first introduced by Liang and Pardee (1992), the reverse transcription reactions were primed with one of twelve specific primers of the form $T_{12}VN$, where V can be A, G, or C and N can be any of the four possible nucleotides. This anchored primer divides the mRNA population into different fractions based on the nucleotide sequence flanking the 5' end of the poly (A) tail. Assuming that approximately 15,000 different genes are expressed in any given cell at one time then the use of these twelve different anchored 3' primers theoretically allows the division of the mRNA population into twelve different fractions of 1,250 different transcripts. It has also been suggested by Liang and Pardee (1992), that the anchored primer $T_{12}MN$ could be used, where M is a degenerate mixture of A, C and G and N is A, C, G, or T, as the terminal 3' base of the primer provides most of the specificity. Further work by Liang *et al.* (1994) found that the use of three one base anchored primers, $T_{12}A$, $T_{12}C$ and $T_{12}G$ could be used to eliminate degenerate annealing of the specific two base anchored primers. Use of a degenerate primer set or a single base primer set reduces the number of cDNA fractions to be analyzed because, theoretically, each primer should sample 3,750 or 5,000 different mRNAs respectively. Bertioli *et al.*, (1995) have shown, however, that using a degenerate primer or one base anchored primer does not reduce the number of PCR reactions required to display most cDNA species present in the pool.

The degenerate and one base anchored primers reduce the chance of identifying any cDNA species that is present in a given PCR reaction. In studies with tomato, it was shown that using the one base anchored primer set generated a template that was too complex for examination (Benito *et al.*, 1996). The complex template from DD performed on tomato plants was much simpler when the twelve specific primers were used. This suggests that reducing the template complexity increases the sensitivity of this technique by making it easier to distinguish different cDNAs. Furthermore, reducing the template complexity also has the benefit of reducing the probability of amplification of two different but similar length cDNA fragments, thereby increasing the resolving power of DD.

It has recently been shown that the DD technique is much more sensitive than the hybridization techniques because often cDNAs do not produce a detectable signal on blots and some that produce signals do not appear to be differentially expressed. It was reported by Bauer *et al.* (1994), that upon the isolation of 50 differentially expressed cDNA fragments from the DD gel, only 5 were found to be differentially expressed by northern blot hybridization. In another study, Warthoe *et al.* (1995), detected 11 differentially regulated cDNAs by DD, of which 8 were found to produce a detectable signal on northern blots. Five of the 8 were differentially expressed. When the more sensitive technique of nuclear runoff transcription was performed, 10 of the 11 were found to be differentially expressed. These results further demonstrate that the DD technique is very sensitive for the detection of differential gene expression. Complementary DNA banding patterns have been found to be > 90 % reproducible

(Liang and Pardee, 1992) while others such as Bauer *et al.* 1994 have reported as low as 60 %.

The high sensitivity and specificity of DD can be attributed to the high specificity and amplification power of PCR, the high resolution of polyacrylamide gel electrophoresis and the ease of detection of radiolabelled nucleotides. The PCR reaction used in DD gives the technique its high sensitivity levels but it also potentially enables the production of artifacts. Artifacts may be generated when cDNA fragments are amplified by either the decamer or the oligo(dT) primer alone or possibly even amplification independent of the primers added to the PCR reaction. Artifacts are eliminated when the fragments are sequenced or when their expression levels are verified by northern blot hybridization.

The evaluation of quantitative differences between cDNAs from two different tissues has also been reported to be one of the major difficulties with DD. In principle, the difference between both strong versus weak, or present versus absent bands, needs to be confirmed. It has been reported by Warthoe *et al.* (1995), that cDNAs displaying differential expression in two separate DD repetitions have a 90 % probability of being differentially expressed in further repetitions. Further analysis has shown that cDNAs found to be differentially regulated by DD for three repetitions were always confirmed by northern blot analysis or nuclear runoff transcription (Warthoe *et al.*, 1995).

MATERIALS AND METHODS

Plant Material

The microspore derived cell suspension culture of winter oilseed rape (*B. napus* L. cv Jet Neuf) was maintained according to Orr *et al.*, (1986) except that cells were grown with sucrose concentrations of 2, 6 and 14 %. The cells were grown for two weeks, rinsed with distilled water over a sterile nylon sieve, gently squeezed in the nylon sieve to remove water, weighed, wrapped in aluminum foil, frozen in liquid nitrogen and stored at -80^{o}C until needed.

Diacylglycerol Acyltransferase Extraction, Assay and Protein Determination

Fresh cells were macerated with acid washed silica in 4 volumes of ice-cold grinding medium per g of cells using a cold mortar and pestle. The homogenate was filtered through a 60 μm nylon mesh. In preparing extract for measurement of DGAT activity, the grinding medium was 0.2 M Hepes-NaOH buffer (pH 7.4) containing 0.5 M sucrose. The resulting homogenate was centrifuged at 10 000 g for 20 minutes and the supernatant was centrifuged at 100 000 g for 1 hour to obtain a microsomal fraction (10 000 - 100 000 g sediment). Microsomes were usually washed once and resuspended in grinding medium to a volume equal to one-tenth of the tissue weight.

Microsomal DGAT was assayed at 30^{o}C for 10 minutes as described by Little *et al.*, (1994) using 60 μL reaction mixture consisting of 0.2 M Hepes-NaOH (pH 7.4), 330 μM *sn*-1,2-diolein, 15 μM [1-^{14}C] oleoyl-CoA (54 Ci/mole), 0.1 % (w/v) Tween-20,

5 mg BSA/mL and 10 μL tissue homogenate. The reaction was terminated by the addition of 10 μL of 5 % (w/v) sodium dodecyl sulfate. Fifty microliters of the reaction mixture was spotted onto a silica gel H (0.5 mm) plate, dried and resolved by thin layer chromatography according to Weselake *et al.*, (1991). Silica gel sections containing radiolabelled TAG were transferred to scintillation vials and combined with 5 mL scintillant for determination of radioactivity.

The protein content of the plant tissue extracts was determined using the Bio-Rad protein microassay based on the Bradford (1976) procedure, using BSA as a standard.

RNA Isolation

Total RNA was isolated using the TRIzol™ reagent with the provided protocol (Canadian Life Technologies, Burlington, Canada). Following RNA isolation, samples were treated with DNAse I (amplification grade) (Canadian Life Technologies) to remove any potentially contaminating DNA, extracted with an equal volume of phenol : chloroform : isoamyl alcohol (25:24:1, v/v/v), followed by two extractions with an equal volume of chloroform : isoamyl alcohol (24:1, v/v), ethanol precipitated, dried and redissolved in diethylpyrocarbonate treated water. A Beckman DU-65 spectrophotometer was used to quantify and assess the purity of the RNA samples using absorbances at 260 and 280 nm where 1 unit of absorbance at 260 nm was equal to 40 μg/ml. The integrity of the total RNA was further assessed by both ethidium bromide stained denaturing agarose gel electrophoresis and northern blotting with a ribosomal RNA probe. The poly(A)+ RNA was purified using the poly A Track kit (Promega), following the protocol provided and quantified as above.

Triacylglycerol Isolation and Fatty Acid Analysis

Lipids were extracted from 1 g samples of cells by the hexane/isopropanol procedure of Hara and Radin (1978). The lipids were dissolved in hexane at a concentration of 10 mg/mL. For analysis of TL, 1 mg of an internal standard, pentadecanoic acid, was added to 250 µL of hexane-dissolved lipid. Hexane was removed using N_2 gas and 5 mL of methanolic-HCl, prepared by the method of Christie (1992), were added. Methylation was allowed to proceed at 50°C for 24 hours. Fatty acid methyl esters (FAMES) were extracted with two portions (5 mL each) of hexane. For analysis of TAG, 250 µL of hexane dissolved lipid (10 mg/mL) was spotted in a narrow band on HPTLC-Fertigplatten Kieselgel 60 plates. Triolein standard was applied in a separate lane. The plates were developed with hexane:diethyl ether:acetic acid (40:10:1). After air drying, the triolein standard was located with iodine vapor and the TAG portions of the sample lanes were scraped into screw cap tubes. One milligram of pentadecanoic acid was added to the TAG solution as an internal standard. Methylation was conducted in the presence of silica according to Browse *et al.* (1986) but with use of 5 mL methanolic-HCl as described above. FAMES representing both TL and TAG were analyzed by gas liquid chromatography using a Varian 3400 GC with a 30-m DB-Wax Megabore column (Chromatographic Specialties Inc., Brockville, Ontario, Canada) according to the method of Pomeroy *et al.*, (1991). The percent area of the peaks and the known concentration of internal standard were used to quantify the proportions of fatty acids present in the TAGs.

Differential Display of mRNA

Differential display (DD) of mRNA was performed according to Liang and Pardee, (1994) (Appendix 2) with a few alterations. The primer 5'-T_{12}AA-3' was used for reverse transcription (RT) and MoMuLV reverse transcriptase was substituted by SuperScript II (Canadian Life Technologies) using the buffer provided with that enzyme. Ten units of placental ribonuclease inhibitor (Canadian Life Technologies) was also added to the reverse transcription cocktail. The same primer previously used for the RT and arbitrarily chosen decamers (Operon Technologies Inc.) were employed for the PCR amplification of the cDNA. The radiolabelled ^{35}S nucleotide dATP was also substituted by 2 μCi of [α-^{32}P]dCTP to a final concentration of 0.033 μM. The PCR amplified cDNA fragments were separated on a denaturing 5.5 % polyacrylamide gel (Long Ranger) in a Tris-borate-EDTA buffer (pH 8.0). The gel was blotted onto Whatman 3MM paper, covered with Saran wrap, placed in a cassette, covered with a X-OMAT™ AR Scientific imaging film (Kodak) and exposed for 1 - 48 hrs depending on the intensity of the labeled fragments. Following identification and recovery of the differentially displayed cDNAs, the isolated DNA was reamplified by PCR, using the same primers as in the DD reaction only with a 10 fold increase in the nucleotide concentration, to verify that the isolation was successful and to size the isolated cDNA.

Recovery and Identification of cDNAs

Once the extracted cDNAs were reamplified and the correct size fragment was verified to be the approximate size of the ones in the DD gel, the remaining half of the

cDNA was cloned using a pGEM T vector system (Promega) following the instructions provided. Clones were transformed into maximum efficiency DH5α competent cells (Canadian Life Technologies) and plated out on LB Agar plates containing 100 μg/ml of ampicillin and 50 μl of 2 % X-gal spread on the surface. To detect the positive colonies containing an insert of the appropriate size, colony PCR was performed (Appendix 3). At least three clones containing an insert of the appropriate size, for each upregulated cDNA fragment were sequenced.

DNA Sequencing and Analysis

Selected clones were sequenced once in each direction using the ABI PRISM™ Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin Elmer) (Appendix 4). Reactions were separated using the ABI 373A automated sequencer and results were analyzed using the Seq Ed™ 675 Sequence Editor Version 1.0.3 (ABI).

RNA Gel Blot analysis and RT-PCR

Northern blots were performed using either the formamide hybridization buffer or the aqueous buffer methods of Brown and Mackey (1997) with similar results from each. Total RNA, (using 20 - 40 μg/lane) or mRNA (using 1 μg/lane) was separated by electrophoresis, vacuum transferred using a VR-25 vacuum transfer system with a VR40 vacuum regulator (Tyler Research Instruments), to maximum strength Nytran membranes, immobilized by UV irradiation and stained with methylene blue to verify transfer and equal loads. The load of RNA and its integrity was further assessed by

probing with a fragment of the nuclear encoded *B. napus* mitochondrial elongation factor -TU (Appendix 6). Membranes were maintained in a sealed plastic bag with a small amount of 0.2 x SSC and 0.1 % SDS to prevent them from drying out. The membranes were stripped by two consecutive twenty minute incubations in a solution of 0.5 % SDS and 0.1 x SSC at 90°C. Reverse transcription was performed on total RNA isolated from two separate cultures grown in the presence of 2, 6 and 14 % sucrose. Reverse transcription was monitored by the incorporation of [α-^{32}P]dCTP into each reaction to verify equal efficiency (Appendix 1). One microlitre of the first strand cDNA was then subjected to PCR amplification (Appendix 7) using oligonucleotide primers, specific to each DD fragment, designed using OLIGO™ version 4.0 (National Biosciences). The PCR products were then separated on a composite 3 % agarose gel consisting of 2 % agarose and 1 % MetaPhor agarose (FMC). Following separation the DNA was stained with ethidium bromide, placed on a 2040 EV transilluminator (Stratagene) and photographed with a SpeedLight Gel Documentation System (Lightools Research). Images were stored on 3.5 inch floppy disks, cropped and printed using Photoshop version 2.5 (Adobe) and a Laser Jet 5P (Hewlett Packard).

Oleosin Expression Monitoring

Reverse transcription was performed as in the RT-PCR section except that the anchored primer $T_{12}AA$ was substituted with T_{15} and the first strand cDNA was diluted eight fold instead of two fold. RT efficiency was monitored (Appendix 1) and PCR was performed using the forward (5'-GACCAGTATTCTATGATCGG-3') and reverse

(5'-AATTCTAAACACCTTATTGC-3') primers designed, using OLIGO™ 4.0 (National Biosciences), to amplify a 579 bp fragment from base pair 28 - 607 of the *B. napus* major oleosin (GenBank accession #X58000). The PCR amplification was performed for 30 cycles. The amplified cDNA fragment was separated out on a 1.5 % agarose gel, stained and visualized as described in the RT-PCR section.

Hybridization Probes

Following PCR amplification, specific DNA fragments were gel purified, ethanol precipitated and redissolved in water. Specific fragments were labeled using a PCR reaction that included 3 μM of dATP, dGTP and dTTP and 0.33 μM [α-^{32}P]dCTP for 50 μCi from a stock of 250 mmol/Ci, 0.26 μM of the particular forward and reverse oligonucleotide primers and 1 μl of the gel purified fragment as template and in a volume of 50 μl. This mix was overlaid with approximately 100 μl of light mineral oil and subjected to 35 cycles of 94°C-1 min, 50-55°C-1 min (depending on the particular fragment and primers used), 72°C-1 min. After amplification the reaction was run through a 1 ml disposable syringe containing Sephadex G-50 (Pharmacia) to remove unincorporated nucleotides. The probe was denatured by the addition 25 μl of 1 M NaOH and heated to 70°C for 2 minutes, neutralized by the addition of 25 μl of 1 M HCl and added directly to 10 ml of hybridization buffer.

5' Controlled Ribonucleotide Tailing of cDNA Ends

Controlled ribonucleotide tailing of cDNA ends was performed according to Schmidt and Mueller, (1996) with a few alterations (Appendix 8). One μg of mRNA from the cell suspension culture of *Brassica napus* L. cv Jet Neuf was reverse transcribed using the primer 5'-T_{12}AA-3' with SuperScript II reverse transcriptase (Canadian Life Technologies) according to the protocol provided. The cDNA was ligated to the adaptor (5'-CTCTTGCTTGAATTCGGACTA-3' phosphorylated at the 5' end and then hybridized to 5'-TAGTCCGAATTCAAGCAAGAGCC-3') and double stranded by placing the cDNA in a PCR reaction and heating to 72°C for 40 minutes. PCR amplification was then performed between the forward adaptor specific primer (5'-TAGTCCGAATTCAAGCAAGAGCC-3') (A1) and the reverse oligonucleotide primers designed to the selected cDNAs. PCR amplification for the CRTC reactions were performed using 2 μl of double stranded cDNA diluted 50, 100, 200, 500, 1000, 2000, 5000 and 10,000 fold. Two μl of each of the first PCRs were diluted fifty fold and then 2 μl of each dilution were added to a second PCR reaction which utilized the same conditions as the first. PCR amplifications were separated on a 1 % agarose and observed as described for the RT-PCR reactions. Amplified cDNAs were extracted from the gel by stabbing a 20 μl pipette tip into the band and transferring the piece of agarose containing the fragment of interest into 100 μl of H_2O. The extracted cDNAs were reamplified in three separate PCR reactions using either the A1 primer, and the gene specific primer alone and both primers. cDNAs that were amplified with the gene specific primer or both primers were cloned into a pGEM T vector using the protocol provided. Cloned cDNAs

were sequenced as described in the sequencing section above.

In an attempt to increase the specificity of the CRTC reaction, the first PCR reaction was performed with only the reverse, gene specific, primer. Further alterations were also made to increase specificity such as increasing the annealing temperature, performing a hot start PCR where the amplification tubes were placed on the thermocycler only after the block had exceeded 80°C and the first three nucleotides were removed from the 5' end of the A1 primer which effectively lowers the melting temperature by 10°C. Because these changes did little to enhance the specificity of the amplification, the CRTC procedure was repeated for each fragment with the substitution of specific reverse oligonucleotide primers for the 5'-T_{12}AA-3' primer. This modification however did not improve the specificity of the amplification.

GenBank Homology Searches

The cDNA fragments BSS1A, BSS1AL1, BSS2C, BSS4A, BSS5B, BSS7B, BSS8A, BSS11A, BSS11AL1 and BSS13A were subjected to homology searches for the last time in GenBank Build 14:27:18 APR 1 1996. This particular build of GenBank actually occurred on March 21, 1997. Each sequence was compared to previously identified sequences using both BLASTN and BLASTX programs from the BLAST network services at the National Center for Biotechnology Information (Gish and States, 1993; Altschul *et al.*, 1990). The BLASTN program compared sequences to the non-redundant nucleotide sequence database which includes the April 1995 release of the Brookhaven Protein Data Bank, release 91 of GenBank (R), the October 15, 1995,

GenBank (R) database having cumulative daily updates to the major release, the June 1994 EMBL Data Library, Release 43.0 and the cumulative daily updates to the EMBL Data Library. The parameters used for the BLASTN homology were the defaults set in the system: V=100, B=50, H=1, -qtype, -ctxfactor=2.00 and E=10. These sequences were also compared to others reported in the database of expressed sequence tags (cumulative daily update) using the BLASTN program and the same parameters as the previous BLASTN search. The BLASTX program was used to compare a six frame translation of each sequence to the non-redundant peptide sequence databases which includes the April 1995 Release of the Brookhaven Protein Data Bank (pdb), the December 1995 SWISS-PRODDED Release 32.0, (swissprot), the June 30, 1995 PIR Release 45.0, (pir), the cumulative weekly update to the major release of SWISS-PRODDED (spupdate), the October 15, 1995 CDS translations from GenBank (R) release 91, (genpept) and the cumulative daily updates to the major release of genpept (gpupdate). The parameters for the BLASTX homology search were: V=100, B=50, H=1, -qtype, -ctxfactor=5.95 and E=10.

RESULTS

Effects of Increasing Concentrations of Sucrose in the Growth Media on Oil Accumulation in the Cell Suspension Culture

TAG content and DGAT activity were altered when the MD cell suspension culture of *B. napus* L. cv Jet Neuf was grown in media containing different sucrose concentrations as a carbon source. Increasing media sucrose concentration from 2 - 14 % (w/v) resulted in a DGAT total activity increase of approximately 6 fold while the specific activity of DGAT increased by approximately 3 fold (Table 1). TAG production expressed on a fresh weight (FW) basis increased three fold in the culture and total lipid (TL) production doubled in cultures grown in 14 % sucrose when compared to those grown in 2 % sucrose (Table 1).

The fatty acid content of TAG and the TL fraction were monitored to determine if major changes were occurring as a result of the elevated media sucrose concentration. Fatty acid profiles showed increases in the amount of 16:0, 16:1, 18:3 and 24:1 in the total lipid (TL) fraction while 16:0, 18:3, and 24:1 increased in the TAG fraction as sucrose was increased from 2 % to 6 % to 14 % (Table 2). The fatty acids 20:0, 22:0, and 24:0 in the TL fraction and 18:1 and 24:0 in the TAG fraction decreased when the sucrose was increased. The fatty acid 18:0 and 18:1 in the TL fraction and 18:0 and 20:0 in the TAG fraction were maximal in the 6 % sucrose cultures. Linoleic acid (18:2) in the TL and TAG fractions was minimal in the 6 % sucrose cultures at higher amounts in the 2 % cultures and at much higher levels in the 14 % sucrose culture. The fatty acids 16:1, 20:1 and 22:0 remained unchanged as the media sucrose concentration was increased from 2 % to 6 % sucrose but in the 14 % cultures 20:1 and 22:0 decreased while 16:1 increased.

Table 1. Characteristics of the cell suspension culture of *Brassica napus* L cv Jet Neuf used in the study on gene expression. The DGAT activity assays and the TAG and the TL analyses were performed as described in **MATERIALS AND METHODS**. The results of the TAG and TL analysis are the average of four cultures while the DGAT assays were performed on the cells which were used for gene expression analyses.

Sucrose Concentration (w/v)	DGAT Total Activity (pmol TAG/ min/gFW)	DGAT Specific Activity (pmol TAG/ min/mg protein)	Average of Four Cultures % TAG/FW	TL/FW
2%	25.4	12.2	0.3	0.8
6%	99.0	35.7	0.8	1.2
14%	151.7	31.5	0.9	1.5

Table 2. The fatty acid composition expressed as a percentage of the entire triacylglycerol (TAG) or total lipid (TL) fraction in the microspore derived cell suspension culture of *Brassica napus* L cv Jet Neuf cultured in 2, 6, and 14% (w/v) sucrose. Isolation of the TAG fraction, methylation of TL and TAG and separation of FAMES by GLC were performed as described in Materials & Methods. Lipid preparations from two independent cultures were analyzed by GLC in duplicate and the values were averaged.

Sucrose conc. (w/v)	Lipid Class	Fatty Acid Composition, Wt %										
		16:0	16:1	18:0	18:1	18:2	18:3	20:0	20:1	22:0	24:0	24:1
2%	TL	10.8	2.4	3.4	41.6	10.3	7.0	5.6	0.6	13.0	4.8	0.8
	TAG	13.7	3.3	3.7	56.6	7.2	4.1	3.2	0.8	3.2	2.9	1.6
6%	TL	12.9	2.8	4.4	51.5	7.6	7.3	4.1	tr	5.7	2.4	1.0
	TAG	13.9	3.3	4.8	55.7	5.5	5.2	3.5	0.8	3.2	2.0	1.9
14%	TL	13.8	2.9	3.7	44.1	13.6	9.0	3.4	tr	5.4	2.2	1.5
	TAG	14.5	3.6	3.7	48.8	12.4	7.2	2.5	tr	2.5	1.5	2.4

tr, Trace, <0.5%

The fatty acid 20:1 in the TL fraction was maximal in the 2 % sucrose cultures and in 6 and 14 % cultures it was present at similar amounts.

Reverse transcription-PCR analysis showed that the expression of the mRNA encoding the *Brassica napus* major oleosin protein (GenBank accession #X58000) was enhanced by increasing media sucrose from 2 % to 6 % to 14 % (Figure 2), when compared to the expression of the mitochondrial elongation factor - TU (EF-TU) (Figure 3), as a control. These results obtained from the cell suspension culture grown in the presence of increasing sucrose concentration show that higher DGAT activity coupled with the enhanced accumulation of TAG and oil bodies represent a biological system in which storage lipid biosynthesis is induced. We decided to use this system to identify upregulated mRNAs because some would likely be involved in the process of lipid biosynthesis.

Figure 2. Reverse Transcription - PCR of mRNAs encoding the major oleosin (nap II) protein of *Brassica napus* (GenBank accession # X58000) using RNA extracted from cells grown in 2, 6 and 14 % (w/v) sucrose. RT reactions were performed on two separate RNA preparations for each of the three different cultures. Lanes 1 and 2 are from cells cultured in 2 % sucrose, lanes 3 and 4 are from cells cultured in 6 % sucrose and lanes 5 and 6 are from cells cultured in 14 % sucrose. Lane seven represents a 100 bp ladder. The forward (5'-GACCAGTATTCTATGATCGG-3') and reverse (5'-AATTCTAAACACCTTATTGC-3') primers amplified a 579 bp fragment from base pair 28 to 607 using 30 cycles.

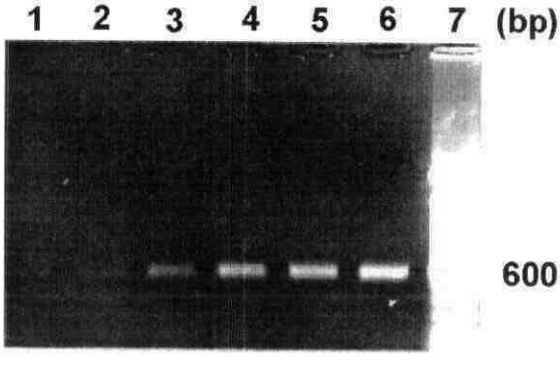
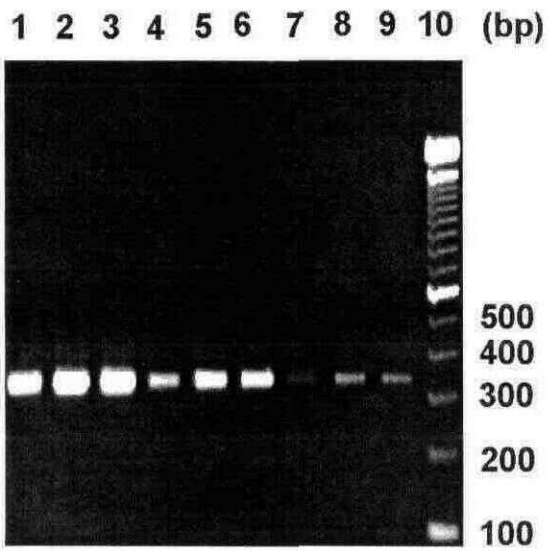

1
2
3
4
5
6
7
(bp)
600

Figure 3. Reverse Transcription - PCR analysis of mRNAs encoding the mitochondrial elongation factor - TU (MTEF-TU) using RNA extracted from cells grown in 2, 6 and 14 % (w/v) sucrose. RT was performed on total RNA from each of the three different cultures. Lanes 1, 4 and 7 are from cells cultured in 2 % sucrose, lanes 2, 5 and 8 are from cells cultured in 6 % sucrose and lanes 3, 6 and 9 are from cells cultured in 14 % sucrose. Lane ten represents a 100 bp ladder. Lanes 1, 2 and 3 represent PCR reactions of 29 cycles, lanes 4, 5 and 6 represent PCR reactions of 27 cycles and lanes 7, 8 and 9 represent PCR reactions of 25 cycles. The PCR reaction employed the forward (5'-GTAGGACTTCTTCTGCGTGG-3') and the reverse (5'-AACTGTTCTACCTCCTTCCC-3') primers to amplify a 330 bp fragment from nucleotide 1063 to 1392. These primers were designed from the sequence of the *Arabidopsis thaliana* sequence for the nuclear encoded mitochondrial elongation factor - TU (GenBank accession #T04276).


1 2 3 4 5 6 7 8 9 10 (bp)
500
400
300
200
100

Identification of cDNAs Representing Upregulated mRNAs

Screening the mRNA populations isolated from each of the 2, 6 and 14 % sucrose cultures with twenty-two arbitrarily chosen decamers (Figure 4) in combination with the anchored primer 5'-T_{12}AA-3' revealed that many transcripts displayed similar expression in the cell suspension culture grown under the three different sucrose concentrations. The similar signal strength of the transcripts suggests that gene expression in these cells is not affected for the most part by the increasing amount of sucrose in the growth media (data not shown). Complementary DNA corresponding to mRNAs that increased, decreased or were unaffected, as sucrose concentration was increased in the growth media, were observed after amplification with the primers OPA1, OPE3 OPA4 and OPA6 in combination with 5'-T_{12}AA-3'. Other cDNA corresponding to mRNAs that had higher levels of expression in the 2 and 14 % cultures, or high in the 6 % cultures but lower in the others were also observed. Some cDNA fragments were observed that appeared to be only present in one of the 2, 6 or 14 % cultures respectively. Some amplified cDNAs from a given culture produced slightly different signal intensities in different PCR reactions using cDNA from different RNA preparations and different RT reactions originating from the same cell source.

The cDNA fragments of interest in this study were the ones which exhibited enhanced expression in the cultures as the sucrose concentration was increased. Thirteen cDNA fragments, *BSS*1-13 (*Brassica napus* sucrose stimulated), were observed to have increased abundance in the cultures grown in the presence of 6 and 14 % sucrose when compared to their abundance in the 2 % sucrose cultures. Most of the thirteen cDNA

Figure 4. Primers used for differential display of mRNA in combination with the anchored primer 5'-T_{12}AA-3'. Primers 1 - 18 were from primer kits A,C,D,E and N from Operon Technologies Inc. These primers have been designed to have ≥60 % G+C and no significant self-complementarity. Primers #19 - 22 were designed by Bauer *et al.*, (1993) to have 50 % G+C content and no uninterrupted self-complementarity of more than two bases. The anchored primer was used to prime the reverse transcription, and then fragments were amplified by PCR using the anchored primer in combination with a decamer at an annealing temperature of 40°C (Appendix 2).

Decamers

1.	OPA1	5'-CAGGCCCTTC-3'	12.	OPD5	5'-TGAGCGGACA-3'
2.	OPA2	5'-TGCCGAGCTG-3'	13.	OPD10	5'-GGTCTACACC-3'
3.	OPA4	5'-AATCGGGCTG-3'	14.	OPE3	5'-CCAGATGCAC-3'
4.	OPA5	5'-AGGGGTCTTG-3'	15.	OPE4	5'-GTGACATGCC-3'
5.	OPA6	5'-GGTCCCTGAC-3'	16.	OPE13	5'-CCCGATTCGG-3'
6.	OPC15	5'-GACGGATCAG-3'	17.	OPE14	5'-TGCGGCTGAG-3'
7.	OPC17	5'-TTCCCCCCAG-3'	18.	OPN16	5'-AAGCGACCTG-3'
8.	OPC18	5'-TGAGTGGGTG-3'	19.	Decamer1	5'-TACAACGAGG-3'
9.	OPC19	5'-GTTGCCAGCC-3'	20.	Decamer2	5'-TGGATTGGTC-3'
10.	OPC20	5'-ACTTCGCCAC-3'	21.	Decamer3	5'-CTTTCTACCC-3'
11.	OPD2	5'-GGACCCAACC-3'	22.	Decamer4	5'-TTTTGGCTCC-3'

fragments exhibited different signal intensities and different degrees of upregulation as the sucrose concentration in the growth media was increased. Assuming roughly equal amplification efficiency for all fragments, then the signal intensity would reflect the different expression levels of the corresponding mRNAs.

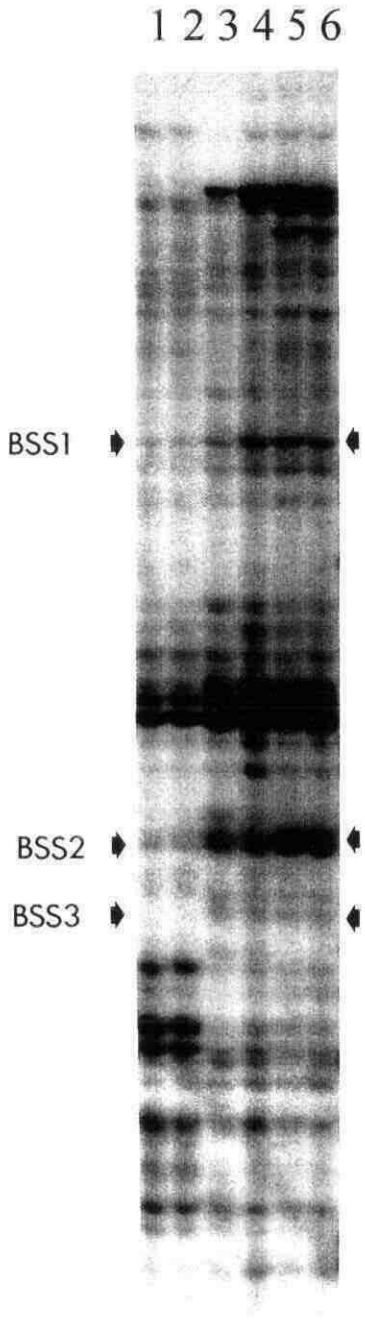
It is possible that the expression levels of TAG biosynthetic enzymes are relatively low since they do not represent major cellular proteins, suggesting that cDNAs displaying weak signal strength and upregulation could represent sequences of interest in this study. However since little is known about the expression of TAG biosynthetic enzymes and since this is a preliminary study of gene expression, in this sucrose stimulated cell suspension culture, the selection of upregulated cDNA fragments was not restricted by their degree of upregulation or by their signal strength. Some isolated cDNAs were not detectable in the 2 % sucrose cultures but were upregulated to varying degrees in the 6 % and 14 % cultures producing signal strengths anywhere from just above the detectable limit to being so intense that the autoradiographic film was overexposed. Other cDNAs displayed moderately strong signal strength in the 2 % cultures with only slight upregulation in the 6 % and 14 % cultures.

The relative signal strength of a cDNA fragment was established by comparing the intensities of all the cDNA fragments during one exposure period. Some cDNAs had signal intensities that were below the limits of detection on the first exposure. Multiple exposure times for three independent DD-PCR reactions were used to further examine the weaker signals.

PCR amplification employing the decamer OPA1 yielded three upregulated

cDNA fragments (BSS1, BSS2 and BSS3) (Figure 5). The first fragment, BSS1, was approximately 300 nucleotides in length. The intensity of BSS1 was weak when cells were grown in the presence of 2 % sucrose, while the intensity of the fragment was much stronger in the 6 % sucrose culture and the cDNA was present in slightly larger amounts in the 14 % sucrose cultures. Duplicate amplifications using cDNA from different RNA preparations of the same culture produced some variability in the signal strength from the 6 % culture but the signal from this culture was always stronger than that from the 2 % culture and weaker or similar to that of the 14 % culture. The signal intensity of BSS1 in the 2 % cultures suggested that it was expressed at quite low levels since the cDNA was almost at the lower limit of detection whereas in the 6 % and 14 % sucrose cultures the cDNA displayed signal intensity that suggested that it was expressed at a moderate level. The cDNA BSS2 was approximately 220 nucleotides in length (Figure 5). The signal strength of BSS2 was very weak in the 2 % sucrose cultures. In the 6 % sucrose culture, however, the signal strength of BSS2 was strongly upregulated producing one of the most intense signals observed. The signal strength was further upregulated in the 14 % sucrose culture. Unlike BSS1 the signal strength for BSS2 was uniform within each of the two different RNA preparations. The signal strength observed for BSS2 suggested that the corresponding mRNA was expressed at relatively low levels in the 2 % sucrose cultures but in the 6 % and 14 % cultures the expression was greatly enhanced. The fragment BSS3 was approximately 190 nucleotides in length (Figure 5). The signal strength of BSS3 in the 2 % sucrose culture was below the limits of detection but in the 6 % and 14 % sucrose cultures the signal strength was slightly stronger. The low level of signal

Figure 5. Differential display of mRNA (via PCR) from MD cell suspension cultures grown in sucrose concentrations of 2, 6 and 14 % (w/v). Lanes 1 and 2 are from cells grown in 2 % sucrose, lanes 3 and 4 are from cells grown in 6 % sucrose and lanes 5 and 6 are from cells grown in 14 % sucrose. All DNA fragments were the result of using the specific anchored primer 5'-T_{12}AA-3' in combination with the primer OPA1 (5'-CAGGCCCTTC-3'). RNA isolation, reverse transcription, PCR and electrophoresis were as described in the **MATERIALS AND METHODS** section. Autoradiography was conducted for 2 hours.


1 2 3 4 5 6
BSS1
BSS2
BSS3

strength for BSS3 suggested that the corresponding mRNA was expressed at relatively low levels when compared to the other fragments observed.

PCR amplification employing the decamer OPE3 yielded one upregulated cDNA fragment, BSS4, of approximately 150 nucleotides in length (data not shown). The fragment BSS4 was present in relatively large amounts in the 2 % culture, when compared to BSS1, BSS2 and BSS3 and displayed only minor upregulation in the 6 % and 14 % sucrose cultures. Based on signal strength it appeared that BSS4 is moderately expressed in the 2 %, 6 % and 14 % sucrose cultures and displayed relatively minor upregulation as media sucrose concentration was increased.

PCR amplification employing the decamer OPA4 yielded six upregulated cDNAs BSS5, BSS6, BSS7, BSS11, BSS12 and BSS13 (data not shown). The fragment BSS5 was approximately 170 nucleotides in length. The signal strength for BSS5 was similar to BSS3 in that it was below the limit of detection in the 2 % culture, stronger but just at the lower limits of detection in the 6 % culture and slightly stronger in the 14 % culture. Based on signal intensity, the expression of BSS5 was relatively low and possibly represented a weakly upregulated mRNA as the media sucrose concentration was increased. The cDNA fragment BSS6 was approximately 330 nucleotides in length. Although the signal intensity for BSS6 was barely detectable in the 2 % sucrose cultures, in the 6 % culture the signal strength was so intense that the X-ray film was overexposed (data not shown). In the 14 % culture the signal was even stronger as it overexposed a larger region of the film (data not shown). The intense upregulation of this cDNA fragment suggested that it encodes a mRNA which exhibited strong upregulation of expression in the 6 % and 14 % sucrose cultures. The cDNA fragment BSS7 was

approximately 140 nucleotides in length. The BSS7 fragment displayed relatively weak signal strength in all three cultures, being close to the lower limit of detection in the 2 % cultures and being only slightly stronger in the 6 % and 14 % sucrose cultures (data not shown). From the weak signal strength and weak upregulation it was apparent that BSS7 encoded part of a mRNA which was expressed at relatively low levels and is only slightly upregulated in the 6 % and 14 % sucrose cultures. The fragment BSS11 was approximately 280 nucleotides in length. The signal strength of BSS11 was moderate in the 2 % sucrose cultures and only slightly stronger in the 6 % and 14 % tissues (data not shown). The intensity of BSS11 suggested that it encoded part of a mRNA which exhibited relatively low expression levels in the 2 % cultures and with a slight expression enhancement in the 6 % and 14 % cultures. The cDNA fragment BSS12 was approximately 270 nucleotides in length. Like BSS11 the signal intensity of BSS12 was low in the 2 % culture whereas in the 6 % and 14 % cultures, it was slightly stronger. From the signal intensity of BSS12, it appeared that the corresponding mRNA exhibited relatively weak expression in the 2 % cultures with minor upregulation in the 6 % and 14 % cultures (data not shown). The cDNA BSS13 is approximately 340 nucleotides in length, the longest upregulated cDNA observed in this experiment. The signal intensity of BSS13 was relatively weak in the 2 % sucrose cultures with a strong upregulation in the 6 % cultures and a slightly stronger signal in the 14 % cultures. From the signal intensity it appeared that BSS13 encoded part of a mRNA which exhibits a strong increase in expression in the 6 % and 14 % sucrose cultures when compared to the 2 % cultures (data not shown).

PCR amplification employing the decamer OPA6 yielded three upregulated cDNA fragments, BSS 8, BSS9 and BSS10. The cDNA BSS8 was approximately 220

nucleotides in length. The signal strength of BSS8 was strong in the 2 %, 6 % and 14 % sucrose cultures with a minor upregulation detected in the 6 % and 14 % cultures (Figure 6). From the signal strength it appeared that BSS8 encoded part of a mRNA which was expressed at relatively high levels in all the cultures with minor expression stimulation occurring when media sucrose was increased from 2 % to 6 % to 14 %. The fragment BSS9 was approximately 230 nucleotides in length. The signal strength of BSS9 was intermediate for all the fragments examined and displayed a minor increase as sucrose was raised from 2 % - 6 % in the culture and a larger increase from the 6 % - 14 % sucrose cultures (Figure 6). Some variability was observed for the expression levles of BSS9 from the different cultures. From the signal strength it appeared that BSS9 encoded part of a mRNA which was expressed at moderate levels in all the cultures but experiences minor stimulation of expression when media sucrose is increased from 2 % to 6 % and in one of the 14 % cultures this mRNA appears to be expressed at higher levels but the other appears to be weaker than in the 6 % cultures. The fragment BSS10 was approximately 90 nucleotides in length (Figure 6). The signal strength for BSS10 was variable and relatively weak in the 2 % culture, variable and again weak in the 6 % culture and for the 14 % it was still weak but a minor increase from the 6 % cultures. From the signal strength of BSS10 it appears that this fragment encoded part of a mRNA which was expressed at low levels in the 2 % culture, only slightly higher levels in the 6 % cultures and only slightly higher in the 14 % cultures.

PCR amplification employing the decamer OPA5 yielded a cDNA of approximately 250 bp (Figure 7). This cDNA was termed BSC (*Brassica napus* sucrose control) - 100, as it appeared to be present in equal amounts in the 2 %, 6 % and 14 % sucrose cultures.

Figure 6. Differential display of mRNA (via PCR) from MD cell suspension cultures grown in sucrose concentrations of 2, 6 and 14 % (w/v). Lanes 1 and 2 are from cells grown in 2 % sucrose, lanes 3 and 4 are from cells grown in 6 % sucrose and lanes 5 and 6 are from cells grown in 14 % sucrose. All bands were the result of using the specific anchored primer 5'-T_{12}AA-3' in combination with the primer OPA6 (5'-GGTCCCTGAC-3'). RNA isolation, reverse transcription, PCR and electrophoresis were as described in the **MATERIALS AND METHODS** section. Autoradiography was conducted for 2 hours.

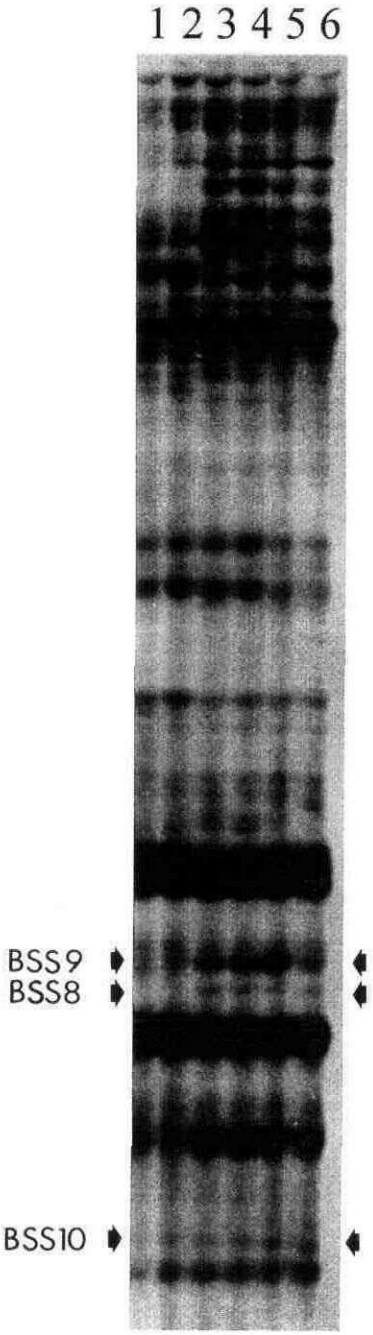
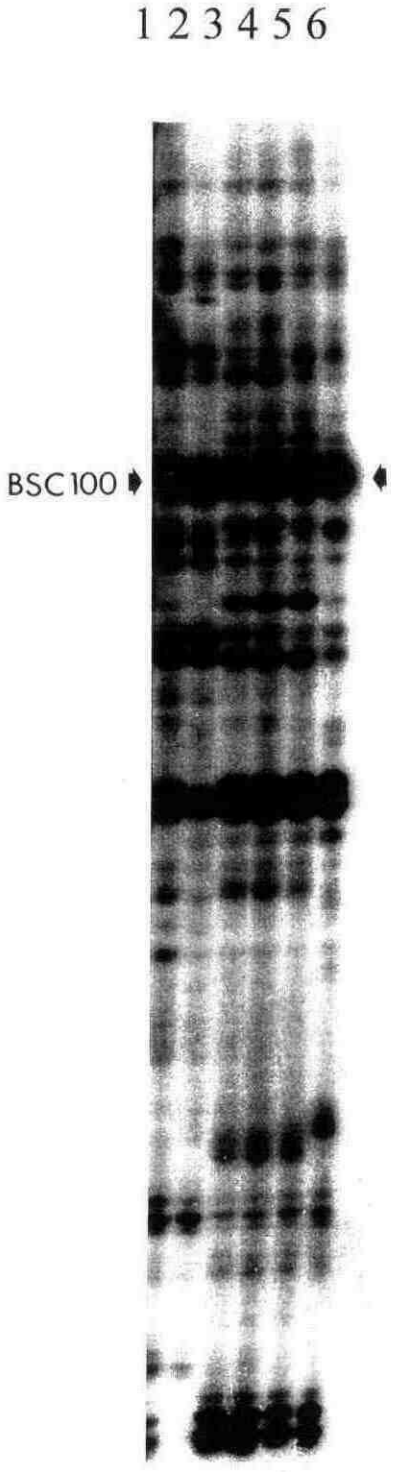

1 2 3 4 5 6
BSS9
BSS8
BSS10

Figure 7. Differential display of mRNA (via PCR) from MD cell suspension cultures grown in sucrose concentrations of 2, 6 and 14 % (w/v). Lanes 1 and 2 are from cells grown in 2 % sucrose, lanes 3 and 4 are from cells grown in 6 % sucrose and lanes 5 and 6 are from cells grown in 14 % sucrose. All bands were the result of using the specific anchored primer 5'-T_{12}AA-3' in combination with the primer OPA5 (5'-AGGGGTCTTG-3'). RNA isolation, reverse transcription, PCR and electrophoresis were as described in the **MATERIALS AND METHODS** section. Autoradiography was conducted for 2 hours.


1 2 3 4 5 6
BSC100

Cloning and Sequencing of cDNA fragments

All the BSS cDNAs but one were successfully cloned and sequenced, as summarized in Table 3. Individual sequence data is presented in figures 8 to 73, except for BSS10 which was eliminated from further examination after three separate cloning attempts failed. At least three clones were sequenced for each DD fragment as the DD process has been found to produce multiple sequences of the same length. Before sequencing, individual cDNA clones were analyzed by colony PCR to determine if they were of the appropriate size based on the approximate expected size determined from the DD polyacrylamide gel. The colony PCR amplification was usually a good indicator of cDNA size but in a few cases the size indicated was much larger than the actual size of the fragment when sequenced. Another difficulty with the use of colony PCR to estimate the insert cDNA length was that the PCR amplification produced large variations in the amount of amplified product. Some reactions produced extremely large amounts of amplified product which caused an overloading of the agarose gel, while others produced much less. It is difficult to get accurate sizing of small cDNA fragments on an agarose gel especially if a large amount of DNA is present as the bands are more dispersed. These sizing difficulties resulted in size predictions with about a 20 - 30 % variation when the clones were sequenced.

Six independent clones from BSS1 were sequenced and identified as BSS1A, BSS1B, BSS1C, BSS1D, BSS1E and BSS1F. When using colony PCR the size of the

Table 3. Characteristics of cDNA fragments obtained by differential display of mRNAs from a *Brassica napus* cell suspension culture grown in the presence of 2, 6 and 14 % (w/v) sucrose.

cDNA Fragments On DD Gel	Apparent Size (Bases)	Decamer Used In PCR	cDNA Fragments Sequenced	Variants	Upregulated cDNAs By RT-PCR Analysis	Individual Sequences Presented In Figures
BSS 1	300	OPA1	6	5	1	8-13 and 91-93
BSS 2	220	OPA1	6	4	1	15-20
BSS 3*	190	OPA1	3	2	1	23-25
BSS 4	150	OPE3	6	3	1	27-32
BSS 5	170	OPA4	3	2	1	35-37
BSS 6	330	OPA4	5	3	0	39-43
BSS 7	140	OPA4	6	5	1	45-50
BSS 8*	220	OPA6	3	2	1	52-54
BSS 9	230	OPA6	3	3	0	56-59
BSS 10	90	OPA6	0	0	0	
BSS 11	280	OPA4	3	1	1	60-62 and 95
BSS 12	270	OPA4	3	2	0	64-66
BSS 13	340	OPA4	3	1	1	68-70
BSC 100	250	OPA5	2	2	0	72-73

*Fragments BSS3A and BSS8A were nearly identical despite being amplified by two different decamers (Figures 23 and 52).

Figure 8. Nucleotide sequence of the cDNA BSS1A, expressed as mRNA sequence. BSS1A was produced by reverse transcription of mRNA primed with 5'-T_{12}AA-3' followed by PCR amplification with the same oligo(dT) primer and the arbitrarily chosen decamer OPA1 (5'-CAGGCCCTTC-3'). The primers used for the DD PCR are singly underlined while the specific primers used in the RT-PCR reactions are double underlined.

```
  1  CAGGCCCTTC  TCATAGTAAC  CATGGATCTT  CAATCTCTGT  CTTGTCACTT   50
 51  GCAATCAGCG  GCGCATTGGT  CATAATGTTC  ACCCGTATCT  AACACCCCTA  100
101  TCATTTTTAG  TTAAGAGTTT  GCTTTTATCT  TGTGAGTAAC  TATGGAGTTC  150
151  TCTTGTTGAG  ACCGCTTATT  TTTAGTTATG  ACATTATAAA  GACTTGGTTA  200
201  AAAAAAAAAA  A                                            211
```

Figure 9. Nucleotide sequence of the cDNA BSS1B, expressed as mRNA sequence. BSS1B was produced by reverse transcription of mRNA primed with 5'-T_{12}AA-3' followed by PCR amplification with the same oligo(dT) primer and the arbitrarily chosen decamer OPA1 (5'-CAGGCCCTTC-3'). The decamer used for the DD PCR is singly underlined but the oligo(dT) primer was not identified.

```
  1   CAGGCCCTTC  AACAAGAATT  CCACCTCTAA  TGCAAAAAAG  CCAACAACGT   50
 51   AAAATAGACC  ATTGCGGTTT  CATGTTCTCG  AATCAGTTTC  CTTGTTGGAC  100
101   CACACAAATT  GTGGGGTTAT  TTTTCTCCAT  TCTTTCTA              138
```

Figure 10. Nucleotide sequence of the cDNA BSS1C, expressed as mRNA sequence. BSS1C was produced by reverse transcription of mRNA primed with 5'-T_{12}AA-3' followed by PCR amplification with the same oligo(dT) primer and the arbitrarily chosen decamer OPA1 (5'-CAGGCCCTTC-3'). The primers used for the DD PCR are singly underlined while the specific primers used in the RT-PCR reactions are double underlined.

```
  1  CAGGCCCTTC  GTAAAGTGCA  GGCCCTTCAA  GATGGTTCAC  CCTTCTTTTT   50
 51  TTTGTTCTAT  TTAGATTTTC  AAATATATTT  TCGCAGAGAC  TAATGTAAGT  100
101  TAATACAAGT  TACAACAGGT  TTCCTTTTGA  CTTGTTTCTT  TGTTCGTTAA  150
151  AAAAAAAAA                                               159
```

Figure 11. Nucleotide sequence of the cDNA BSS1D, expressed as mRNA sequence. BSS1D was produced by reverse transcription of mRNA primed with 5'-T_{12}AA-3' followed by PCR amplification with the same oligo(dT) primer and the arbitrarily chosen decamer OPA1 (5'-CAGGCCCTTC-3'). The primers used for the DD PCR are singly underlined.

```
  1  CAGGCCCTTC  GAAGGATAAT  AATGGGAAGC  GTGAGCAGCT  TCGTGATTCA   50
 51  GCACGCTCCT  TGCCCTGTCA  CCGTTGTCAA  GGATAACGAC  TCTCACTAAA  100
101  TAATGAAGTC  TATCTCATTA  TGTTTTTACC  AAAACAATCA  TCGCTGCGTA  150
151  TGGTTTTAAT  TAAAGAATAA  AAGACCCTTT  CTCTCGTTAA  AAAAAAAAAA  200
```

Figure 12. Nucleotide sequence of the cDNA BSS1E, expressed as mRNA sequence. BSS1E was produced by reverse transcription of mRNA primed with 5'-T_{12}AA-3' followed by PCR amplification with the same oligo(dT) primer and the arbitrarily chosen decamer OPA1 (5'-CAGGCCCTTC-3'). The primers used for the DD PCR were identified and are singly underlined while the specific primers used in the RT-PCR reactions are double underlined.

```
  1  CAGGCCCTTC GGTGGTGGAC GTCCCGGAAG AGTGAAGAGA AGAAATGAGA  50
 51  AGTCTGCCTC CAAGAAAGCT TCTGGTGGTG ATGCAGATGG TGATGACGAA 100
101  GAGTAAATCA AACTCTCACC TGTTTTAGCT ATTATGAAGC GATCTGCGTT 150
151  TTGATACAAT GTAGTCTTGT TCTTTTTCGG TGGATTGTTT TGTGTTTACT 200
201  TTCGCTTTCT CTTAAGAATG TCGAACTTCT GCTTAAAAAA AAAAAA      246
```

Figure 13. Nucleotide sequence of the cDNA BSS1F, expressed as mRNA sequence. BSS1F was produced by reverse transcription of mRNA primed with 5'-T_{12}AA-3' followed by PCR amplification with the same oligo(dT) primer and the arbitrarily chosen decamer OPA1 (5'-CAGGCCCTTC-3'). The primers used for the DD PCR are singly underlined.

```
  1  CAGGCCCTTC  GAAGGATAAT  AATGGGAAGC  GTGAGCAGCT  TCGTGATTCA   50
 51  GCACGCTCCT  TGCCCTGTCA  CCGTTGTCAA  GGATAACGAC  TCTCACTAAA  100
101  TAATGAAGTC  TATCTCATTA  TGTTTTTACC  AAAACAATCA  TCGCTGCGTA  150
151  TGGTTTTAAT  TAAAGAATAA  AAGACCCTTT  CTCTCGTTAA  AAAAAAAAAA  200
```

Figure 14. Sequence homology comparison of BSS1D and BSS1F. The sequences are identical.

```
BSS1D    1 CAGGCCCTTC  GAAGGATAAT  AATGGGAAGC  GTGAGCAGCT  TCGTGATTCA  50
BSS1F    1 CAGGCCCTTC  GAAGGATAAT  AATGGGAAGC  GTGAGCAGCT  TCGTGATTCA  50

BSS1D   51 GCACGCTCCT  TGCCCTGTCA  CCGTTGTCAA  GGATAACGAC  TCTCACTAAA 100
BSS1F   51 GCACGCTCCT  TGCCCTGTCA  CCGTTGTCAA  GGATAACGAC  TCTCACTAAA 100

BSS1D  101 TAATGAAGTC  TATCTCATTA  TGTTTTTACC  AAAACAATCA  TCGCTGCGTA 150
BSS1F  101 TAATGAAGTC  TATCTCATTA  TGTTTTTACC  AAAACAATCA  TCGCTGCGTA 150

BSS1D  151 TGGTTTTAAT  TAAAGAATAA  AAGACCCTTT  CTCTCGTTAA  AAAAAAAAAA 200
BSS1F  151 TGGTTTTAAT  TAAAGAATAA  AAGACCCTTT  CTCTCGTTAA  AAAAAAAAAA 200
```

Figure 15. Nucleotide sequence of the cDNA BSS2A, expressed as mRNA sequence. BSS2A was produced by reverse transcription of mRNA primed with 5'-T_{12}AA-3' followed by PCR amplification with the same oligo(dT) primer and the arbitrarily chosen decamer OPA1 (5'-CAGGCCCTTC-3'). The only identified primer was OPA1 which is singly underlined.

```
  1  CAGGCCCTTC  TCATAGTAAC  CATGGATCTT  CAATCTCTGT  CTTGTCACTT   50
 51  GCAATCAGCG  GCGCATTGGT  CATAATGTTC  ACCCGTATCT  AACACCCCTA  100
101  TCATTTTTAG  TTAAGAGTTT  GCTTTTATCT  TGTGAGTAAC  TATGGAGTTC  150
151  TCTTGTTGAG  ACCGCTTATT  TTTAGTTATG  ACATTATAAA  GACTTGGTTA  200
201  AAAAAGAAGA  AAAAAGAAAA  AATTCAGGCC  CTTCTCATAG  TAACCATGGA  250
```

Figure 16. Nucleotide sequence of the cDNA BSS2B, expressed as mRNA sequence. BSS2B was produced by reverse transcription of mRNA primed with 5'-T_{12}AA-3' followed by PCR amplification with the same oligo(dT) primer and the arbitrarily chosen decamer OPA1 (5'-CAGGCCCTTC-3'). The primers used for the DD PCR are singly underlined.

```
  1  CAGGCCCTTC  TCATAGTAAC  CATGGATCTT  ACAATCTCTG  TACTTGTCAC   50
 51  TTGCAATCAG  CGGCGCATTG  GTCATAATGT  TACACCCGTA  TCTAACACCC  100
101  CTATCATTTT  TAGTTAAGAG  TTTGCTTTTA  TCTTGTGAGT  AACTATGGAG  150
151  TTCTCTTGTT  GAGACCGCTT  ATTTTTAGTT  ATGACATTAT  AAAGACTTGG  200
201  TTAAAAAAAA  AAAA                                          214
```

Figure 17. Nucleotide sequence of the cDNA BSS2C, expressed as mRNA sequence. BSS2C was produced by reverse transcription of mRNA primed with 5'-T_{12}AA-3' followed by PCR amplification with the same oligo(dT) primer and the arbitrarily chosen decamer OPA1 (5'-CAGGCCCTTC-3'). The primers used for the DD PCR are singly underlined while the specific primers used in the RT-PCR reactions are double underlined.

```
  1  CAGGCCCTTC  AAGAGAAAGA  TGTACCCAGC  GACCACAATA  TTTACTTATC   50
 51  ATATGAAGAC  ATCTAATAGA  GCAGTATGGT  AAATCTTACG  CAGTTATTCA  100
101  GACAATTTAT  ATAATATAAA  TTAGTTGTCT  GTAGTGATCG  CTTTTTTTTC  150
151  TATTTGAATG  TCAAAATTTG  GTCAGTTATA  TTGGGGATGT  TTATCGTGTT  200
201  AAAAAAAAAA  AA                                           212
```

Figure 18. Nucleotide sequence of the cDNA BSS2D, expressed as mRNA sequence. BSS2D was produced by reverse transcription of mRNA primed with 5'-T_{12}AA-3' followed by PCR amplification with the same oligo(dT) primer and the arbitrarily chosen decamer OPA1 (5'-CAGGCCCTTC-3'). The primers used for the DD PCR are singly underlined.

```
  1  CAGGCCCTTC  AAGAGAAAGA  TGTACCCAGC  GACCACAATA  TTTACTTATC   50
 51  ATATGAAGAC  ATCTAATAGA  GCAGTATGGT  AAATCTTACG  CAGTTATTCA  100
101  GACAATTTAT  ATAATATAAA  TTAGTTGTCT  GTAGTGATCG  CTTTTTTTTC  150
151  TATTTGAATG  TCAAAATTTG  GTCAGTTATA  TTGGGGATGT  TTATCGTGTT  200
201  AAAAAAAAAA  AA                                           212
```

Figure 19. Nucleotide sequence of the cDNA BSS2E, expressed as mRNA sequence. BSS2E was produced by reverse transcription of mRNA primed with 5'-T_{12}AA-3' followed by PCR amplification with the same oligo(dT) primer and the arbitrarily chosen decamer OPA1 (5'-CAGGCCCTTC-3'). The primers used for the DD PCR are singly underlined.

```
  1  CAGGCCCTTC  AAGAGAAAGA  TGTACCCAGC  GACCACAATA  TTTACTTATC   50
 51  ATATGAAGAC  ATCTAATAGA  GCAGTATGGT  AAATCTTACG  CAGTTATTCA  100
101  GACAATTTAT  ATAATATAAA  TTAGTTGTCT  GTAGTGATCG  CTTTTTTTTC  150
151  TATTTGAATG  TCAAAATTTG  GTCAGTTATA  TTGGGGATGT  TTATCGTGTT  200
201  AAAAAAAAAA  AA                                           212
```

Figure 20. Nucleotide sequence of the cDNA BSS2F, expressed as mRNA sequence. BSS2F was produced by reverse transcription of mRNA primed with 5'-T_{12}AA-3' followed by PCR amplification with the same oligo(dT) primer and the arbitrarily chosen decamer OPA1 (5'-CAGGCCCTTC-3'). The oligo(dT) primer used for the DD PCR is singly underlined but the decamer was not identified.

```
  1  TTCATAGTAA  CCATGGATCT  TCAATCTCTG  TCTTGTCACT  TGCAATCAGC   50
 51  GGCGCATTGG  TCATAATGTT  CACCCGTATC  TAACACCCCT  ATCATTTTTA  100
101  GTTAAGAGTT  TGCTTTTATC  TTGTGAGTAA  CTATGGAGTT  CTCTTGTTGA  150
151  GACCGCTTAT  TTTTAGTTAT  GACATTATAA  AGACTTGGTT  AAAAAAAAAA  200
201  AA                                                      202
```

Figure 21. Sequence homology comparison of BSS2C, BSS2D and BSS2E. These sequences are identical.

```
BSS2C    1 CAGGCCCTTC AAGAGAAAGA TGTACCCAGC GACCACAATA TTTACTTATC  50
BSS2D    1 CAGGCCCTTC AAGAGAAAGA TGTACCCAGC GACCACAATA TTTACTTATC  50
BSS2E    1 CAGGCCCTTC AAGAGAAAGA TGTACCCAGC GACCACAATA TTTACTTATC  50

BSS2C   51 ATATGAAGAC ATCTAATAGA GCAGTATGGT AAATCTTACG CAGTTATTCA 100
BSS2D   51 ATATGAAGAC ATCTAATAGA GCAGTATGGT AAATCTTACG CAGTTATTCA 100
BSS2E   51 ATATGAAGAC ATCTAATAGA GCAGTATGGT AAATCTTACG CAGTTATTCA 100

BSS2C  101 GACAATTTAT ATAATATAAA TTAGTTGTCT GTAGTGATCG CTTTTTTTTC 150
BSS2D  101 GACAATTTAT ATAATATAAA TTAGTTGTCT GTAGTGATCG CTTTTTTTTC 150
BSS2E  101 GACAATTTAT ATAATATAAA TTAGTTGTCT GTAGTGATCG CTTTTTTTTC 150

BSS2C  151 TATTTGAATG TCAAAATTTG GTCAGTTATA TTGGGGATGT TTATCGTGTT 200
BSS2D  151 TATTTGAATG TCAAAATTTG GTCAGTTATA TTGGGGATGT TTATCGTGTT 200
BSS2E  151 TATTTGAATG TCAAAATTTG GTCAGTTATA TTGGGGATGT TTATCGTGTT 200

BSS2C  201 AAAAAAAAAA AA                                        212
BSS2D  201 AAAAAAAAAA AA                                        212
BSS2E  201 AAAAAAAAAA AA                                        212
```

Figure 22. Sequence alignment of BSS1A and BSS2B. The / symbol represents a nucleotide that is absent from that particular sequence but present in the other. All other nucleotides are identical.

```
BSS1A    1 CAGGCCCTTC TCATAGTAAC CATGGATCTT /CAATCTCTG T/CTTGTCAC  48
BSS2B    1 CAGGCCCTTC TCATAGTAAC CATGGATCTT ACAATCTCTG TACTTGTCAC  50

BSS1A   51 TTGCAATCAG CGGCGCATTG GTCATAATGT T/CACCCGTA TCTAACACCC  97
BSS2B   51 TTGCAATCAG CGGCGCATTG GTCATAATGT TACACCCGTA TCTAACACCC 100

BSS1A  101 CTATCATTTT TAGTTAAGAG TTTGCTTTTA TCTTGTGAGT AACTATGGAG 147
BSS2B  101 CTATCATTTT TAGTTAAGAG TTTGCTTTTA TCTTGTGAGT AACTATGGAG 150

BSS1A  151 TTCTCTTGTT GAGACCGCTT ATTTTTAGTT ATGACATTAT AAAGACTTGG 197
BSS2B  151 TTCTCTTGTT GAGACCGCTT ATTTTTAGTT ATGACATTAT AAAGACTTGG 200

BSS1A  201 TTAAAAAAAA AAAA                                      211
BSS2B  201 TTAAAAAAAA AAAA                                      214
```

Figure 23. Nucleotide sequence of the cDNA BSS3A, expressed as mRNA sequence. BSS3A was produced by reverse transcription of mRNA primed with 5'-T_{12}AA-3' followed by PCR amplification with the same oligo(dT) primer and the arbitrarily chosen decamer OPA1 (5'-CAGGCCCTTC-3'). Only the oligo(dT) primer was identified and is singly underlined. The decamer was not identified.

```
  1  CCCTGACAAA  GAAGCTGACA  AGATCGACTC  TTGCTCTCTT  GCCATTGAAT   50
 51  ACGTTTTCAA  TCATCCTATG  TCTAGCTAAA  CACACCTGTG  TGCTATCTCT  100
101  CTCATGTTTT  TTGTTCTTTC  CTGATCATGG  GACTTCCAAA  TAAATTCAGT  150
151  TCTGTTTTGA  GAGTCATTCT  TGACTCTTTT  AACAGTGTCC  TTAAGAAACT  200
201  CTTATGCCTT  AAAAAAAAAA  AA                               222
```

Figure 24. Nucleotide sequence of the cDNA BSS3B, expressed as mRNA sequence. BSS3B was produced by reverse transcription of mRNA primed with 5'-T_{12}AA-3' followed by PCR amplification with the same oligo(dT) primer and the arbitrarily chosen decamer OPA1 (5'-CAGGCCCTTC-3'). The primers used for the DD PCR are singly underlined.

```
  1  CAGGCCCTTC  GAAGGATAAT  AATGGGAAGC  GTGAGCAGCT  TCGTGATTCA   50
 51  GCACGCTCCT  TGCCCTGTCA  CCGTTGTCAA  GGATAACGAC  TCTCACTAAA  100
101  TAATGAAGTC  TATCTCATTA  TGTTTTTACC  AAAACAATCA  TCGCTGCGTA  150
151  TGGTTTTAAT  TAAAGAATAA  AAGACCCTTT  CTCTCGTTAA  AAAAAAAAAA  200
```

Figure 25. Nucleotide sequence of the cDNA BSS3C, expressed as mRNA sequence. BSS3C was produced by reverse transcription of mRNA primed with 5'-T_{12}AA-3' followed by PCR amplification with the same oligo(dT) primer and the arbitrarily chosen decamer OPA1 (5'-CAGGCCCTTC-3'). The primers used for the DD PCR are singly underlined.

```
  1  CAGGCCCTTC  GAAGGATAAT  AATGGGAAGC  GTGAGCAGCT  TTCGTGATTC   50
 51  AGCACGCTCC  TTGCCCTGTC  ACCGTTGTCA  AGGATAACGA  CTCTCACTAA  100
101  ATAATGAAGT  CTATCTCATT  ATGTTTTTAC  CAAAACAATC  ATCGCTGCFT  150
151  ATGGTTTTAA  TTAAAGAATA  AAAGACCCTT  TCTCTCGTTA  AAAAAAAAAA  200
201  A                                                       201
```

Figure 26. Sequence homology comparison of BSS1D, BSS1F, BSS3B and BSS3C. The / symbol indicates that a nucleotide is absent from that particular sequence but present in another. All other nucleotides are identical.

```
BSS1D    1 CAGGCCCTTC GAAGGATAAT AATGGGAAGC GTGAGCAGCT T/CGTGATTC  49
BSS1F    1 CAGGCCCTTC GAAGGATAAT AATGGGAAGC GTGAGCAGCT T/CGTGATTC  49
BSS3B    1 CAGGCCCTTC GAAGGATAAT AATGGGAAGC GTGAGCAGCT T/CGTGATTC  49
BSS3C    1 CAGGCCCTTC GAAGGATAAT AATGGGAAGC GTGAGCAGCT TTCGTGATTC  50

BSS1D   50 AGCACGCTCC TTGCCCTGTC ACCGTTGTCA AGGATAACGA CTCTCACTAA  99
BSS1F   51 AGCACGCTCC TTGCCCTGTC ACCGTTGTCA AGGATAACGA CTCTCACTAA  99
BSS3B   50 AGCACGCTCC TTGCCCTGTC ACCGTTGTCA AGGATAACGA CTCTCACTAA  99
BSS3C   51 AGCACGCTCC TTGCCCTGTC ACCGTTGTCA AGGATAACGA CTCTCACTAA 100

BSS1D  101 ATAATGAAGT CTATCTCATT ATGTTTTTAC CAAAACAATC ATCGCTGCGT 149
BSS1F  101 ATAATGAAGT CTATCTCATT ATGTTTTTAC CAAAACAATC ATCGCTGCGT 149
BSS3B  101 ATAATGAAGT CTATCTCATT ATGTTTTTAC CAAAACAATC ATCGCTGCGT 149
BSS3C  101 ATAATGAAGT CTATCTCATT ATGTTTTTAC CAAAACAATC ATCGCTGCGT 150

BSS1D  151 ATGGTTTTAA TTAAAGAATA AAAGACCCTT TCTCTCGTTA AAAAAAAAAA 199
BSS1F  151 ATGGTTTTAA TTAAAGAATA AAAGACCCTT TCTCTCGTTA AAAAAAAAAA 199
BSS3B  151 ATGGTTTTAA TTAAAGAATA AAAGACCCTT TCTCTCGTTA AAAAAAAAAA 199
BSS3C  151 ATGGTTTTAA TTAAAGAATA AAAGACCCTT TCTCTCGTTA AAAAAAAAAA 200

BSS1D  200 A                                                     200
BSS1F  200 A                                                     200
BSS3B  200 A                                                     200
BSS3C  201 A                                                     201
```

Figure 27. Nucleotide sequence of the cDNA BSS4A, expressed as mRNA sequence. BSS4A was produced by reverse transcription of mRNA primed with 5'-T_{12}AA-3' followed by PCR amplification with the same oligo(dT) primer and the arbitrarily chosen decamer OPE3 (5'-CCAGATGCAC-3'). The primers used for the DD PCR are singly underlined while the specific primers used in the RT-PCR reactions are double underlined.

```
  1  CCAGATGCAC  TGTGAAGCAA  TGCAAGACAT  TGTTTTTGGC  GTATTTGCAA   50
 51  TTTTCTTTGG  ACTTGAAATA  ACCATGATGT  GAGACATAAA  AGTTATTATA  100
101  GTGATGTACT  TTTGCTATTC  GTTTGTTAAA  AAAAAAAAA               139
```

Figure 28. Nucleotide sequence of the cDNA BSS4B, expressed as mRNA sequence. BSS4B was produced by reverse transcription of mRNA primed with 5'-T_{12}AA-3' followed by PCR amplification with the same oligo(dT) primer and the arbitrarily chosen decamer OPE3 (5'-CCAGATGCAC-3'). The primers used for the DD PCR are singly underlined.

```
  1  CCAGATGCAC  TGTGAAGCAA  TGCAAGACAT  TGTTTTTGGC  GTATTTGCAA    50
 51  TTTTCTTTGG  ACTTGAAATA  ACCATGATGT  GAGACATAAA  AGTTATTATA   100
101  GTGATGTACT  TTTGCTATTC  GTTTGTTAAA  AAAAAAAAA               139
```

Figure 29. Nucleotide sequence of the cDNA BSS4C, expressed as mRNA sequence. BSS4C was produced by reverse transcription of mRNA primed with 5'-T_{12}AA-3' followed by PCR amplification with the same oligo(dT) primer and the arbitrarily chosen decamer OPE3 (5'-CCAGATGCAC-3'). The primers used for the DD PCR are singly underlined.

```
  1  CCAGATGCAC  TGTGAAGCAA  TGCAAGACAT  TGTTTTTGGC  GTATTTGCAA   50
 51  TTTTCTTTGG  ACTTGAAATA  ACCATGATGT  GAGACATAAA  AGTTATTATA  100
101  GTGATGTACT  TTTGCTATTC  GTTTGTTAAA  AAAAAAAAA              139
```

Figure 30. Nucleotide sequence of the cDNA BSS4D, expressed as mRNA sequence. BSS4D was produced by reverse transcription of mRNA primed with 5'-T_{12}AA-3' followed by PCR amplification with the same oligo(dT) primer and the arbitrarily chosen decamer OPE3 (5'-CCAGATGCAC-3'). The oligo(dT) primer used for the DD PCR is singly underlined. The decamer was not identified.

```
 1  AAAAAAAAAA  AAAAAAAATG  TTTTGATGTT  AGAACTGAGA  AACTAAGTTT      50
51  TATCCAAGCA  CATGCACCTC  TCGTTAAAAA  AAAAAAA                     87
```

Figure 31. Nucleotide sequence of the cDNA BSS4E, expressed as mRNA sequence. BSS4E was produced by reverse transcription of mRNA primed with 5'-T_{12}AA-3' followed by PCR amplification with the same oligo(dT) primer and the arbitrarily chosen decamer OPE3 (5'-CCAGATGCAC-3'). The oligo(dT) primer used for the DD PCR is singly underlined. The decamer was not identified.

```
 1  AAAAAAAAAA  AAAAAAAATG  TTTTGATGTT  AGAACTGAGA  AACTAAGTTT    50
51  TATCCAAGCA  CATGCACCTC  TCGTTAAAAA  AAAAAAA                   86
```

Figure 32. Nucleotide sequence of the cDNA BSS4F, expressed as mRNA sequence. BSS4F was produced by reverse transcription of mRNA primed with 5'-T_{12}AA-3' followed by PCR amplification with the same oligo(dT) primer and the arbitrarily chosen decamer OPE3 (5'-CCAGATGCAC-3'). Neither of the DD primers were identified.

```
  1 GCTGATACCG  CTCGCCGCAG  CCGAACCGAC  CGAGCGCAGC  GAGTCAGTGA    50
 51 GCGAGGAAGC  GGAAGAGCGC  CCAATACGCA  AACCGCCTCT  CCCCGCGCGT   100
101 TGGCCGATTC  ATTAATGCAG  CTGGCACGAC  AGGTTTCCCG  ACTGGAAAGC   150
151 GGGCAGTGAG  CGCAACGCAA  TTAATGTGAG  TTAGCTCACT  CATTAGGCAC   200
201 CCCAGGCTTT  ACACTTTATG  CTTCCGGCTC  GTATGTTGTG  TGGAATCCCG   250
251 CGGCCATGGC  GGCCGGGAGC  ATGCGACGTC  GGGGGGC               287
```

Figure 33. Sequence homology comparison of BSS4A, BSS4B and BSS4C. These sequences are identical.

```
BSS4A     1 CCAGATGCAC TGTGAAGCAA TGCAAGACAT TGTTTTTGGC GTATTTGCAA  50
BSS4B     1 CCAGATGCAC TGTGAAGCAA TGCAAGACAT TGTTTTTGGC GTATTTGCAA  50
BSS4C     1 CCAGATGCAC TGTGAAGCAA TGCAAGACAT TGTTTTTGGC GTATTTGCAA  50

BSS4A    51 TTTTCTTTGG ACTTGAAATA ACCATGATGT GAGACATAAA AGTTATTATA 100
BSS4B    51 TTTTCTTTGG ACTTGAAATA ACCATGATGT GAGACATAAA AGTTATTATA 100
BSS4C    51 TTTTCTTTGG ACTTGAAATA ACCATGATGT GAGACATAAA AGTTATTATA 100

BSS4A   101 GTGATGTACT TTTGCTATTC GTTTGTTAAA AAAAAAAAA            139
BSS4B   101 GTGATGTACT TTTGCTATTC GTTTGTTAAA AAAAAAAAA            139
BSS4C   101 GTGATGTACT TTTGCTATTC GTTTGTTAAA AAAAAAAAA            139
```

Figure 34. Sequence homology comparison of BSS4D and BSS4E. These sequences are identical.

```
BSS4D    1 AAAAAAAAAA  AAAAAAAATG  TTTTGATGTT  AGAACTGAGA  AACTAAGTTT  50
BSS4E    1 AAAAAAAAAA  AAAAAAAATG  TTTTGATGTT  AGAACTGAGA  AACTAAGTTT  50

BSS4D   51 TATCCAAGCA  CATGCACCTC  TCGTTAAAAA  AAAAAAA                  87
BSS4E   51 TATCCAAGCA  CATGCACCTC  TCGTTAAAAA  AAAAAAA                  86
```

Figure 35. Nucleotide sequence of the cDNA BSS5A, expressed as mRNA sequence. BSS5A was produced by reverse transcription of mRNA primed with 5'-T_{12}AA-3' followed by PCR amplification with the same oligo(dT) primer and the arbitrarily chosen decamer OPA4 (5'-AATCGGGCTG-3'). The primers used for the DD PCR are singly underlined while the specific primers used in the RT-PCR reactions are double underlined.

```
  1  AATCGGGCTG  ATCGATAAGA  TTGTTAGACC  ACCGCGCATC  AAAGCGGACG   50
 51  CTCCTCGCCA  AGACGAAACC  TCAGGTCTCG  GTTAATCTGT  TTGTTTGTTT  100
101  TTGGTAATGT  AATAACGTCT  TTGAAGTTTT  TTATACAATG  GTTGAAGTCG  150
151  TTGGTTCTTG  TTAAAAAAAA  AAAA                              174
```

Figure 36. Nucleotide sequence of the cDNA BSS5B, expressed as mRNA sequence. BSS5B was produced by reverse transcription of mRNA primed with 5'-T_{12}AA-3' followed by PCR amplification with the same oligo(dT) primer and the arbitrarily chosen decamer OPA4 (5'-AATCGGGCTG-3'). The primers used for the DD PCR are singly underlined while the specific primers used in the RT-PCR reactions are double underlined.

```
  1  AATCGGGCTG  GAGAGTGCGA  TGGAGAGCTT  GAGTGTGAGT  GGTGACAGGA   50
 51  TTCAAGGAAG  TGTTTGATGA  AAAGGTTGAA  GGTTGGGAAC  ATGTGGGAGT  100
101  TTGAGAAAGT  TGTTATCTTT  TATGTTTTGT  CTCGATATAT  CGTTAGCGTT  150
151  TGTGTTAAAA  AAAAAAAA                                      168
```

Figure 37. Nucleotide sequence of the cDNA BSS5C, expressed as mRNA sequence. BSS5C was produced by reverse transcription of mRNA primed with 5'-T_{12}AA-3' followed by PCR amplification with the same oligo(dT) primer and the arbitrarily chosen decamer OPA4 (5'-AATCGGGCTG-3'). The primers used for the DD PCR are singly underlined.

```
  1 AATCGGGCTG  GAGAGTGCGA  TGGAGAGCTT  GAGTGTGAGT  GGTGACAGGA    50
 51 TTCAAGGTAA  GTGTTTGATG  AAAAGGTTGA  AGGTTGGGAA  CATGTGGGAG   100
101 TTTGAGAAAG  TTGTTATCTT  TTATGTTTTG  TCTCGATATA  TCGTTAGCGT   150
151 TTGTGTTAAA  AAAAAAAAA                                      169
```

Figure 38. Sequence homology comparison of BSS5B and BSS5C. The / symbol indicates that a base is absent from that sequence but present in the other. All other nucleotides are identical.

```
BSS5B   1 AATCGGGCTG GAGAGTGCGA TGGAGAGCTT GAGTGTGAGT GGTGACAGGA  50
BSS5C   1 AATCGGGCTG GAGAGTGCGA TGGAGAGCTT GAGTGTGAGT GGTGACAGGA  50

BSS5B  51 TTCAAGG/AA GTGTTTGATG AAAAGGTTGA AGGTTGGGAA CATGTGGGAG  99
BSS5C  51 TTCAAGGTAA GTGTTTGATG AAAAGGTTGA AGGTTGGGAA CATGTGGGAG 100

BSS5B 101 TTTGAGAAAG TTGTTATCTT TTATGTTTTG TCTCGATATA TCGTTAGCGT 149
BSS5C 101 TTTGAGAAAG TTGTTATCTT TTATGTTTTG TCTCGATATA TCGTTAGCGT 150

BSS5B 151 TTGTGTTAAA AAAAAAAAA                                  168
BSS5C 151 TTGTGTTAAA AAAAAAAAA                                  169
```

Figure 39. Nucleotide sequence of the cDNA BSS6A, expressed as mRNA sequence. BSS6A was produced by reverse transcription of mRNA primed with 5'-T_{12}AA-3' followed by PCR amplification with the same oligo(dT) primer and the arbitrarily chosen decamer OPA4 (5'-AATCGGGCTG-3'). The decamer primer was identified and is singly underlined.The oligo(dT) primer was not identified.

```
  1  AATCGGGCTG  CAACCCACAG  ATTCATCTGT  TCAATAAATG  TGTGTATACC   50
 51  AAAACCTATA  ACCATAAAAG  AAGTAAAAAA  GAATCTCACG  CTGCACAAAG  100
101  CACCACCACT  ACTAGTAGTA  TATGGGTTAG  AAAGAACCAC  CAACTCTTAA  150
151  ACCCAAATAA  GACTCAAGAG  AGCAAAAACT  GGTAACACCA  AGAGAAGAAA  200
201  TGTGGAGACA  CGTCCTTTGT  GACCATTGCT  AGGTAGTATA  GAAAGAAAGC  250
251  GAAACATTCC  TGACCGTTGA  AAGAAACACT  TTGGTCCGGA  AACCCATCTC  300
301  CAGCCCCGAA  TT                                            312
```

Figure 40. Nucleotide sequence of the cDNA BSS6B, expressed as mRNA sequence. BSS6B was produced by reverse transcription of mRNA primed with 5'-T_{12}AA-3' followed by PCR amplification with the same oligo(dT) primer and the arbitrarily chosen decamer OPA4 (5'-AATCGGGCTG-3'). The primers used for the DD PCR are singly underlined while the specific primers used in the RT-PCR reactions are double underlined.

```
  1  AATCGGGCTG  CAAAGCAAAG  GGAGAAGGAC  TTACCGTGGC  CCACGGGAAG   50
 51  GAATCAATCC  TTAACATGTT  CAAAACCCAT  GCCAACATTG  GAGTTGATTT  100
101  TGTTCTGGAG  AAAGGAAGGA  GTCTGTCAAG  GAAAGAGCCG  GAGACGCAGT  150
151  TGGCAGCCAA  GTCTAAGAAA  TCAGCAGCTT  AAGCTTTCTT  TGTTTTTTTT  200
201  AGTCTCCTTT  TTGTGTTGTT  GAAAGAGATT  TTGTTTCATT  TTTCTCCATC  250
251  TTTGCAAGAC  GATAGATATG  TTTTTTGTTG  GATCTTTTGT  TCAGTGCTGC  300
301  TGTTTGATGA  TGGTTAAAAA  AAAAAAA                            327
```

Figure 41. Nucleotide sequence of the cDNA BSS6C, expressed as mRNA sequence. BSS6C was produced by reverse transcription of mRNA primed with 5'-T_{12}AA-3' followed by PCR amplification with the same oligo(dT) primer and the arbitrarily chosen decamer OPA4 (5'-AATCGGGCTG-3'). The decamer was identified and is singly underlined. The oligo(dT) primer was not identified.

```
  1  AATCGGGCTG  CAACCCACAG  ATTCATCTGT  TCAATAAATG  TGTGTATACC   50
 51  AAAACCTATA  ACCATAAAAG  AAGTAAAAAA  GAATCTCACG  CTGCACAAAG  100
101  CACCACCACT  ACTAGTAGTA  TATGGGTTAG  AAAGAACCAC  CAACTCTTAA  150
151  ACCCAAATAA  GACTCAAGAG  AGCAAAAACT  GGTAACACCA  AGAGAAGAAA  200
201  TGTGGAGACA  CGTCCTTTGT  GACCATTGCT  AGGTAGTATA  GAAAGAAGCG  250
251  AACATTCCTG  ACCGTTGAAA  GAACACTTTG  GTCCGAAACC  CATCTCCAGC  300
301  CCGATT                                                    306
```

Figure 42. Nucleotide sequence of the cDNA BSS6D, expressed as mRNA sequence. BSS6D was produced by reverse transcription of mRNA primed with 5'-T_{12}AA-3' followed by PCR amplification with the same oligo(dT) primer and the arbitrarily chosen decamer OPA4 (5'-AATCGGGCTG-3'). The oligo(dT) primer was identified and is singly underlined. The decamer was not identified.

```
  1  GGAAACTAAA  CCGGGAGGAG  AGACTAGTTG  CTGATATGGC  TGGGATGGAA   50
 51  ACTAAACCCA  GAGGAGAGAC  TAGTTGCTGA  TATGGCTGGG  ATGGAAACTA  100
101  AACCGGAGGA  GAAAAAGACA  AGTTAACCGG  GAATTACGCA  GACAAATTGG  150
151  TCTGTGCTTG  GGACTATGAT  CGATCTCTGA  TTGTAATTCT  TGTGAACTAA  200
201  GATCATATGC  TTTCTGTTGT  ATCGAATTGT  GCAACTTCAT  TTCTTATGTT  250
251  AATATGATGA  AACCTTAATT  AAAGGTGTTT  GCTGGGTTAA  AAAAAAAAAA  300
```

Figure 43. Nucleotide sequence of the cDNA BSS6E, expressed as mRNA sequence. BSS6E was produced by reverse transcription of mRNA primed with 5'-T_{12}AA-3' followed by PCR amplification with the same oligo(dT) primer and the arbitrarily chosen decamer OPA4 (5'-AATCGGGCTG-3'). The decamer was identified and is singly underlined. The oligo(dT) primer was not identified.

```
  1  AATCGGGCTG  CAAACCCACA  GGATTCATTC  TGTTCAATAA  ATGTGTGTAT   50
 51  ACCAAAACCT  ATAACCATAA  AAGGAAGTAA  AAAAGGAATT  CTCACGCTGC  100
101  ACAAAGCACC  ACCACTACTA  GTAGTATATG  GGTTAGAAAG  AACCACCAAC  150
151  TCTTAAACCC  AAATAAGACT  CAAGAGAGCA  AAAACTGGTA  ACACCAAGAG  200
201  AAGAAATGTA  GAGACACGTC  CTTTGTGACC  ATTGCTAGGT  AGTATAGAAG  250
251  GAAGCGAACA  TTCCTGACCG  TTGAAGAACA  CTTTGGTCGG  AAACCCATCT  300
301  CCAGCCCGAT  T                                            311
```

Figure 44. Sequence homology comparison of BSS6A, BSS6C and BSS6E. The / symbol indicates that a nucleotide was absent from that sequence but identified in another. The mismatched nucleotide is in bold text. All other nucleotides are identical.

```
BSS6A    1 AATCGGGCTG  CAA/CCCACA  /GATTCAT/C  TGTTCAATAA  ATGTGTGTAT  47
BSS6C    1 AATCGGGCTG  CAA/CCCACA  /GATTCAT/C  TGTTCAATAA  ATGTGTGTAT  47
BSS6E    1 AATCGGGCTG  CAAACCCACA  GGATTCATTC  TGTTCAATAA  ATGTGTGTAT  50

BSS6A   48 ACCAAAACCT  ATAACCATAA  AAG/AAGTAA  AAAAG/AATC  /TCACGCTGC  95
BSS6C   48 ACCAAAACCT  ATAACCATAA  AAG/AAGTAA  AAAAG/AATC  /TCACGCTGC  95
BSS6E   51 ACCAAAACCT  ATAACCATAA  AAGGAAGTAA  AAAAGGAATT  CTCACGCTGC 100

BSS6A  101 ACAAAGCACC  ACCACTACTA  GTAGTATATG  GGTTAGAAAG  AACCACCAAC 145
BSS6C  101 ACAAAGCACC  ACCACTACTA  GTAGTATATG  GGTTAGAAAG  AACCACCAAC 150
BSS6E  101 ACAAAGCACC  ACCACTACTA  GTAGTATATG  GGTTAGAAAG  AACCACCAAC 150

BSS6A  151 TCTTAAACCC  AAATAAGACT  CAAGAGAGCA  AAAACTGGTA  ACACCAAGAG 195
BSS6C  151 TCTTAAACCC  AAATAAGACT  CAAGAGAGCA  AAAACTGGTA  ACACCAAGAG 195
BSS6E  151 TCTTAAACCC  AAATAAGACT  CAAGAGAGCA  AAAACTGGTA  ACACCAAGAG 200

BSS6A  201 AAGAAATGTG  GAGACACGTC  CTTTGTGACC  ATTGCTAGGT  AGTATAGAAA 245
BSS6C  201 AAGAAATGTG  GAGACACGTC  CTTTGTGACC  ATTGCTAGGT  AGTATAGAAA 245
BSS6E  201 AAGAAATGTA  GAGACACGTC  CTTTGTGACC  ATTGCTAGGT  AGTATAGAAG 250

BSS6A  251 GAAAGCGAAA  CATTCCTGAC  CGTTGAAAGA  AACACTTTGG  TCCGGAAACC 295
BSS6C  251 GAA/GCGAA/-  CATTCCTGAC  CGTTGAAAGA  A/CACTTTGG  TCC/GAAACC 291
BSS6E  251 GAA/GCGAA/-  CATTCCTGAC  CGTTGAA/GA  A/CACTTTGG  TC/GGAAACC 295

BSS6A  296 CATCTCCAGC  CCCGAATT                                312
BSS6C  292 CATCTCCAG/  CCCGA/TT                                306
BSS6E  296 CATCTCCAG/  CCCGA/TT                                311
```

Figure 45. Nucleotide sequence of the cDNA BSS7A, expressed as mRNA sequence. BSS7A was produced by reverse transcription of mRNA primed with 5'-T_{12}AA-3' followed by PCR amplification with the same oligo(dT) primer and the arbitrarily chosen decamer OPA4 (5'-AATCGGGCTG-3'). The primers used for the DD PCR are singly underlined and the primers used for the RT-PCR reactions are double underlined.

```
  1  AATCGGGCTG  GAAAAGAAAA  TGTATCTGAA  AATTCAAACC  ACTAAGTGAA   50
 51  TTCGTATAGA  TCTTGTTTTA  TAGTATTTTG  GATTTTATGT  TTTACTTGAA  100
101  CTAAATCCAA  ACTCAAATGG  ATATTGATTA  AAAAAAAAAA              140
```

Figure 46. Nucleotide sequence of the cDNA BSS7B, expressed as mRNA sequence. BSS7B was produced by reverse transcription of mRNA primed with 5'-T_{12}AA-3' followed by PCR amplification with the same oligo(dT) primer and the arbitrarily chosen decamer OPA4 (5'-AATCGGGCTG-3'). The primers used for the DD PCR are singly underlined while the specific primers used in the RT-PCR reactions are double underlined.

```
  1  AATCGGGCTG  GAGGGTATTA  AATGACTTCT  TATCCAAATT  TTGAAACTTT   50
 51  GTTTGTTTCT  TTTGTAATGT  AGATGCTCCT  TAAAACTGAG  TGCTTTTAAT  100
101  TTTTATCAAG  ATCTTTGAAT  TTTGCGTTAA  AAAAAAAAAA              140
```

Figure 47. Nucleotide sequence of the cDNA BSS7C, expressed as mRNA sequence. BSS7C was produced by reverse transcription of mRNA primed with 5'-T_{12}AA-3' followed by PCR amplification with the same oligo(dT) primer and the arbitrarily chosen decamer OPA4 (5'-AATCGGGCTG-3'). The primers used for the differential display PCR are singly underlined.

```
  1  AATCGGGCTG  GAGGGTATTA  AATGACTTCT  TATCCAAATT  TTGAAACTTT   50
 51  GTTTGTTTCT  TTTGTAATGT  AGATGCTCCT  TAAAACTGAG  TGCTTTTAAT  100
101  TTTTATCAAG  ATCTTTGAAT  TTTGCGTTAA  AAAAAAAAAA              140
```

Figure 48. Nucleotide sequence of the cDNA BSS7D, expressed as mRNA sequence. BSS7D was produced by reverse transcription of mRNA primed with 5'-T_{12}AA-3' followed by PCR amplification with the same oligo(dT) primer and the arbitrarily chosen decamer OPA4 (5'-AATCGGGCTG-3'). The primers used for the DD PCR are singly underlined while the specific primers used in the RT-PCR reactions are double underlined.

```
  1  AATCGGGCTG  TGTTGAAGTA  TTGAAGTAAA  GGATGATCCA  ACTTGTGAAA   50
 51  AAGAATAATT  TTTGGATAAA  TGAGAAGTTA  CTTGAGAACT  TTGAAGTTCA  100
101  CATGAGTGAG  ATAGAAGTAG  TTGCTTAAAA  AAAAAAAA               138
```

Figure 49. Nucleotide sequence of the cDNA BSS7E, expressed as mRNA sequence. BSS7E was produced by reverse transcription of mRNA primed with 5'-T_{12}AA-3' followed by PCR amplification with the same oligo(dT) primer and the arbitrarily chosen decamer OPA4 (5'-AATCGGGCTG-3'). The primers used for the DD PCR are singly underlined.

```
  1  AATCGGGCTG  ATGATGAGTG  AAAGTGTCTC  TTTTTTACTA  CTACAAATGA    50
 51  TGGAATAAAT  TAGAAAATGA  TTTTATTTTT  CCTTGTCGTA  ATTGTGTTGG   100
101  ACTTTTTGGA  ATAAATGATT  TTGGTTAAAA  AAAAAAAA                 138
```

Figure 50. Nucleotide sequence of the cDNA BSS7F, expressed as mRNA sequence. BSS7F was produced by reverse transcription of mRNA primed with 5'-T_{12}AA-3' followed by PCR amplification with the same oligo(dT) primer and the arbitrarily chosen decamer OPA4 (5'-AATCGGGCTG-3'). Neither the decamer not the oligo(dT) primer was identified.

```
  1  AATGCCTGGG  GTGCCTAATG  AGTGAGCTAA  CTCACATTAA  TTGCGTTGCG   50
 51  CTCACTGCCC  GCTTTCCAGT  CGGGAAACCT  GTCGTGCCAG  CTGCATTAAT  100
101  GAAACGGCCA  ACGCGCGGGG  AGAGGCGGTT  TGCGTATTGG  GCGCTCTTCC  150
151  GCTTCCTCGC  TCACTGACTC  GCTGCGCTCG                          180
```

Figure 51. Sequence homology comparison of cDNAs BSS7B and BSS7C. These sequences are identical.

```
BSS7B     1 AATCGGGCTG GAGGGTATTA AATGACTTCT TATCCAAATT TTGAAACTTT  50
BSS7C     1 AATCGGGCTG GAGGGTATTA AATGACTTCT TATCCAAATT TTGAAACTTT  50

BSS7B    51 GTTTGTTTCT TTTGTAATGT AGATGCTCCT TAAAACTGAG TGCTTTTAAT 100
BSS7C    51 GTTTGTTTCT TTTGTAATGT AGATGCTCCT TAAAACTGAG TGCTTTTAAT 100

BSS7B   101 TTTTATCAAG ATCTTTGAAT TTTGCGTTAA AAAAAAAAAA            140
BSS7C   101 TTTTATCAAG ATCTTTGAAT TTTGCGTTAA AAAAAAAAAA            140
```

Figure 52. Nucleotide sequence of the cDNA BSS8A, expressed as mRNA sequence. BSS8A was produced by reverse transcription of mRNA primed with 5'-T_{12}AA-3' followed by PCR amplification with the same oligo(dT) primer and the arbitrarily chosen decamer OPA6 (5'-GGTCCCTGAC-3'). The decamer primers used for the DD PCR was identified however three nucleotides were absent from its 5' end. The truncated decamer and the oligo(dT) primers are singly underlined while the specific primers used in the RT-PCR reactions are double underlined.

```
  1  CCCTGACAAA  GAAGCTGACA  AGATCGACTC  TTGCTCTCTT  GCCATTGAAT   50
 51  ACGTTTTCAA  TCATCCTATG  TCTAGCTAAA  CACACCTGTG  TGCTATCTCT  100
101  CTCATGTTTT  TTGTTCTTTC  CTGATCATGG  GACTTCCAAA  TAAATTCAGT  150
151  TCTGTTTTGA  GAGTCATTCT  TGACTCTTTT  AACAGTGTCC  TTAAGAAACT  200
201  CTTATGCCTT  AAAAAAAAAA  AA                               222
```

Figure 53. Nucleotide sequence of the cDNA BSS8B, expressed as mRNA sequence. BSS8B was produced by reverse transcription of mRNA primed with 5'-T_{12}AA-3' followed by PCR amplification with the same oligo(dT) primer and the arbitrarily chosen decamer OPA6 (5'-GGTCCCTGAC-3'). The primers used for the DD PCR are singly underlined.

```
  1  GGTCCCTGAC  AAAGAAGCTG  ACAAGATCGA  CTCTTGCTCT  CTTGCCATTG   50
 51  AATACGTTTT  CAATCATCCT  ATGTCTAGCT  AAACACACCT  GTGTGCTATC  100
101  TCTCTCATGT  TTTTTGTTCT  TTCCTGATCA  TGGGACTTCC  AAATAAATTC  150
151  AGTTCTGTTT  TGAGAGTCAT  TCTTGACTCT  TTTAACAGTG  TCCTTAAGAA  200
201  ACTCTTATGC  CTTAAAAAAA  AAAAA                             225
```

Figure 54. Nucleotide sequence of the cDNA BSS8C, expressed as mRNA sequence. BSS8C was produced by reverse transcription of mRNA primed with 5'-T_{12}AA-3' followed by PCR amplification with the same oligo(dT) primer and the arbitrarily chosen decamer OPA6 (5'-GGTCCCTGAC-3'). The oligo(dT) primer was identified and is singly underlined. The decamer was not identified.

```
  1  GTGTTTACTG  TGCATTGCGG  AACTACATTT  CTTTTATCAT  TTTTATTTTC   50
 51  TTGTTGATAA  CAACGCTTGG  TTTGTTGTAA  GAAGAATTAG  TAGTTCACAT  101
101  GAATGGACAT  TCAAGTTTGG  TTAAAAAAAA  AAAA                    134
```

Figure 55. Sequence Homology Comparison of cDNAs BSS8A, BSS8B and BSS3A. These sequences are identical except for the three extra nucleotides at the 5' end of BSS8B.

```
BSS8A   1    CCCTGACAAA GAAGCTGACA AGATCGACTC TTGCTCTCTT GCCATTGAAT  50
BSS8B   1GGTCCCTGACAAA GAAGCTGACA AGATCGACTC TTGCTCTCTT GCCATTGAAT  53
BSS3A   1    CCCTGACAAA GAAGCTGACA AGATCGACTC TTGCTCTCTT GCCATTGAAT  50

BSS8A  51  ACGTTTTCAA TCATCCTATG TCTAGCTAAA CACACCTGTG TGCTATCTCT 100
BSS8B  54  ACGTTTTCAA TCATCCTATG TCTAGCTAAA CACACCTGTG TGCTATCTCT 103
BSS3A  51  ACGTTTTCAA TCATCCTATG TCTAGCTAAA CACACCTGTG TGCTATCTCT 100

BSS8A 101  CTCATGTTTT TTGTTCTTTC CTGATCATGG GACTTCCAAA TAAATTCAGT 150
BSS8B 104  CTCATGTTTT TTGTTCTTTC CTGATCATGG GACTTCCAAA TAAATTCAGT 153
BSS3A 101  CTCATGTTTT TTGTTCTTTC CTGATCATGG GACTTCCAAA TAAATTCAGT 150

BSS8A 151  TCTGTTTTGA GAGTCATTCT TGACTCTTTT AACAGTGTCC TTAAGAAACT 200
BSS8B 151  TCTGTTTTGA GAGTCATTCT TGACTCTTTT AACAGTGTCC TTAAGAAACT 203
BSS3A 151  TCTGTTTTGA GAGTCATTCT TGACTCTTTT AACAGTGTCC TTAAGAAACT 200

BSS8A 201  CTTATGCCTT AAAAAAAAAA AA                              222
BSS8B 201  CTTATGCCTT AAAAAAAAAA AA                              225
BSS3A 201  CTTATGCCTT AAAAAAAAAA AA                              223
```

Figure 56. Nucleotide sequence of the cDNA BSS9A, expressed as mRNA sequence. BSS9A was produced by reverse transcription of mRNA primed with 5'-T_{12}AA-3' followed by PCR amplification with the same oligo(dT) primer and the arbitrarily chosen decamer OPA4 (5'-AATCGGGCTG-3'). The primers used for the differential display PCR were not identified.

```
 1   TTGTTGTAAA  ACGACGGCCA  GTGAATTGTA  ATACGACTCA  CTATAGGGCG  50
51   AATTGGGCCC  GACGTCGCAT  GCTCCCGGCC  GCCATGGCCG  CGGGAT      96
```

Figure 57. Nucleotide sequence of the cDNA BSS9B, expressed as mRNA sequence. BSS9B was produced by reverse transcription of mRNA primed with 5'-T_{12}AA-3' followed by PCR amplification with the same oligo(dT) primer and the arbitrarily chosen decamer OPA4 (5'-AATCGGGCTG-3'). The primers used for the differential display PCR were not identified.

```
 1   AAAAAAAAAA  AAAAAAACCA  AAGGGTTTGG  ATTATGGTCC  CTGACGTTAG   50
51   TATA                                                        54
```

Figure 58. Nucleotide sequence of the cDNA BSS9C, expressed as mRNA sequence. BSS9C was produced by reverse transcription of mRNA primed with 5'-T_{12}AA-3' followed by PCR amplification with the same oligo(dT) primer and the arbitrarily chosen decamer OPA4 (5'-AATCGGGCTG-3'). The oligo(dT) primer was identified and is singly underlined. The decamer was not identified.

```
  1  TATTTTTAAG  ATGAAAGCCC  CCGTTCTTGG  ATTTGGGAGG  GCGTTGGCTC   50
 51  TTTGGTTCAG  AGTTAGGGTT  ATTTGGATTT  TCGGGTACAG  TGTTTCTATG  100
101  TTGAAAAATA  TATATTTTTT  AATATATTCT  ACTCTCTTCT  CGTTAAAAAA  150
151  AAAAAA                                                   156
```

Figure 59. Nucleotide sequence of the cDNA BSS9D, expressed as mRNA sequence. BSS9D was produced by reverse transcription of mRNA primed with 5'-T_{12}AA-3' followed by PCR amplification with the same oligo(dT) primer and the arbitrarily chosen decamer OPA4 (5'-AATCGGGCTG-3'). Two oligo(dT) primers have been identified and are singly underlined. The decamer was not identified.

```
  1  AATACTCAAG  CTATGCATCC  AACGCGTTGG  GAGCTCTCCC  ATATGGTCGA   50
 51  CCTGCAGGCG  GCGGTGGCGG  ATGAGACGGT  GGTGGAGAGC  GGAGGAGGAG  100
101  GATCGCCGAG  AGGAAAGTGT  TTGAAGTCTT  GTTTTGTTGG  TATGATAGTT  150
151  CGAGAGATCA  GACCAGTTCT  TAGTTGATTG  CTATTGTTAA  AAAAAAAAAA  200
201  AACTTATTTT  TAAGATGAAA  GCCCCCGTTC  TTGGATTTGG  GAGGGCGTTG  250
251  GCTTTTTGGT  TCAGANTTAG  GGTTATTTGG  ATTTTCGGGT  ACAGTGTTTC  300
301  TATGTTTGAA  AAATATATAT  NTTTTAATAT  ATTCTACTCT  CTTCTCGTTA  350
351  AAAAAAAAAA  A                                            361
```

Figure 60. Nucleotide sequence of the cDNA BSS11A, expressed as mRNA sequence. BSS11A was produced by reverse transcription of mRNA primed with 5'-T_{12}AA-3' followed by PCR amplification with the same oligo(dT) primer and the arbitrarily chosen decamer OPA4 (5'-AATCGGGCTG-3'). The primers used for the DD PCR are singly underlined while the specific primers used in the RT-PCR reactions are double underlined.

```
  1  AATCGGGCTG  AAGAACGGGA  ACCATAGCTC  ATGGAACCAC  CACTTCTATT   50
 51  CGGTGTTTAG  GGTTTATTCC  TTTAAATCTA  CGACTCTTCA  TGTACCTTGG  100
101  AACCTGTCTT  AGCTTCTCAG  TTGAGTATTA  ACAAATTCTT  AGTTGTAATC  150
151  TTGATTTATT  TTATCTTTGT  CCTAGGAATG  ATTTGTGCCT  GAGTGAATAC  200
201  TGGTTTAACA  GTTAAGCTTG  TTTCTGTTTA  TGAGTTTAGT  TTCAGCAGTG  250
251  GAGGAGGTTT  GTTCTGTTAA  AAAAAAAAAA                         280
```

Figure 61. Nucleotide sequence of the cDNA BSS11B, expressed as mRNA sequence. BSS11B was produced by reverse transcription of mRNA primed with 5'-T_{12}AA-3' followed by PCR amplification with the same oligo(dT) primer and the arbitrarily chosen decamer OPA4 (5'-AATCGGGCTG-3'). The primers used for the DD PCR are singly underlined.

```
  1  AATCGGGCTG  AAGAACGGGA  ACCATAGCTC  ATGGAACCAC  CACTTCTATT   50
 51  CGGTGTTTAG  GGTTTATTCC  TTTAAATCTA  CGACTCTTCA  TGTACCTTGG  100
101  AACCTGTCTT  AGCTTCTCAG  TTGAGTATTA  ACAAATTCTT  AGTTGTAATC  150
151  TTGATTTATT  TTATCTTTGT  CCTAGGAATG  ATTTGTGCCT  GAGTGAATAC  200
201  TGGTTTAACA  GTTAAGCTTG  TTTCTGTTTA  TGAGTTTAGT  TTCAGCAGTG  250
251  GAGGAGGTTT  GTTCTGTTAA  AAAAAAAAAA                         280
```

Figure 62. Nucleotide sequence of the cDNA BSS11C, expressed as mRNA sequence. BSS11C was produced by reverse transcription of mRNA primed with 5'-T_{12}AA-3' followed by PCR amplification with the same oligo(dT) primer and the arbitrarily chosen decamer OPA4 (5'-AATCGGGCTG-3'). The primers used for the DD PCR are singly underlined.

```
  1  AATCGGGCTG  AAGAACGGAA  CCATAGCTCA  TGGAACCACC  ACTTCTATTC   50
 51  GGTGTTTAGG  GTTTATTCCT  TTAAATCTAC  GACTCTTCAT  GTACCTTGGA  100
101  ACCTGTCTTA  GCTTCTCAGT  TGAGTATTAA  CAAATTCTTA  GTTGTAATCT  150
151  TGATTTATTT  TATCTTTGTC  CTAGGAATGA  TTTGTGCCTG  AGTGAATACT  200
201  GGTTTAACAG  TTAAGCTTGT  TTCTGTTTAT  GAGTTTAGTT  TCAGCACTGG  250
251  AAGAGTTTGT  TCTGTTAAAA  AAAAAAAA                          278
```

Figure 63. Sequence homology comparison of cDNAs BSS11A, BSS11B and BSS11C. The / symbol indicates that a base was absent in one sequence but identified in another. All other nucleotides are identical.

```
BSS11A    1 AATCGGGCTG  AAGAACGGGA  ACCATAGCTC  ATGGAACCAC  CACTTCTATT     50
BSS11B    1 AATCGGGCTG  AAGAACGGGA  ACCATAGCTC  ATGGAACCAC  CACTTCTATT     50
BSS11C    1 AATCGGGCTG  AAGAACGG/A  ACCATAGCTC  ATGGAACCAC  CACTTCTATT     49

BSS11A   51 CGGTGTTTAG  GGTTTATTCC  TTTAAATCTA  CGACTCTTCA  TGTACCTTGG    100
BSS11B   51 CGGTGTTTAG  GGTTTATTCC  TTTAAATCTA  CGACTCTTCA  TGTACCTTGG    100
BSS11C   50 CGGTGTTTAG  GGTTTATTCC  TTTAAATCTA  CGACTCTTCA  TGTACCTTGG     99

BSS11A  101 AACCTGTCTT  AGCTTCTCAG  TTGAGTATTA  ACAAATTCTT  AGTTGTAATC    150
BSS11B  101 AACCTGTCTT  AGCTTCTCAG  TTGAGTATTA  ACAAATTCTT  AGTTGTAATC    150
BSS11C  100 AACCTGTCTT  AGCTTCTCAG  TTGAGTATTA  ACAAATTCTT  AGTTGTAATC    149

BSS11A  151 TTGATTTATT  TTATCTTTGT  CCTAGGAATG  ATTTGTGCCT  GAGTGAATAC    200
BSS11B  151 TTGATTTATT  TTATCTTTGT  CCTAGGAATG  ATTTGTGCCT  GAGTGAATAC    200
BSS11C  150 TTGATTTATT  TTATCTTTGT  CCTAGGAATG  ATTTGTGCCT  GAGTGAATAC    199

BSS11A  201 TGGTTTAACA  GTTAAGCTTG  TTTCTGTTTA  TGAGTTTAGT  TTCAGCAGTG    250
BSS11B  201 TGGTTTAACA  GTTAAGCTTG  TTTCTGTTTA  TGAGTTTAGT  TTCAGCAGTG    250
BSS11C  200 TGGTTTAACA  GTTAAGCTTG  TTTCTGTTTA  TGAGTTTAGT  TTCAGCACTG    249

BSS11A  251 GA/GGAGGTT  TGTTCTGTTA  AAAAAAAAAA  A                        280
BSS11B  251 GA/GGAGGTT  TGTTCTGTTA  AAAAAAAAAA  A                        280
BSS11C  250 GAAG/AG/TT  TGTTCTGTTA  AAAAAAAAAA  A                        278
```

Figure 64. Nucleotide sequence of the cDNA BSS12A, expressed as mRNA sequence. BSS12A was produced by reverse transcription of mRNA primed with 5'-T_{12}AA-3' followed by PCR amplification with the same oligo(dT) primer and the arbitrarily chosen decamer OPA4 (5'-AATCGGGCTG-3'). The primers used for the DD PCR are singly underlined while the specific primers used in the RT-PCR reactions are double underlined.

```
  1  AATCGGGCTG  GGTCGGATAG  CTTGCTCACG  TTAGGCTCGT  TTAGGAAGCT   50
 51  CAAGGAGCGT  TATTTCCCTG  GGTCGACTGA  GAAGTACACT  GGAGTGTTGT  100
101  ATGGTCTAGG  TGTGGAAGAT  TCTGCTACTA  GTACTGCTAT  TGCTAATTAA  150
151  GTTTAAGAGG  GGATTTGCAT  TAGGTTTTTT  CAGTCTTTGT  GAGTGTGAGA  200
201  CCAATTAGAC  TTTGTGTTTG  TGTAAATTGG  TTTTAGTAAG  TGAATGTTGT  250
251  TTCCTTTGTT  AAAAAAAAAA  AA                               262
```

Figure 65. Nucleotide sequence of the cDNA BSS12B, expressed as mRNA sequence. BSS12B was produced by reverse transcription of mRNA primed with 5'-T_{12}AA-3' followed by PCR amplification with the same oligo(dT) primer and the arbitrarily chosen decamer OPA4 (5'-AATCGGGCTG-3'). The primers used for the DD PCR are singly underlined while the specific primers used in the RT-PCR reactions are double underlined.

```
  1  AATCGGGCTG  GTGATGACCC  AAGCTGGAGT  GTCTAAGCCA  AGGGCTGTTA   50
 51  AGGCTCTCAA  GGCTGCTGAT  GGAGATATTG  TATCTGCTAT  CATGGAGCTT  100
101  ACTACCTAAA  ATCCTAAAAC  CTTTTCTCTA  CTTAGATGCT  GCTGTTTCTC  150
151  GTGTGTTTTC  TTTGTCTTGT  TTACCAGTCA  GAATGTAAGT  GACTTTTCAT  200
201  GTTGGGTCAT  TATGAGTTTA  GACTCTTTTA  ATATTACATA  AGATAAATTG  250
251  TTGTTAAAAA  AAAAAAA                                      267
```

Figure 66. Nucleotide sequence of the cDNA BSS12C, expressed as mRNA sequence. BSS12C was produced by reverse transcription of mRNA primed with 5'-T_{12}AA-3' followed by PCR amplification with the same oligo(dT) primer and the arbitrarily chosen decamer OPA4 (5'-AATCGGGCTG-3'). The primers used for the DD PCR are singly underlined.

```
  1  AATCGGGCTG  GTGATGACCC  AAGCTGGAGT  GTCTAAGCCA  AGGGCTGTTA   50
 51  AGGCTCTCAA  GGCTGCTGAT  GGAGATATTG  TATCTGCTAT  CATGGAGCTT  100
101  ACTACCTAAA  ATCCTAAAAC  CTTTTCTCTA  CTTAGATGCT  GCTGTTTCTC  150
151  GTGTGTTTTC  TTTGTCTTGT  TTACCAGTCA  GAATGTAAGT  GACTTTTCAT  200
201  GTTGGGTCAT  TATGAGTTTA  GACTCGTTTT  AATATTACAT  AAGATAAATT  250
251  GTTGTTAAAA  AAAAAAAA                                     268
```

Figure 67. Sequence Alignment Comparison of cDNAs BSS12B and BSS12C. The / symbol indicates that a base was absent from one sequence but identified in another. All other nucleotides are identical.

```
BSS12B     1  AATCGGGCTG GTGATGACCC AAGCTGGAGT GTCTAAGCCA AGGGCTGTTA   50
BSS12C     1  AATCGGGCTG GTGATGACCC AAGCTGGAGT GTCTAAGCCA AGGGCTGTTA   50

BSS12B    51  AGGCTCTCAA GGCTGCTGAT GGAGATATTG TATCTGCTAT CATGGAGCTT  100
BSS12C    51  AGGCTCTCAA GGCTGCTGAT GGAGATATTG TATCTGCTAT CATGGAGCTT  100

BSS12B   101  ACTACCTAAA ATCCTAAAAC CTTTTCTCTA CTTAGATGCT GCTGTTTCTC  150
BSS12C   101  ACTACCTAAA ATCCTAAAAC CTTTTCTCTA CTTAGATGCT GCTGTTTCTC  150

BSS12B   151  GTGTGTTTTC TTTGTCTTGT TTACCAGTCA GAATGTAAGT GACTTTTCAT  200
BSS12C   151  GTGTGTTTTC TTTGTCTTGT TTACCAGTCA GAATGTAAGT GACTTTTCAT  200

BSS12B   201  GTTGGGTCAT TATGAGTTTA GACTCTTTT/ AATATTACAT AAGATAAATT  249
BSS12C   201  GTTGGGTCAT TATGAGTTTA GACTCGTTTT AATATTACAT AAGATAAATT  250

BSS12B   250  GTTGTTAAAA AAAAAAAA                                     267
BSS12C   251  GTTGTTAAAA AAAAAAAA                                     268
```

Figure 68. Nucleotide sequence of the cDNA BSS13A, expressed as mRNA sequence. BSS13A was produced by reverse transcription of mRNA primed with 5'-T_{12}AA-3' followed by PCR amplification with the same oligo(dT) primer and the arbitrarily chosen decamer OPA4 (5'-AATCGGGCTG-3'). The primers used for the DD PCR are singly underlined while the specific primers used in the RT-PCR reactions are double underlined.

```
  1  AATCGGGCTG  ATGGTGACCA  GAAGGAGTTT  GCCAAGCAAG  AGCTTCAATT   51
 51  AGGAAGCTTC  CCGACGATAC  TTCTCTTCCC  GAAAAGCGCT  CCGCGGGCAA  101
101  TTAAGTACCC  GTCAGAGCAT  AGAGATGTGG  ATTCGCTTAT  GTCATTTGTG  151
151  AATCTTCTCC  GGTGAGTTGT  GAGATTGGAA  AGTTAAACAG  TGTGATGAAG  201
201  AATGAAGAAA  ACTGAGATGA  AAGATGATTT  CATCTTGAGA  TTGTGGATTC  251
251  TAAAAGAATA  AGCAAAAGCT  ATGTGTTCTA  TATGGTATTG  TAGGGTTTAT  301
301  AGACCCCTTT  CCTGTCTCCC  TGTCCGTTAA  AAAAAAAAAA             340
```

Figure 69. Nucleotide sequence of the cDNA BSS13B, expressed as mRNA sequence. BSS13B was produced by reverse transcription of mRNA primed with 5'-T_{12}AA-3' followed by PCR amplification with the same oligo(dT) primer and the arbitrarily chosen decamer OPA4 (5'-AATCGGGCTG-3'). The primers used for the DD PCR are singly underlined.

```
  1  AATCGGGCTG  ATGGTGACCA  GAAGGAGTTT  GCCAAGCAAG  AGCTTCAATT   50
 51  AGGAAGCTTC  CCGACGATAC  TTCTCTTCCC  GAAAAGCGCT  CCGCGGGCAA  100
101  TTAAGTACCC  GTCAGAGCAT  AGAGATGTGG  ATTCGCTTAT  GTCATTTGTG  150
151  AATCTTCTCC  GGTGAGTTGT  GAGATTGGAA  AGTTAAACAG  TGTGATGAAG  200
201  AATGAAGAAA  ACTGAAGATG  AAGGATGATT  TCATCTTGAG  ATTGTGGATC  250
251  TAAAAGAATA  AGCAAAAGCT  ATGTGTTCTA  TATGGTATTG  TAGGGTATAT  300
301  AGACTCTTTT  TCTGCCTTCT  TGTCGTTAAA  AAAAAAAAA              339
```

Figure 70. Nucleotide sequence of the cDNA BSS13C, expressed as mRNA sequence. BSS13C was produced by reverse transcription of mRNA primed with 5'-T_{12}AA-3' followed by PCR amplification with the same oligo(dT) primer and the arbitrarily chosen decamer OPA4 (5'-AATCGGGCTG-3'). The primers used for the DD PCR are singly underlined.

```
  1  AATCGGGCTG  ATGGTGACCA  GAAGGAGTTT  GCCAAGCAAG  AGCTTCAATT   50
 51  AGGAGCTTCC  CAACGGATAC  TTCTCTTTCC  CAAAAGCGCT  CCACGCGCAA  100
101  TTAAGTACCC  GTCAGAGCAT  AGAGATGTCG  ATTCGCTTAT  GTCATTTGTG  150
151  AATCTTCTCC  GGTGAGTTGT  GAGATTGGAA  AGTTAAACAG  TGTGATGAAG  200
201  AATGAAGAAA  ACTAAGATGA  AGGATGATTT  CATCTTGAGA  TTGTGGATCT  250
251  AAAAGAATAA  GCAAAAGCTA  TGTGTTCTAT  ATGGTATTGT  AGGGTATATA  300
301  GACTCTTTTT  CTGCCTTCTT  GTCGTTAAAA  AAAAAAAA              338
```

Figure 71. Sequence homology comparison of cDNAs BSS13A, BSS13B and BSS13C. The / symbol indicates that a nucleotide was absent from one sequence but identified in another. Mismatched nucleotides are in bold text. All other nucleotides are identical.

```
BSS13A    1 AATCGGGCTG ATGGTGACCA GAAGGAGTTT GCCAAGCAAG AGCTTCAATT   50
BSS13B    1 AATCGGGCTG ATGGTGACCA GAAGGAGTTT GCCAAGCAAG AGCTTCAATT   50
BSS13C    1 AATCGGGCTG ATGGTGACCA GAAGGAGTTT GCCAAGCAAG AGCTTCAATT   50

BSS13A   51 AGGAAGCTTC CCGACG/ATA CTTCTCTTCC CGAAAAGCGC TCCGCGGGCA   99
BSS13B   51 AGGAAGCTTC CCGACG/ATA CTTCTCTTCC CGAAAAGCGC TCCGCGGGCA   99
BSS13C   51 AGGA/GCTTC CCAACGGATA CTTCTCTTTC CCAAAAGCGC TCCACGCGCA   99

BSS13A  100  ATTAAGTACC CGTCAGAGCA TAGAGATGTG GATTCGCTTA TGTCATTTGT  149
BSS13B  100  ATTAAGTACC CGTCAGAGCA TAGAGATGTG GATTCGCTTA TGTCATTTGT  149
BSS13C  100  ATTAAGTACC CGTCAGAGCA TAGAGATGTC GATTCGCTTA TGTCATTTGT  149

BSS13A  150  GAATCTTCTC CGGTGAGTTG TGAGATTGGA AAGTTAAACA GTGTGATGAA  199
BSS13B  150  GAATCTTCTC CGGTGAGTTG TGAGATTGGA AAGTTAAACA GTGTGATGAA  199
BSS13C  150  GAATCTTCTC CGGTGAGTTG TGAGATTGGA AAGTTAAACA GTGTGATGAA  199

BSS13A  200  GAATGAAGAA AACTGA/GAT GAAAG/ATGA TTTCATCTTG AGATTGTGGA  247
BSS13B  200  GAATGAAGAA AACTGAAGAT GAA/GGATGA TTTCATCTTG AGATTGTGGA  248
BSS13C  200  GAATGAAGAA AACT/AAGAT GAA/GGATGA TTTCATCTTG AGATTGTGGA  247

BSS13A  248  TTCTAAAAGA ATAAGCAAAA GCTATGTGTT CTATATGGTA TTGTAGGGTT  297
BSS13B  249  T/CTAAAAGA ATAAGCAAAA GCTATGTGTT CTATATGGTA TTGTAGGGTA  297
BSS13C  248  T/CTAAAAGA ATAAGCAAAA GCTATGTGTT CTATATGGTA TTGTAGGGTA  296

BSS13A  298  TATAGACCCC TTTCCTGTCT CCCTGTCCGT TAAAAAAAAA AAA        340
BSS13B  298  TATAGACTCT TTTTCTGCCT TCTTGTC/GT TAAAAAAAAA AAA        339
BSS13C  297  TATAGACTCT TTTTCTGCCT TCTTGTC/GT TAAAAAAAAA AAA        338
```

Figure 72. Nucleotide sequence of *Brassica* sucrose control (BSC) 100A, expressed as mRNA sequence, which appeared to be present at similar amounts in the 2 %, 6 % and 14 % sucrose cultures. BSC100A was produced by reverse transcripton of mRNA with 5'-T_{12}AA-3' followed by PCR amplification using the same oligo(dT) primer and the arbitrarily chosen decamer OPA5 (5'-AGGGGTCTTG-3'). Only the oligo(dT) primer was identified and is singly underlined. The decamer was not identified.

```
  1  GTCTTGTTGT  CTTGGAGTCT  AGGGGTCTTG  GAGTGTTGTA  GTACTTGTAG   50
 51  TACTTGTGTC  ACGGAGTAAA  AGTTTCTAGT  GGAGTCTAGT  AGTCTTGTAG  100
101  TGTTGTTGTA  GTCTTCTACT  ACTTGTGTCA  CGGGTCTTGT  ATCAGACTTT  150
151  GGTCCCAATC  TCTATATATT  AAGTCATGTT  GCGTTAAAAA  AAAAAAA     197
```

Figure 73. Nucleotide sequence of *Brassica* sucrose control (BSC) 100B, expressed as mRNA sequence, which appeared to be present at similar amounts in the 2 %, 6 % and 14 % sucrose cultures. BSC100B was produced by reverse transcripton of mRNA with 5'-T_{12}AA-3' followed by PCR amplification using the same oligo(dT) primer and the arbitrarily chosen decamer OPA5 (5'-AGGGGTCTTG-3'). The decamer and oligo(dT) primers were identified and are singly underlined. The specific primers used for the RT-PCR analysis are double underlined.

```
  1  AGGGGGTCTT  GGTGTTAGGT  CAAGGAGATT  TGCTCTGTTG  ATTGATAACC   50
 51  TCAAGGTCAC  TGTTGCCAAC  GTTGAATCTG  GCGGCGAGTT  CACAATTTCC  100
101  AGCGCCGATG  ATATCCTCAA  GGCTCTCTAA  GAAACTCTTT  ATCGTTTGTG  150
151  TGTATGAAGA  ACCGCTGCAT  GTGATAGTAG  TAAAGAGTGT  ATCTCTGGCC  200
201  TGATTTCTGA  CGATAAATTA  GGAATAATAA  TAAAGCGGTT  TAAGTTGCTT  250
251  AAAAAAAAAA  AA                                           262
```

insert in all these clones was approximately 300 bp. The values obtained for length after sequencing were, however, significantly shorter as they were 211, 138, 159, 200, 246 and 200 nucleotides in length (Figures 8 - 13). The appropriate primer combination (decamer at one end and 5'-T_{12}AA-3' at the other) was identified in five, BSS1A, BSS1C, BSS1D, BSS1E and BSS1F of the six clones. The decamer OPA1 was identified in BSS1B but the oligo(dT) primer was not detected. The clones BSS1A, BSS1B, BSS1C, BSS1D, BSS1E and BSS1F were all significantly shorter than the 300 bases predicted from the differential display gel. The lack of specific primer sequences in BSS1B suggests that it is an artifact of the PCR reaction. The clones BSS1D and BSS1F were found to be identical sequences (Figure 14), and therefore these two cDNAs must represent the same mRNA.

Six independent clones were sequenced for fragment BSS2 and identified as BSS2A, BSS2B, BSS2C, BSS2D, BSS2E and BSS2F (Figures 15 - 20). When using colony PCR the size of the insert in all the clones was approximately 220 bases. Upon sequencing the BSS2 fragments were all about the correct size as they were 250, 214, 212, 212, 212 and 202 bases in length. The clones BSS2B, BSS2C, BSS2D and BSS2E all encoded OPA1 and 5'-T_{12}AA-3'. The clone BSS2F did not encode the decamer OPA1 while BSS2A did not encode 5'-T_{12}AA-3' suggesting that these two fragments most likely represented PCR artifacts. The three cDNA clones BSS2C, BSS2D and BSS2E were all identical (Figure 21) suggesting that they all represent the same mRNA sequence. The clone BSS2B was identical to BSS1A (Figure 22). It remains to be determined whether or not this could have been the result of contamination during the cloning process.

Three clones were sequenced for BSS3 and are identified as BSS3A, BSS3B and BSS3C. When using colony PCR the insert size in all three clones was approximately 200 bases. Sequencing analysis revealed that the length for these fragments was 222, 200 and 201 bases in length (Figures 23 - 25). Althought the correct primer combination of OPA1 and 5'-T_{12}AA-3' was identified for the cDNA fragments BSS3B and BSS3C, the cDNA BSS3A lacked the decamer sequence, suggesting that it was an artifact of the PCR reaction. The sequences encoded by fragments BSS3B, BSS3C, BSS1D and BSS1F were nearly identical (Figure 26) suggesting that they all represent the same mRNA.

Six independant clones were sequenced for BSS4 and are identified as BSS4A, BSS4B, BSS4C, BSS4D, BSS4E and BSS4F. When using colony PCR to determine the sizes of these clones, all were approximately 150 bases. Sequencing analysis revealed that the actual sizes were 139, 139, 139, 87, 86 and 287 bases (Figure 27 - 32). The clones BSS4A, BSS4B and BSS4C were identical (Figure 33), had both primers, OPE3 and 5'-T_{12}AA-3' and were close to the approximated size of 150 bases. The fragments BSS4D and BSS4E were identical (Figure 34), but this sequence had poly A stretches at each end and were significantly shorter than expected. The sequences BSS4D and BSS4E were likely artifacts of the PCR reaction. The clone BSS4F was significantly longer than expected and had neither the correct decamer sequence nor 5'-T_{12}AA-3', indicating that it was also a PCR artifact.

Three independant clones were sequenced for BSS5 and were identified as BSS5A, BSS5B and BSS5C of 174, 168 and 169 bases respectively (Figure 35 - 37). All three clones sequenced for BSS5 had the decamer OPA4 and 5'-T_{12}AA-3' and were very

close to the 170 base length approximated from the differential display gel and the colony PCR reaction. BSS5B and BSS5C were nearly identical and therefore likely encoded the same mRNA sequence (Figure 38).

Five clones were sequenced for BSS6 and were identified as BSS6A, BSS6B, BSS6C, BSS6D and BSS6E of 312, 327, 306, 300 and 311 bases (Figures 39 - 43). All the BSS6 clones sequenced had the correct decamer OPA4, except for BSS6D. Only BSS6B and BSS6D, however, had the anchored primer 5'-T_{12}AA-3'. These five BSS6 clones were close to the 330 base pair length estimated from the differential display gel. Fragments BSS6A, BSS6C and BSS6E exhibited strong sequence homology (Figure 44) but these three fragments and BSS6D were all considered as PCR artifacts as each was missing one of the primers used for their amplification. Only the fragment BSS6B was apparently a legitimate differential display fragment having the correct primer combination.

Six clones were sequenced for BSS7 and are identified as BSS7A, BSS7B, BSS7C, BSS7D, BSS7E and BSS7F of 140, 140, 140, 138, 138 and 180 bases respectively (Figures 45 - 50). All the BSS7 cDNAs had the appropriate primer combination and length except BSS7F which was significantly longer than the 140 bases estimated from the differential display gel and had neither the primers 5'-T_{12}AA-3' nor the decamer OPA. BSS7B and BSS7C were identical sequences suggesting that each represented the same mRNA sequence (Figure 51).

Three independant clones were sequenced for BSS8 and are identified as BSS8A, BSS8B and BSS8C, of 222, 225 and 134 bases, respectively (Figures 52 - 54). The

fragments BSS8A and BSS8B encoded the decamer OPA6 and 5'-T_{12}AA-3' and are both close to the predicted size on the differential display gel. The clone BSS8C was likely a PCR artifact as the primer OPA6 was not detected in its sequence and it was significantly shorter than the 220 nucleotides predicted from the differential display gel. The fragments BSS8A, BSS8B and BSS3A exhibited strong sequence homology (Figure 55) suggesting that these cDNA fragments represented the same mRNA. Interestingly BSS3A and BSS8A were both truncated.

Four clones were sequenced for BSS9 and are identified as BSS9A, BSS9B, BSS9C and BSS9D of 96, 54, 156 and 361 bases, respectively (Figures 56 - 59). All of the fragments sequenced for BSS9 were PCR artifacts as none encoded the appropriate primers and none were close to the predicted size of 230 bases. Furthermore BSS9D encoded two 5'-T_{12}AA-3' sequences. Consequently, these clones were not analyzed further.

Three clones were sequenced for BSS11 and are identified as BSS11A, BSS11B and BSS11C of 280, 280 and 278 nucleotides in length, respectively (Figures 60 - 62). The fragments BSS11A, BSS11B and BSS11C all contained the decamer OPA4 and 5'-T_{12}AA-3'. BSS11A, BSS11B and BSS11C were all nearly identical sequences suggesting that they each represented the same mRNA sequence (Figure 63).

Three fragments were sequenced for BSS12 and are identified as BSS12A, BSS12B and BSS12C of 262, 267 and 268 bases, respectively (Figures 64 - 66). All three clones sequenced for BSS12 encoded OPA4 and 5'-T_{12}AA-3' and were close to the predicted size of 270 bases. The clones BSS12B and BSS12C were nearly identical sequences suggesting that these clones represented the same mRNA (Figure 67).

Three clones were sequenced for BSS13 and are identified as BSS13A, BSS13B and BSS13C of 340, 339 and 338 bases (Figures 68 - 70). All three sequences had the appropriate primer combination of OPA4 and 5'-T_{12}AA-3' and were close to the size predicted from the differential display gel. BSS13A, BSS13B and BSS13C were all nearly identical suggesting that they all represented the same mRNA (Figure 71).

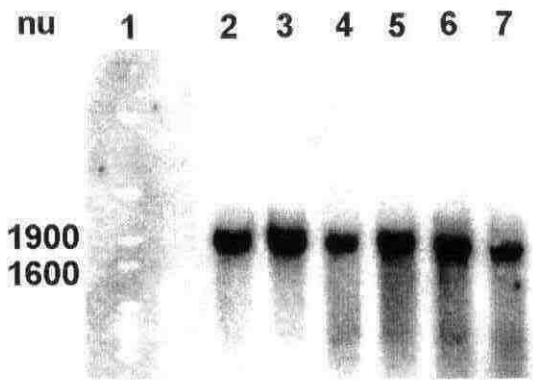
One cDNA, called *Brassica* sucrose control (BSC100), appeared to be expressed equally in the 2 %, 6 % and 14 % cultures. Two clones were sequenced for BSC100 and are identified as BSC100A and BSC100B of 197 and 261 bases respectively (Figures 72 and 73). BSC100B was approximately the predicted size of 250 bases and had OPA5 and 5'-T_{12}AA-3'. The fragment BSC100A might have been a PCR artifact as it was significantly shorter than predicted and although it encoded 5'-T_{12}AA-3', the decamer OPA5 was not detected.

Cloning and sequencing of BSS cDNAs revealed 18 different cDNA sequences which are apparently upregulated in parallel to the increased media sucrose concentration when examined by differential display. The fragments of further interest were: BSS1A, BSS1C, BSS1D, BSS1E, BSS2C, BSS4A, BSS5A, BSS5B, BSS6B, BSS7A, BSS7B, BSS7D, BSS7E, BSS8A, BSS11A, BSS12A, BSS12B and BSS13A. These clones, except for BSS7D and BSS7E, were subjected to northern blot and/or RT-PCR expression analysis and the upregulated fragments were used to search for homologous genes or proteins in GenBank. Attempts were made to isolate full length clones from cDNAs encoding upregulated mRNA sequences. PCR artifacts and fragments of divergent size were not subjected to further analysis with the exception of BSS1 where fragments shorter than expected were further analyzed.

Expression of the "BSS" mRNAs in Cultures Exhibiting Increased Oil Accumulation

Specific primers were designed for the sixteen selected cDNA fragments, so that each could be specifically amplified by PCR. These primers were indicated by the double underlining in Figures 8 - 73 where appropriate. The cDNA clones representing upregulated mRNAs from the differential display gel were used to probe a northern blot of RNA isolated from cultures grown in media containing 2 %, 6 %, or 14 % sucrose. cDNA fragments were labeled using PCR, in the presence [α-^{32}P]dCTP, producing probes containing at least 10 million counts per minute (Appendix 5). The RNA blots were initially probed with a labeled 329 bp fragment of the nuclear encoded mitochondrial elongation factor -TU (EF-TU), to verify equal loading and transfer of RNA (Wilkinson *et al.*, 1995). This probe always resulted in a strong and sharp signal to a template of approximately 1900 nucleotides (Figure 74). The signal from the blot of the EF-TU fragment was approximately equal in all the lanes, but the RNA from the 2 % culture produced a slightly stronger signal than that of the 6 % and 14 % cultures which differed from the RT-PCR analysis where the 2 % cultures produced a slightly weaker signal than the 6 and 14 % cultures. The minor differences in signal strength from the different cultures could have been a result of differences in the amount of loaded RNA, differences in the efficiency of transfer between the lanes, or possibly differences in expression of the EF-TU fragment. Because of the minor signal strength variations observed for the EF-TU transcript, the BSS fragments needed to have significantly larger signal strength increases than the standard in order to be considered as upregulated. Following the analysis of the northern blots containing the EF-TU fragment the membranes were placed

Figure 74. Northern blot of the nuclear encoded mitochondrial elongation factor - TU to verify equal load and transfer of RNA, from the three fractions, to the membrane. Twenty μg of total RNA was loaded from the 2 %, 6 % and 14 % sucrose cultures. Lanes 2 and 3 represent RNA from the 2 % cultures, lanes 4 and 5 are RNA from the 6 % cultures and lanes 6 and 7 are RNA from the 14 % cultures. Lane 1 is the RNA ladder of 300 - 7400 nucleotides. The mitochondrial elongation factor was labeled by PCR in the presence of [α-^{32}P]dCTP as described in **MATERIALS AND METHODS** using the primers listed in the legend of Figure 3.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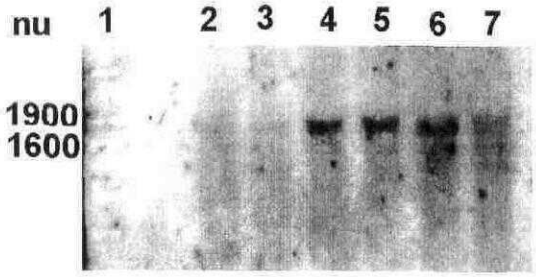
in a stripping solution to remove the probe. Membranes stripped of excess ^{32}P were used for subsequent blots. Membranes were reprobed with different BSS fragments however, at best, hybridization signal strengths were very weak, if detectable. A few fragments were used to probe blots prior to blotting with the elongation factor - TU. This was done to determine the effects of stripping on subsequent hybridizations. All of these probes did not produce detectable signals, but when the membranes were stripped and reprobed with the EF - TU, a strong hybridization signal was produced. The lack of signal from the BSS fragments indicated that these sequences were possibly expressed at relatively low abundance when compared to that of the EF-TU fragment. A weak hybridization signal was detectable only for BSS11A (Figure 75). The cDNA BSS11A hybridized to a mRNA of about 1600 nucleotides. The signal strength of BSS11A was strongly upregulated when the growth media sucrose concentration was increased. In the 2 % sucrose cultures, the transcript was barely detectable, but it became stronger in the 6 % sucrose culture. The hybridization signal was of similar or slightly lower intensity in the 14 % sucrose culture versus the 6 % sucrose culture.

Northern blot experiments generally resulted in weak signals. Therefore, the more sensitive method of reverse transcription-polymerase chain reaction (RT-PCR) was employed to investigate the expression of the BSS fragments under different sucrose concentrations. As for the northern blot analysis, the RT-PCR reactions were normalized by amplification of the nuclear encoded mitochondrial elongation factor - TU (329 nucleotides) and / or the BSC100B fragment (226 nucleotides). The EF-TU fragment was amplified on first strand cDNA made by reverse transcription of total RNA with the

Figure 75. Northern blot analysis of BSS11A. Twenty μg of total RNA was loaded from the 2 %, 6 % and 14 % sucrose cultures. Lanes 2 and 3 represent RNA from the 2 % cultures, lanes 4 and 5 are RNA from the 6 % cultures and lanes 6 and 7 are RNA from the 14 % cultures. Lane 1 is the RNA molecular weight ladder (300 - 7400 nucleotides). A 245 bp fragments of BSS11A was amplified by PCR using the forward (5'-ATAGCTCATGGAACCACCAC-3') and reverse (5'-TAACAGAACAAACCTCCTCC-3') primers in the presence of [α-^{32}P]dCTP as described in **MATERIALS AND METHODS**. Northern blot hybridization was carried out as described in **MATERIALS AND METHODS**.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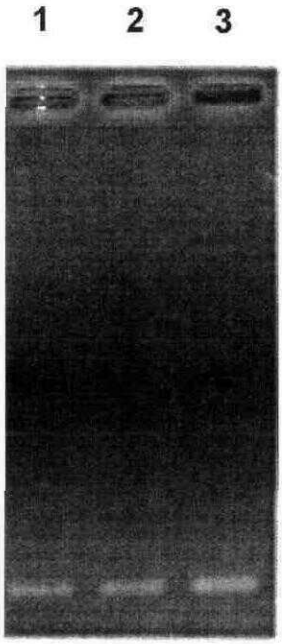
1600

primer 5'-T_{15}-3'. The RT-PCR analysis of the EF-TU fragment displayed near equal intensity for cDNA from the 2 %, 6 % and 14 % cultures. Amplifications were performed for the EF-TU fragment using 25, 27, 29 and 30 cycles. In the 25 and 27 cycle amplifications, there was a minor reduction in the signal intensity for the 2 % culture (Figure 3). The BSC100B fragment was amplified using first strand cDNA made by reverse transcription using the anchored oligo(dT) primer 5'-T_{12}AA-3'. In all cases the amplification of this fragment was of near equal intensity in the 2, 6 and 14 % sucrose cultures, although in amplifications using 30 cycles, there was a slightly stronger signal from the 14 % culture (Figure 76). As with the EF-TU fragment the difference observed was minimal. Although the slight differences observed for these controls could have reflected the relative expression differences, they nevertheless do give a base line with which to compare the other fragments.

In order to be considered as a fragment corresponding to an upregulated mRNA, the clones tested by RT-PCR had to display much greater signal intensity differences than those observed for the EF-TU and BSC100B fragments when examined in the 2 %, 6 % and 14 % cultures. Some anomalous behavior was observed for the relative expression differences between cultures of a given cDNA. The final expression levels for such fragments were determined by the most commonly occurring expression levels from different RNA preparations and RT reactions. The intensity of the EF-TU fragment was much stronger than that of the BSC100B fragment. This intensity difference could reflect the relative expression levels of each or alternatively it could be because the more accurate sequence available for the EF-TU cDNA allowed production of primers which

Figure 76. Reverse transcription - PCR analysis of BSC100B. BSC100B was produced by reverse transcription of mRNA from each of the 2 %, 6 % and 14 % sucrose cultures using the anchored oligo(dT) primer 5'-T_{12}AA-3' followed by PCR amplification using the forward (5'-GTTAGGTCAAGGAGATTTGC-3') and reverse (5'-AACCGCTTTATTATTATTCC-3') primers to amplify a 226 nucleotide fragment of BSC100B. Following the PCR amplification the cDNA was separated on a 3 % composite gel composed of 2 % agarose and 1 % MetaPhor agarose, stained with ethidium bromide and visualized by irradiation of the ethidium bromide stained DNA with UV light. Lanes 1, 2 and 3 represent a 30 cycle PCR amplification of the BSC100B cDNA fragment from the 2 %, 6 % and 14 % sucrose cultures. All the PCR reactions used 2 µl of a four fold dilution of first strand cDNA produced by the reverse transcription of 0.2 µg of total RNA in a 20 µl reaction. The temperatures for the PCR reaction were 94°C for 1 minute, 50°C for 1 minute and 72°C for 1 minute.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are much more efficient for PCR amplification. The BSC100B fragment was only sequenced once in each direction so the possibility of errors is much greater that of the EF-TU fragment which was reported in GenBank and therefore should be a much more accurate sequence. The signal strength of EF-TU was much more intense than any of the other fragments analyzed while the signal strength of BSC100B was similar to the average expression level when compared to the other fragments.

The BSS1A fragment (130 nucleotides) was amplified by PCR, using the forward (5'-ATCTCTGTCTTGTCACTTGC-3') and reverse (5'-GGTCTCAACAAGAGAACTCC-3') primers with first strand cDNA from the 2 %, 6 % and 14 % sucrose cultures. PCR amplification of BSS1A using 31 cycles displayed a weak signal in the 2 % sucrose culture. The signal was slightly stronger in the 6 % sucrose culture. In the 14 % culture, however, the signal was much stronger than the 6 % culture (Figure 77). Similar relative signal strengths were observed for subsequent amplification using 25, 27 and 29 cycles as all displayed increasing signal intensity as media sucrose was increased from 2 % to 6 % to 14 %. These results indicated that BSS1A encoded part of a mRNA that exhibits enhanced expression in the 6 % and 14 % sucrose cultures when compared to that of the 2 % cultures. Because BSS1A appeared to be expressed in larger amounts in the 6 % and 14 % cultures, it was of further interest to this study. The expression level of BSS1A, based on the signal strength obtained during the RT-PCR analysis was slightly more than that of BSC-100.

The BSS1C fragment (123 nucleotides) was amplified by PCR, using the forward (5'-TTCAAGATGGTTCACCCTTC-3') and reverse

Figure 77. Reverse transcription - PCR analysis of BSS1A. BSS1A was produced by reverse transcription of mRNA from each of the 2 %, 6 % and 14 % sucrose cultures using the anchored oligo(dT) primer 5'-T_{12}AA-3' followed by PCR amplification using the forward (5'-ATCTCTGTCTTGTCACTTGC-3') and reverse (5'-GGTCTCAACAAGAGAACTCC-3') primers to amplify a 130 nucleotide fragment of BSS1A. Following the PCR amplification the cDNA was separated on a 3 % composite gel composed of 2 % agarose and 1 % Metaphor agarose, stained with ethidium bromide and visualized by irradiation of the ethidium bromide stained DNA with UV light. Lane 1 is the 100 bp ladder while lanes 2 and 5, 3 and 6 and 4 and 7 are PCR amplification of BSS1A from the 2 %, 6 % and 14 % sucrose cultures. Lanes 2, 3 and 4 were from a 31 cycle PCR reaction while lanes 5, 6 and 7 were from a 29 cycle PCR reaction. All the PCR reactions used 2 µl of a four fold dilution of first strand cDNA produced by the reverse transcription of 0.2 µg of total RNA in a 20 µl reaction. The temperatures for the PCR reaction were 94^{o}C for 1 minute, 50^{o}C for 1 minute and 72^{o}C for 1 minute.

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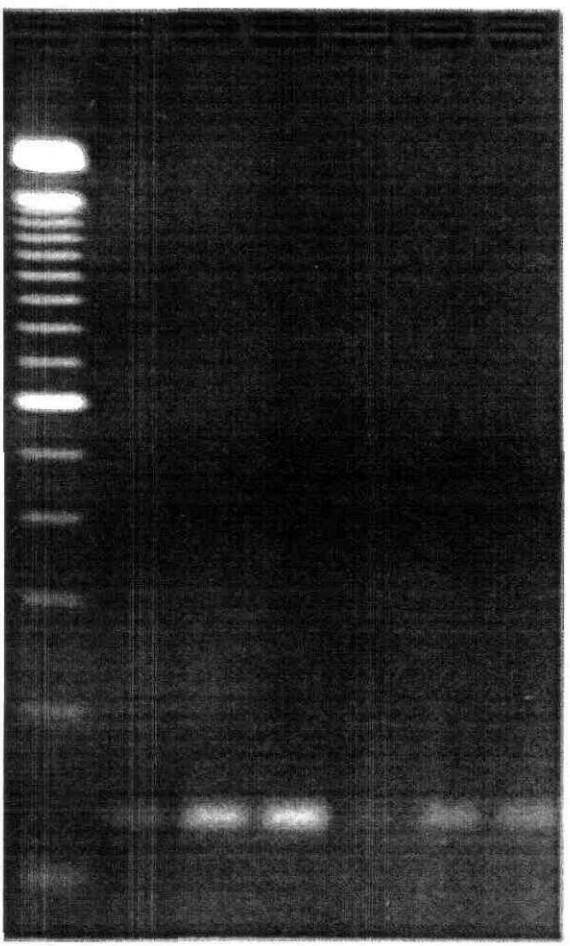

(5'-ACGAACAAAGAAACAAGTC-3') primers with first strand cDNA from the 2 %, 6 % and 14 % sucrose cultures. PCR amplification of BSS1C using 29 cycles displayed weak signal intensity in the 2 % sucrose culture, and approximately the same signal intensity in the 6 % and 14 % sucrose cultures (Figure 78). Two further amplifications, performed using 27 and 25 cycles, displayed the same results. The detection of similar RT-PCR signal strength from the 2 %, 6 % and 14 % sucrose cultures suggested that BSS1C encoded part of a mRNA which does not exhibit enhanced expression when the media sucrose concentration is increased from 2 % to 6 % to 14 %. The expression of BSS1C was significantly weaker than that of BSC100B. Therefore BSS1C was not of further interest to this study.

The BSS1D fragment (177 nucleotides) was amplified by PCR, using the forward (5'-CTGGCGATGAATCTCGTAAC-3') and reverse (5'-CTTTGAAGAGCACTGAGTCC-3') primers with first strand cDNA from the 2 %, 6 % and 14 % sucrose cultures. These particular primers were designed to an upstream region of this fragment from other work. PCR amplifications were performed using 25, 27, 29, 29 and 30 cycles. All amplifications displayed a relatively strong signal from the 2 % sucrose culture, while the 6 % culture had a much weaker signal and the 14 % sucrose culture was stronger than the 6 % culture but still much weaker than the 2 % culture (Figure 79). The decrease in signal intensity for BSS1D indicated that the corresponding mRNA is expressed at the highest levels in the 2 % culture, the lowest in

Figure 78. Reverse transcription - PCR analysis of BSS1C. BSS1C was produced by reverse transcription of mRNA from each of the 2 %, 6 % and 14 % sucrose cultures using the anchored oligo(dT) primer 5'-T_{12}AA-3' followed by PCR amplification using the forward (5'-TTCAAGATGGTTCACCCTTC-3') and reverse (5'-AACGAACAAAGAAACAAGTC-3') primers to amplify a 122 nucleotide fragment of BSS1C. Following the PCR amplification the cDNA was separated on a 3 % composite gel composed of 2 % agarose and 1 % MetaPhor agarose, stained with ethidium bromide and visualized by irradiation on a UV transilluminator. Lanes 1, 2 and 3 are PCR amplifications of BSS1C from the 2 %, 6 % and 14 % sucrose cultures. Lanes 1, 2 and 3 were from a 25 cycle PCR reaction. All the PCR reactions used 2 µl of a four fold dilution of first strand cDNA produced by the reverse transcription of 0.2 µg of total RNA in a 20 µl reaction. The temperatures for the PCR reaction were 94°C for 1 minute, 50°C for 1 minute and 72°C for 1 minute.

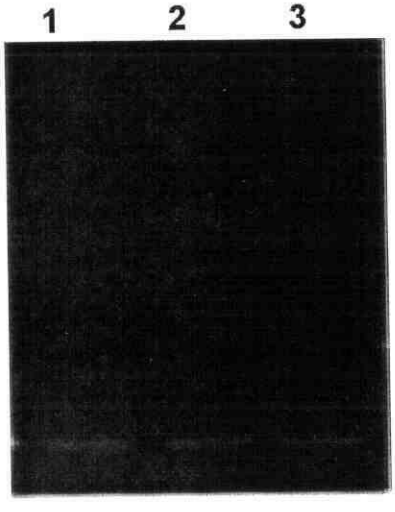
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Figure 79. Reverse transcription - PCR analysis of BSS1D. BSS1D was produced by reverse transcription of mRNA from each of the 2 %, 6 % and 14 % sucrose cultures using the anchored oligo(dT) primer 5'-T_{12}AA-3' followed by PCR amplification using the forward (5'-CTGGCGATGAATCTCGTAAC-3') and reverse (5'-CTTTGAAGAGCACTGAGTCC-3') primers to amplify a 290 nucleotide fragment of a region of BSS1D upstream from the sequence of the DD fragment. Following the PCR amplification the cDNA was separated on a 3 % composite gel composed of 2 % agarose and 1 % MetaPhor agarose, stained with ethidium bromide and visualized by irradiation on a UV transilluminator. Lanes 1, 2 and 3 are PCR amplifications of BSS1D from the 2 %, 6 % and 14 % sucrose cultures. Lanes 1, 2 and 3 were from a 29 cycle PCR reaction. All the PCR reactions used 2 µl of a 4 fold dilution of first strand cDNA produced by the reverse transcription of 0.2 µg of total RNA in a 20 µl reaction. The temperatures for the PCR reaction were 94^{o}C for 1 minute, 55^{o}C for 1 minute and 72^{o}C for 1 minute.


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the 6 % culture and moderately in the 14 % culture. The BSS1D fragment appeared to be repressed by increased media sucrose and therefore was not of further interest to this study.

The BSS1E fragment (206 nucleotides) was amplified by PCR using the forward (5'-GAGTGAAGAGAAGAAATGAG-3') and reverse (5'-TAAGCAGAAGTTCGACATTC-3') primers with first strand cDNA from the 2 %, 6 % and 14 % sucrose cultures. Four independent PCR amplifications of BSS1E using 25, 27, 29 and 30 cycles displayed relatively strong signal strength in the 2 % sucrose cultures, and approximately the same signal strength from the 6 % and 14 % sucrose cultures (Figure 80). These results indicated that BSS1E encoded part of a mRNA which did not display over-expression as the media sucrose concentration was increased from 2 % to 6 % to 14 %. Consequently BSS1E was not of further interest to this study.

The BSS2C fragment (194 nucleotides) was amplified by PCR, using the forward (5'-TTCAAGAGAAAGATGTACCC-3') and reverse (5'-TTAACACGATAAACATCCCC-3') primers with first strand cDNA from the 2, 6 and 14 % sucrose cultures. Five independent PCR amplifications of BSS2C using 25, 27,29, 29 and 31 cycles all displayed a relatively weak signal strength in the 2 % sucrose cultures, a much stronger signal in the 6 % culture and a strong signal from the 14 % culture which was slightly weaker than the 6 % culture (Figure 81). From the signal strength it appeared that BSS2C encoded part of a mRNA which was upregulated as media sucrose was increased from 2 % to 14 %. Since this cDNA encoded part of a mRNA that was apparently expressed in larger amounts in the 6 % and 14 % sucrose

Figure 80. Reverse transcription - PCR analysis of BSS1E. BSS1E was produced by reverse transcription of mRNA from each of the 2 %, 6 % and 14 % sucrose cultures using the anchored oligo(dT) primer 5'-T_{12}AA-3' followed by PCR amplification using the forward (5'-GAGTGAAGAGAAGAAATGAG-3') and reverse (5'-TAAGCAGAAGTTCGACATTC-3') primers to amplify a 206 nucleotide fragment of BSS1E. Following the PCR amplification the cDNA was separated on a 3 % composite gel composed of 2 % agarose and 1 % MetaPhor agarose, stained with ethidium bromide and visualized by irradiation on a UV transilluminator. Lanes 1, 2 and 3 are PCR amplifications of BSS1E from the 2 %, 6 % and 14 % sucrose cultures. Lanes 1, 2 and 3 were from a 29 cycle PCR reaction. All the PCR reactions used 2 µl of a 4 fold dilution of first strand cDNA produced by the reverse transcription of 0.2 µg of total RNA in a 20 µl reaction. The temperatures for the PCR reaction were 94^{O}C for 1 minute, 50^{O}C for 1 minute and 72^{O}C for 1 minute.


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Figure 81. Reverse transcription - PCR analysis of BSS2C. BSS2C was produced by reverse transcription of mRNA from each of the 2 %, 6 % and 14 % sucrose cultures using the anchored oligo(dT) primer 5'-T_{12}AA-3' followed by PCR amplification using the forward (5'-TTCAAGAGAAAGATGTACCC-3') and reverse (5'-TTAACACGATAAACATCCCC-3') primers to amplify a 194 nucleotide fragment of BSS2C. Following the PCR amplification the cDNA was separated on a 3 % composite gel composed of 2 % agarose and 1 % MetaPhor agarose, stained with ethidium bromide and visualized by irradiation of the ethidium bromide stained DNA with UV light. Lanes 1, 2 and 3 are PCR amplifications of BSS2C from the 2 %, 6 % and 14 % sucrose cultures respectively. This PCR amplification was for 29 cycles. All the PCR reactions used 2 µl of a four fold dilution of first strand cDNA produced by the reverse transcription of 0.2 µg of total RNA in a 20 µl reaction. The temperatures for the PCR reaction were 94°C for 1 minute, 50°C for 1 minute and 72°C for 1 minute.

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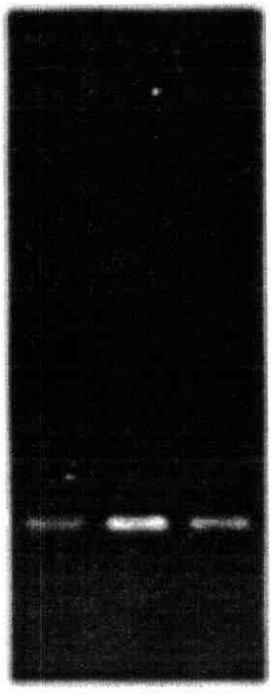

cultures when compared to the 2 % culture, it was of further interest to this study. The expression level of BSS2C in the 2 % culture was significantly weaker than that of BSC100B while in the 6 and 14 % sucrose cultures expression levels were close to that of BSC100B.

The BSS4A fragment (106 nucleotides) was amplified by PCR, using the forward (5'-GCAAGACATTGTTTTTGGCG-3') and reverse (5'-TAACAAACGAATAGCAAAAG-3') primers on first strand cDNA from the 2 %, 6 % and 14 % sucrose cultures. Two independent PCR amplifications, performed using 25 and 27 cycles, both displayed a very weak signal in the 2 % sucrose culture with a stronger signal from the 6 % culture (Figure 82). The signal from the 14 % culture was approximately equal to that from the 6 % culture. The signal strength in the PCR reactions at 27 and 25 cycles suggested that BSS4A encoded part of a mRNA that was upregulated as media sucrose concentration was increased. Since the BSS4A fragment encoded part of a mRNA that had enhanced expression in the stimulated cultures, this fragment was of further interest to this study. The expression level of BSS4A is substantially lower than that of BSC-100 for the 2 %, 6 % and 14 % cultures.

The BSS5A fragment (155 nucleotides) was amplified by PCR, using the forward (5'-CGGGCTGATCGATAAGATTG-3') and reverse (5'-AAGAACCAACGACTTCAACC-3') primers with first strand cDNA from the 2, 6 and 14 % sucrose cultures. Two PCR amplifications, using 29 and 30 cycles, displayed a relatively weak signal in the 2 % culture, with a weak but stronger signal from the 6 % culture (Figure 83). In the 14 % culture, the signal was still weak but stronger than the 6 % culture. Further investigation of BSS5A was not performed as the RT-PCR

Figure 82. Reverse transcription - PCR analysis of BSS4A. BSS4A was produced by reverse transcription of mRNA from each of the 2 %, 6 % and 14 % sucrose cultures using the anchored oligo(dT) primer 5'-T_{12}AA-3' followed by PCR amplification using the forward (5'-GCAAGACATTGTTTTTGGCG-3') and reverse (5'-TAACAAACGAATAGCAAAAG-3') primers to amplify a 106 nucleotide fragment of BSS4A. Following PCR amplification, the cDNA was separated on a 3 % composite gel composed of 2 % agarose and 1 % MetaPhor agarose, stained with ethidium bromide and visualized by irradiation on a UV transilluminator. Lanes 1, 2 and 3 are PCR amplifications of BSS4A from the 2 %, 6 % and 14 % sucrose cultures. Lanes 1, 2 and 3 were from a 27 cycle PCR reaction. All the PCR reactions used 2 µl of a 4 fold dilution of first strand cDNA produced by the reverse transcription of 0.2 µg of total RNA in a 20 µl reaction. The temperatures for the PCR reaction were 94°C for 1 minute, 50°C for 1 minute and 72°C for 1 minute.

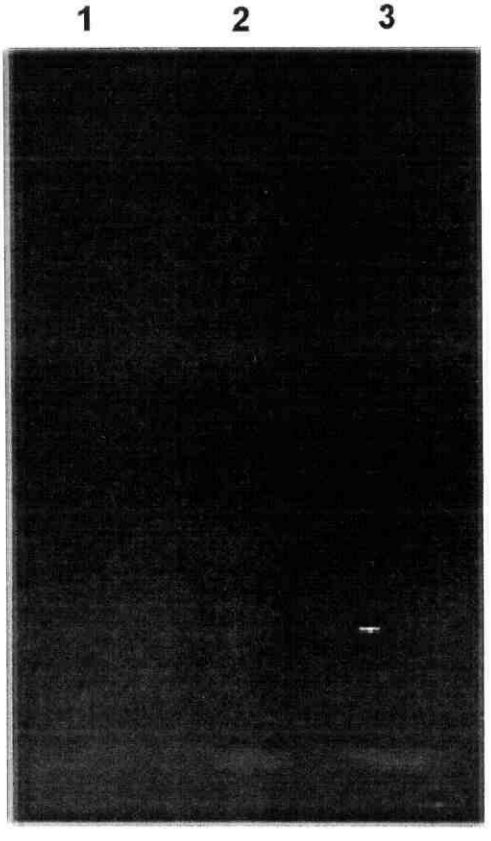
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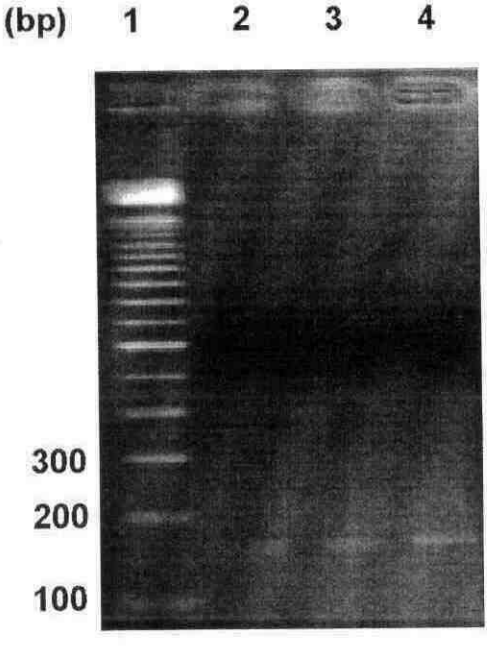
Figure 83. Reverse transcription - PCR analysis of BSS5A. BSS5A was produced by reverse transcription of mRNA from each of the 2 %, 6 % and 14 % sucrose cultures using the anchored oligo(dT) primer 5'-T_{12}AA-3' followed by PCR amplification using the forward (5'-CGGGCTGATCGATAAGATTG-3') and reverse (5'-AAGAACCAACGACTTCAACC-3') primers to amplify a 155 nucleotide fragment of BSS5A. Following the PCR amplification the cDNA was separated on a 3 % composite gel composed of 2 % agarose and 1 % MetaPhor agarose, stained with ethidium bromide and visualized by irradiation on a UV transilluminator. Lane 1 is the 100 bp ladder. Lanes 2, 3 and 4 are PCR amplifications of BSS5A from the 2 %, 6 % and 14 % sucrose cultures. Lanes 2, 3 and 4 were from a 29 cycle PCR reaction. All the PCR reactions used 2 µl of a 4 fold dilution of first strand cDNA produced by the reverse transcription of 0.2 µg of total RNA in a 20 µl reaction. The temperatures for the PCR reaction were 94°C for 1 minute, 50°C for 1 minute and 72°C for 1 minute.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analysis did not suggest that BSS5A was upregulated in the stimulated cultures.

The BSS5B fragment (104 nucleotides) was amplified by PCR using the forward (5'-TGGAGAGTGCGATGGAGAGC-3') and reverse (5'-CAACTTTCTCAAACTCCCAC-3') primers with first strand cDNA from the 2, 6 and 14 % sucrose cultures. Two PCR amplifications of BSS5B, using 29 and 30 cycles, displayed a relatively weak signal from the 2 % culture, with a weak but stronger signal from the 6 % culture and a slightly stronger signal in the 14 % culture (Figure 84). The signal strength of the PCR reactions at 30 and 29 cycles suggested that BSS5B encoded part of a mRNA which was upregulated as the media sucrose concentration was increased from 2 % to 6 % to 14 %. Assuming similar PCR amplification efficiency as the other fragments, this one appeared to encode a mRNA which was expressed in relatively low amounts because it took 29 cycles of PCR to produce a detectable signal. Thus BSS5B was of further interest to this study.

The BSS6B fragment (290 nucleotides) was amplified by PCR using the forward (5'-AGGGAGAAGGACTTACCGTG-3') and reverse (5'-ATCAAACAGCAGCACTGAAC-3') primers on first strand cDNA from the 2, 6 and 14 % sucrose cultures. PCR amplifications, using 27, 29, 30 and 31 cycles, displayed nearly the same signal strength the 2 %, 6 % and 14 % sucrose cultures (Figure 85). The signal strength results suggested that BSS6B encoded part of a mRNA that was expressed in approximately equal amounts in the 2 %, 6 % and 14 % cultures. Since BSS6B was not clearly upregulated when examined by RT-PCR analysis, the fragment was not of further interest in this study.

The BSS7A fragment (109 nucleotides) was amplified by PCR using the

Figure 84. Reverse transcription - PCR analysis of BSS5B. BSS5B was produced by reverse transcription of mRNA from each of the 2 %, 6 % and 14 % sucrose cultures using the anchored oligo(dT) primer 5'-T_{12}AA-3' followed by PCR amplification using the forward (5'-TGGAGAGTGCGATGGAGAGC-3') and reverse (5'-CAACTTTCTCAAACTCCCAC-3') primers to amplify a 104 nucleotide fragment of BSS5B. Following the PCR amplification the cDNA was separated on a 3 % composite gel composed of 2 % agarose and 1 % MetaPhor agarose, stained with ethidium bromide and visualized by irradiation on a UV transilluminator. Lanes 1, 2 and 3 are PCR amplifications of BSS5A from the 2 %, 6 % and 14 % sucrose cultures. Lanes 1, 2 and 3 were from a 30 cycle PCR reaction. All the PCR reactions used 2 µl of a 4 fold dilution of first strand cDNA produced by the reverse transcription of 0.2 µg of total RNA in a 20 µl reaction. The temperatures for the PCR reaction were 94°C for 1 minute, 50°C for 1 minute and 72°C for 1 minute.

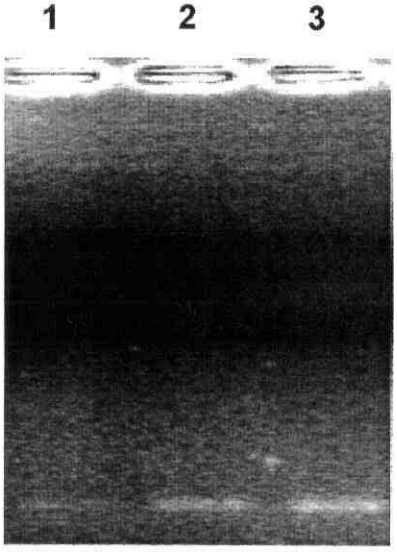
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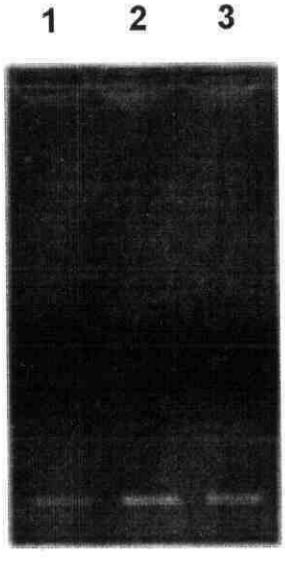
Figure 85. Reverse transcription - PCR analysis of BSS6B. BSS6B was produced by reverse transcription of mRNA from each of the 2 %, 6 % and 14 % sucrose cultures using the anchored oligo(dT) primer 5'-T_{12}AA-3' followed by PCR amplification using the forward (5'-AGGGAGAAGGACTTACCGTG-3') and reverse (5'-ATCAAACAGCAGCACTGAAC-3') primers to amplify a 290 nucleotide fragment of BSS6B. Following the PCR amplification the cDNA was separated on a 3 % composite gel composed of 2 % agarose and 1 % MetaPhor agarose, stained with ethidium bromide and visualized by irradiation on a UV transilluminator. Lanes 1, 2 and 3 are PCR amplifications of BSS6B from the 2 %, 6 % and 14 % sucrose cultures. Lanes 1, 2 and 3 were from a 29 cycle PCR reaction. All the PCR reactions used 2 μl of a 4 fold dilution of first strand cDNA produced by the reverse transcription of 0.2 μg of total RNA in a 20 μl reaction. The temperatures for the PCR reaction were 94°C for 1 minute, 50°C for 1 minute and 72°C for 1 minute.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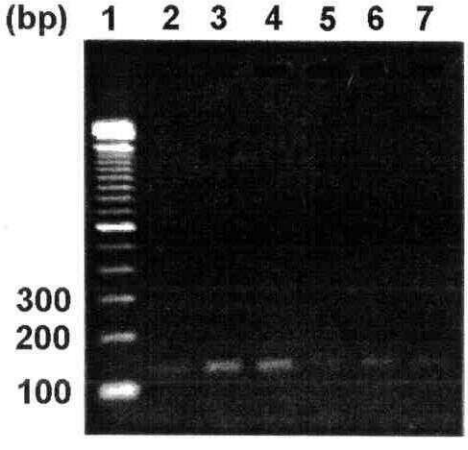
forward (5'-TGGAAAAGAAAATGTATCTG-3') and reverse (5'-TTTGAGTTTGGATTTAGTTC-3') primers on first strand cDNA from the 2, 6 and 14 % sucrose cultures. PCR amplifications of BSS7A using 30, 31, 33 and 35 cycles all produced a signal strength that was too weak to be detected (data not shown). The lack of signal detected for the PCR reactions using up to 35 cycles suggested that BSS7A was either expressed at very low levels or that there was a problem with the primers used in the amplification. PCR amplification using 40 cycles displayed signal strength that remained constant when media sucrose concentration was increased from 2 % to 6 % to 14 % (data not shown). The signal strength of the PCR reactions suggest that BSS7A encoded part of a mRNA which had expression levels that appeared to be unaffected by alteration of the media sucrose concentration. Since BSS7A was not clearly upregulated when examined by RT-PCR analysis, the fragment is not of further interest in this study.

The BSS7B fragment (123 nucleotides) was amplified by PCR using the forward (5'-GCTGGAGGGTATTAAATGAC-3') and reverse (5'-TAACGCAAAATTCAAAGATC-3') primers on first strand cDNA from the cell culture grown in the presence of 2 %, 6 % and 14 % sucrose. PCR amplifications using 27, 29 and 30 cycles displayed weak signal strength in the 2 % culture, stronger signal strength in the 6 % culture and approximately the same signal strength in the 14 % culture (Figure 86). The signal strength of the PCR reactions suggested that this fragment encoded part of a mRNA which was expressed in larger amounts as sucrose was increased from 2 % to 6 % to 14 %. Consequently BSS7B was of further interest to this study. The expression level of BSS7B in the 2 % sucrose cultures was slightly weaker

Figure 86. Reverse transcription - PCR analysis of BSS7B. BSS7B was produced by reverse transcription of mRNA from each of the 2 %, 6 % and 14 % sucrose cultures using the anchored oligo(dT) primer 5'-T_{12}AA-3' followed by PCR amplification using the forward (5'-GCTGGAGGGTATTAAATGAC-3') and reverse (5'-TAACGCAAAATTCAAAGATC-3') primers to amplify a 123 nucleotide fragment of BSS1A. Following the PCR amplification the cDNA was separated on a 3 % composite gel composed of 2 % agarose and 1 % Metaphor agarose, stained with ethidium bromide and visualized by irradiation of the ethidium bromide stained DNA with UV light. Lane 1 is the 100 bp ladder, while lanes 2 and 5, 3 and 6 and 4 and 7 are PCR amplification of BSS7B from the 2 %, 6 % and 14 % sucrose cultures. Lanes 2, 3 and 4 were from a 29 cycle PCR reaction, while lanes 5, 6 and 7 were from a 27 cycle PCR reaction. All the PCR reactions used 2 µl of a four fold dilution of first strand cDNA produced by the reverse transcription of 0.2 µg of total RNA in a 20 µl reaction. The temperatures for the PCR reaction were 94°C for 1 minute, 50°C for 1 minute and 72°C for 1 minute.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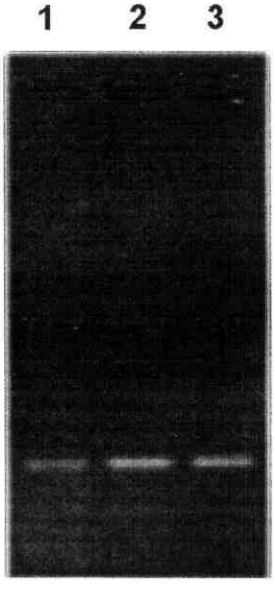
than that of BSC-100B.

The BSS8A fragment (166 nucleotides) was amplified by PCR using the forward (5'-TTGAATACGTTTTCAATCATCC-3') and reverse (5'-AAGGCATAAGAGTTTCTTAAGG-3') primers on first strand cDNA from the culture grown in the presence of 2 %, 6 % and 14 % sucrose. PCR amplifications using 29, 30 cycles displayed a relatively weak signal in the 2 % culture, a stronger signal in the 6 % culture. In the 14 % culture the signal was slightly weaker than the 6 % culture (Figure 87). From the signal strength it appeared that this fragment encoded part of a mRNA which was expressed in larger amounts in the 6 % and 14 % sucrose cultures when compared to the 2 % cultures. Consequently, BSS8A was of further interest to this study. The expression level of BSS8A in the 2 % sucrose cultures was slightly weaker than that of BSC100B.

The BSS11A fragment (246 nucleotides) was amplified by PCR, using the forward (5'-ATAGCTCATGGAACCACCAC-3') and reverse (5'-TAACAGAACAAACCTCCTCC-3') primers on first strand cDNA from the culture grown in the presence of 2, 6 and 14 % sucrose. PCR amplifications using 27, 29 and 30 cycles produced a signal that was almost undetectable in the 2 % culture. In the 6 % culture, the signal was very strong whereas in the 14 % sucrose culture the signal was similar to that of the 6 % culture (Figure 88). Based on this information BSS11A encoded part of a mRNA that was expressed much more abundantly in the 6 % and 14 % cultures than in the 2 % cultures. Consequently BSS11A was of further interest to this study. The expression level of BSS11A for the 2 % culture was significantly lower than that of BSC100B but in the 6 % and 14 % cultures the expression levels exceeded that of

Figure 87. Reverse transcription - PCR analysis of BSS8A. BSS8A was produced by reverse transcription of mRNA from each of the 2 %, 6 % and 14 % sucrose cultures using the anchored oligo(dT) primer 5'-T_{12}AA-3' followed by PCR amplification using the forward (5'-TTGAATACGTTTTCAATCATCC-3') and reverse (5'-AAGGCATAAGAGTTTCTTAAGG-3') primers to amplify a 166 nucleotide fragment of BSS8A. Following the PCR amplification, the cDNA was separated on a 3 % composite gel composed of 2 % agarose and 1 % MetaPhor agarose, stained with ethidium bromide and visualized by irradiation on a UV transilluminator. Lanes 1, 2 and 3 are PCR amplifications of BSS8A from the 2 %, 6 % and 14 % sucrose cultures. Lanes 1, 2 and 3 were from a 30 cycle PCR reaction. All the PCR reactions used 2 μl of a 4 fold dilution of first strand cDNA produced by the reverse transcription of 0.2 μg of total RNA in a 20 μl reaction. The temperatures for the PCR reaction were 94^{o}C for 1 minute, 50^{o}C for 1 minute and 72^{o}C for 1 minute.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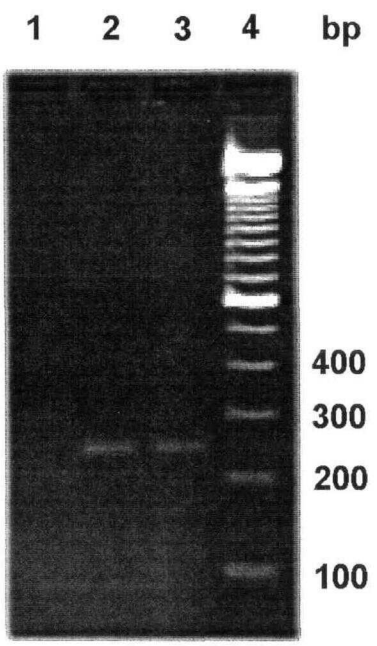
2
3

Figure 88. Reverse transcription - PCR analysis of BSS11A. BSS11A was produced by reverse transcription of mRNA from each of the 2 %, 6 % and 14 % sucrose cultures using the anchored oligo(dT) primer 5'-T_{12}AA-3' followed by PCR amplification using the forward (5'-ATAGCTCATGGAACCACCAC-3') and reverse (5'-TAACAGAACAAACCTCCTCC-3') primers to amplify a 246 nucleotide fragment of BSS11A. Following the PCR amplification, the cDNA was separated on a 3 % composite gel composed of 2 % agarose and 1 % MetaPhor agarose, stained with ethidium bromide and visualized by irradiation on a UV transilluminator. Lanes 1, 2 and 3 are PCR amplifications of BSS11A from the 2 %, 6 % and 14 % sucrose cultures and lane 4 is the 100 bp ladder. Lanes 1, 2 and 3 were from a 29 cycle PCR reaction. All the PCR reactions used 2 μl of a 4 fold dilution of first strand cDNA produced by the reverse transcription of 0.2 μg of total RNA in a 20 μl reaction. The temperatures for the PCR reaction were 94^{o}C for 1 minute, 50^{o}C for 1 minute and 72^{o}C for 1 minute.


1
2
3
4
bp
400
300
200
100

BSC100B.

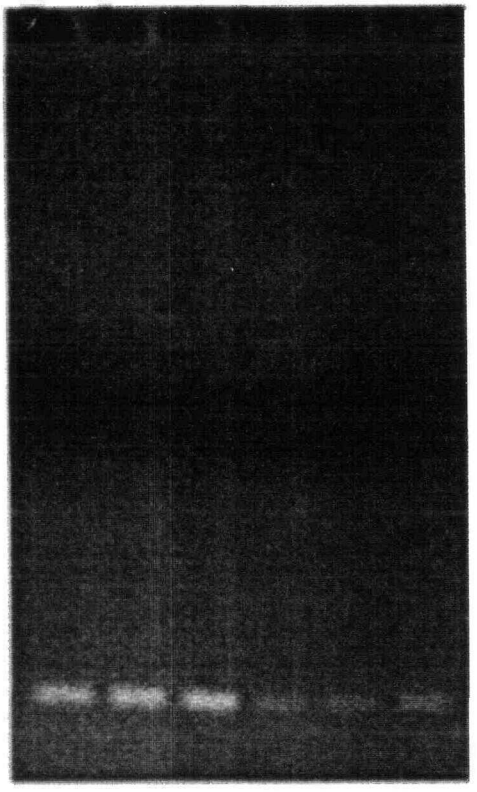
The BSS12A fragment (241 nucleotides) was amplified by PCR using the forward (5'-GGATAGCTTGCTCACGTTAG-3') and reverse (5'-AGGAAACAACATTCACTTAC-3') primers on first strand cDNA. The PCR reaction amplified BSS12A as well as an artifact approximately 40 bases longer than the expected size of BSS12A (data not shown). Increasing the annealing temperature of the PCR did not eliminate the undesired cDNA artifact suggesting that this mRNA is potentially a member of a gene family and that the primers were annealing to conserved regions of the cDNAs. The expression level of BSS12A appeared to be the same in the 2 %, 6 % and 14 % cultures. Further investigation into the expression levels of BSS12A was not pursued. Due to the difficulties in amplification of this fragment, it may be reexamined in the future but not in this study.

The BSS12B fragment (155 nucleotides) was amplified by PCR using the forward (5'-CTGCTGATGGAGATATTGTA-3') and reverse (5'-ACTCATAATGACCCAACATG-3') primers on first strand cDNA from the 2 %, 6 % and 14 % sucrose cultures. PCR amplification using 29 and 31 cycles displayed a strong and similar signal in the 2 %, 6 % and 14 % sucrose cultures (Figure 89). Since BSS12B was not expressed at higher levels in the 6 % and 14 % cultures, this fragment was not of further interest to this study.

The BSS13A fragment (135 nucleotides) was amplified by PCR using the forward (5'-GTGACCAGAAGGAGTTTGCC-3') and reverse (5'-CAAATGACATAAGCGAATCC-3') primers on first strand cDNA from the 2 %, 6 % and 14 % sucrose cultures. PCR amplifications using 29 and 31 cycles

Figure 89. Reverse transcription - PCR analysis of BSS12B. BSS12B was produced by reverse transcription of mRNA from each of the 2 %, 6 % and 14 % sucrose cultures using the anchored oligo(dT) primer 5'-T_{12}AA-3' followed by PCR amplification using the forward (5'-CTGCTGATGGAGATATTGTA-3') and reverse (5'-ACTCATAATGACCCAACATG-3') primers to amplify a 155 nucleotide fragment of BSS12B. Following PCR amplification, the cDNA was separated on a 3 % composite gel composed of 2 % agarose and 1 % Metaphor agarose, stained with ethidium bromide and visualized by irradiation of the ethidium bromide stained DNA with UV light. Lane 1 and 4, 2 and 5 and 3 and 6 are PCR amplifications of BSS12B from the 2 %, 6 % and 14 % sucrose cultures. Lanes 1, 2 and 3 are from a 31 cycle PCR reaction, while lanes 4, 5 and 6 were from a 29 cycle PCR amplification. All the PCR reactions used 2 µl of a four fold dilution of first strand cDNA produced by the reverse transcription of 0.2 µg of total RNA in a 20 µl reaction. The temperatures for the PCR reaction were 94^{o}C for 1 minute, 50^{o}C for 1 minute and 72^{o}C for 1 minute.

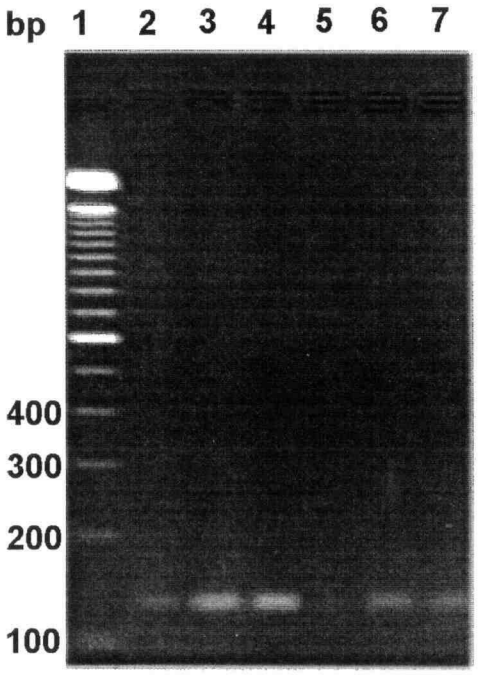
1 2 3 4 5 6



displayed a weak signal strength in the 2 % sucrose cultures while the 6 % cultures displayed a much stronger signal (Figure 90). In the 14 % cultures the signal strength was approximately the same as the 6 % cultures. The stimulation of expression observed for BSS13A suggests that this fragment encoded part of a mRNA that was upregulated in the tissues exhibiting increased oil accumulation. Consequently BSS13A was of further interest to this study. The expression level of BSS13A was slightly stronger than that of BSC100B.

In summary, RT-PCR was used to show that eight (BSS1A, BSS2C, BSS4A, BSS5B, BSS7B, BSS8A, BSS11A and BSS13A) of the cDNA fragments tested represented fragments of mRNAs that were upregulated when the media sucrose concentration was increased from 2 % to 6 % to 14 %. Often the cDNAs isolated by DD represented multiple fragments which revealed enhanced, repressed, or constant expression levels as media sucrose concentration was increased.

Figure 90. Reverse transcription - PCR analysis of BSS13A. BSS13A was produced by reverse transcription of mRNA from each of the 2 %, 6 % and 14 % sucrose cultures using the anchored oligo(dT) primer 5'-T_{12}AA-3' followed by PCR amplification using the forward (5'-GTGACCAGAAGGAGTTTGCC-3') and reverse (5'-CAAATGACATAAGCGAATCC-3') primers to amplify a 135 nucleotide fragment of BSS13A. Following PCR amplification, the cDNA was separated on a 3 % composite gel composed of 2 % agarose and 1 % Metaphor agarose, stained with ethidium bromide and visualized by irradiation of the ethidium bromide stained DNA with UV light. Lanes 2 and 5, 3 and 6, 4 and 7 are PCR amplifications of BSS13A from the 2 %, 6 % and 14 % sucrose cultures and lane 1 is a 100 bp ladder. Lanes 2, 3 and 4 are from a 31 cycle PCR reaction while lanes 5, 6 and 7 are from a 29 cycle PCR reaction. All the PCR reactions used 2 µl of a four fold dilution of first strand cDNA produced by the reverse transcription of 0.2 µg of total RNA in a 20 µl reaction. The temperatures for the PCR reaction were 94°C for 1 minute, 50°C for 1 minute and 72°C for 1 minute.


bp 1 2 3 4 5 6 7
400
300
200
100

Controlled Ribonucleotide Tailing of cDNA Ends

As the need arose to obtain full length sequences corresponding to the upregulated DD sequences, the controlled ribonucleotide tailing of cDNA ends (CRTC) technique was evaluated. This technique was based on the presence of a specific adaptor which is ligated at the 5' end of first strand cDNA, and amplification using a specific reverse primer based on the DD fragment and a common forward primer corresponding to the ligated adaptor. Under this approach, the specificity of the amplification is related to the specificity of the reverse primer. With this approach only a few results were obtained.

PCR amplification of BSS1A using a specific primer and the CRTC primer produced a cDNA of approximately 700 nucleotides in length. Three clones (BSS1A longer) BSS1AL1 (Figure 91), BSS1AL2 (Figure 92) and BSS1AL3 (Figure 93) of 720, 732 and 728 nucleotides in length were each sequenced once, for this cDNA. Sequencing analysis of this cDNA revealed that the 3' end of BSS1AL1, BSS1AL2 and BSS1AL3 were all homologous to the sequence of BSS1A (Figure 94). A high level of sequence homology was also observed for the clones BSS1AL1, BSS1AL2 and BSS1AL3 (Figure 95). From the sequences of BSS1AL1, BSS1AL2 and BSS1AL3 it was determined that the decamer OPA1 (5'-CAGGCCCTTC-3'), used in the differential display PCR amplification of BSS1A had annealed to the complementary sequence 5'-GAAGGGCTTG-3'. This represents a 90 % match as the third base of OPA1 (G) annealed to a mismatched T on the cDNA fragment which encoded BSS1A. Although the use of CRTC to obtain more sequence data for BSS1A was successful, it is doubtful that the clones obtained represent a full length cDNA.

Figure 91. Nucleotide sequence of CRTC cDNA BSS1AL1 (BSS1A longer) with the 3' end of BSS1A added to the 3' end of BSS1AL1. The BSS1AL1 sequence was obtained by controlled ribonucleotide tailing of cDNA ends and is expressed as mRNA sequence. First strand cDNA, produced by reverse transcription with 5'-T_{12}AA-3', had the double stranded adaptor (5'-TAGTCCGAATTCAAGCAAGAGCC-3' annealed to 5'-CTCTTGCTTGAATTCGGACTA-3') ligated to the 3' end and then PCR was performed between the adaptor primer and a primer specific to BSS1A (double underlined). The singly underlined sequence 5'-CAAGCCCTTC-3', was identified as being 90 % identical to OPA1 (5'-CAGGCCCTTC-3') which provided a strong enough annealing of the decamer to allow the cDNA fragment BSS1A to be amplified by the differential display PCR reaction.

```
  1  GGTCTCAACA  AGAGAACTCC  TCGCAACCTT  AATATTAGTA  AACGCCCCCA   50
 51  AGATATGAAT  CCTCGTATCC  GCAATCACAA  TCCTCGTCTT  CGTCGAGTTC  100
101  TCAATCGCAA  ACTTCGTCTT  CCTCTCTGTT  TCCTCTCCGT  CTGTTAGCGG  150
151  AAACACTGCG  CAGTCCGTCG  AATGCTCCGC  CGTGATAATG  ACCTTGACGA  200
201  AGTCTTTGCC  TTTCGTGACG  ATCGGGAGTC  AAGTGGAGAA  GCCCGAGACC  250
251  GCGTGTTGCT  CTGTTCTCAA  AACCGTGCTC  GACACGGAAA  GCTGAATGTC  300
301  TGTGCGAAGG  GTTGAAGAGC  AGTGCCGCCG  CGGGGAATCA  ATCTGAACTT  350
351  CACTAAAGCC  GGTACTTTTT  CCCGATGCTT  GCCAAGTCAA  AGCTCCTCCC  400
401  ATGCCTGCTT  GTGCATTGTT  TGCTAAACCC  CCTGCTAGTG  CGCCAGCTCC  450
451  AGTTCCTGCT  GCTAGACCAC  TAAATGGATC  GGGACCGAGT  TCAAACTCAG  500
501  CTCCAGCTCC  AAGCCCTCCT  CATAGTAACC  ATGGATCTTC  AATCTCTGTC  550
551  TTGTCACTTG  CAATCAGCGG  CGCATTGGTC  ATAATGTTCA  CCCGTATCTA  600
601  ACACCCCTAT  CATTTTTAGT  TAAGAGTTTG  CTTTTATCTT  GTGAGTAACT  650
651  ATGGAGTTCT  CTTGTTGAGA  CCGCTTATTT  TTAGTTATGA  CATTATAAAG  700
701  ACTTGGTTAA  AAAAAAAAAA                                     720
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Figure 92. Nucleotide sequence of the CRTC cDNA BSS1AL2 (BSS1A longer) with the 3' end of BSS1A added to the 3' end of BSS1AL2. The BSS1AL2 sequence was obtained by controlled ribonucleotide tailing of cDNA ends and is expressed as mRNA sequence. First strand cDNA, produced by reverse transcription with 5'-T_{12}AA-3', had the double stranded adaptor (5'-TAGTCCGAATTCAAGCAAGAGCC-3' annealed to 5'-CTCTTGCTTGAATTCGGACTA-3') ligated to the 3' end and then PCR was performed between the adaptor primer and a primer specific to BSS1A (double underlined). The singly underlined sequence, 5'-CAAGCCCTTC-3', was identified as being 90 % identical to OPA1 (5'-CAGGCCCTTC-3') which provided a strong enough annealing of the decamer to allow the cDNA fragment BSS1A to be amplified by the differential display PCR reaction.

```
  1  GGTCTCAACA  AGAGAACTCC  CCCCACGGGC  GAACAAACCG  GAGAGATCTA   50
 51  ACCCCGACGA  AAACGAAATC  AAATCAAACA  ATGGCCCTCT  TCTCCGCCGC  100
101  GCTTCCCCTT  CTCCTCCTCT  TCCTCTCTGT  TTCCTCTCCG  TCTGTTAGCG  150
151  GAAACACTGC  GCAGTCCGTC  GAATGCTCCG  CCGTGATAAT  GACCATGACG  200
201  AAGTGTTTGC  CTTTCGTGAC  GATCGGTAGT  CACGTGGAGA  AACCCGAGAC  250
251  CGCGTGTTGC  TCTGTTATCA  AAACCGTGCT  CGAACACAAA  AGCTGAATGT  300
301  TCTGTGCGAA  AGGGTTTGAA  GGAGCAGTGC  CGCCGGCGGG  GAATCAATCT  350
351  GGAACCTTCA  CTAAAGCCGG  TACTCTCTCG  GATGCTGGCC  AAATTCAAAG  400
401  CTTCTTCCCA  TGCCTGCTTG  TGCATTGTTT  GGCTAAAACC  CCCTTGCTAG  450
451  TGCGCCAGCT  CCAGTTCCTG  CTGCTAGACC  ACTAAATGGA  TCGGGACCAG  500
501  GTTCAAATTC  TGCTCCAGCT  CCAAGCCCTT  CTCATAGTAA  CCATGGATCT  550
551  TCAATCTCTG  TCTTGTCACT  TGCAATCAGC  GGCGCATTGG  TCATAATGTT  600
601  CACCCGTATC  TAACACCCCT  ATCATTTTTA  GTTAAGAGTT  TGCTTTTATC  650
651  TTGTGAGTAA  CTATGGAGTT  CTCTTGTTGA  GACCGCTTAT  TTTTAGTTAT  700
701  GACATTATAA  AGACTTGGTT  AAAAAAAAAA  AA                     732
```

Figure 93. Nucleotide sequence of the CRTC cDNA BSS1AL3 (BSS1A longer) with the 3' end of BSS1A added to the 3' end of BSS1AL3. The BSS1AL3 sequence was obtained by controlled ribonucleotide tailing of cDNA ends and is expressed as mRNA sequence. First strand cDNA, produced by reverse transcription with 5'-T_{12}AA-3', had the double stranded adaptor (5'-TAGTCCGAATTCAAGCAAGAGCC-3' annealed to 5'-CTCTTGCTTGAATTCGGACTA-3') ligated to the 3' end and then PCR was performed between the adaptor primer and a primer specific to BSS1A (double underlined). The singly underlined sequence 5'-CAAGCCCTTC-3', in bold, was identified as being 90 % identical to OPA1 (5'-CAGGCCCTTC-3') which provided a strong enough annealing of the decamer to allow the cDNA fragment BSS1A to be amplified by the differential display PCR reaction.

```
  1  GGTCTCAACA  AGAGAACTCC  CCCCACGGGC  GAACAAACCG  GAGAGATCTA   50
 51  ACCCCGACGA  AAACGAAATC  AAATCAAACA  ATGGCCCTCT  TCTCCGCCGC  100
101  GCTTCCCCTT  CTCCTCCTCT  TCCTCTCTGT  TTCCTCTCCG  TCTGTTAGCG  150
151  GAAACACTGC  GCAGTCCGTC  GAATGCTCCG  CCGTGATAAT  GACCATGACG  200
201  AAGTGTTTGC  CTTTCGTGAC  GATCGGTAGT  CACGTGGAGA  AACCCGAGAC  250
251  CGCGTGTTGC  TCTGTTATCA  AAACCGTGCT  CGAACACAAA  AGCTGAATGT  300
301  TCTGTGCGAA  AGGGTTTGAA  AGAGCAGTGC  CGCCCGCCGG  GAATCAATCT  350
351  GAACCTCACT  AAAGCCGGTA  CTCTCTCGAT  GCTGGCCAAA  TTCAAAGCTT  400
401  CTTCCCATGC  CTGCTTGTGC  ATTGTTTGGC  TAAAACCCCT  TGCTAGTGCG  450
451  CCAGCTCCAG  TTCCTGCTGC  TAGACCACTA  AATGGATCGG  GACCAGGTTC  500
501  AAATTCTGCT  CCAGCTCCAA  GCCCTTCTCA  TAGTAACCAT  GGATCTTCAA  550
551  TCTCTGTCTT  GTCACTTGCA  ATCAGCGGCG  CATTGGTCAT  AATGTTCACC  600
601  CGTATCTAAC  ACCCCTATCA  TTTTTAGTTA  AGAGTTTGCT  TTTATCTTGT  650
651  GAGTAACTAT  GGAGTTCTCT  TGTTGAGACC  GCTTATTTTT  AGTTATGACA  700
701  TTATAAAGAC  TTGGTTAAAA  AAAAAAAA                          728
```

Figure 94. Sequence homology comparison of cDNAs BSS1AL1, BSS1AL2, BSS1AL3 and BSS1A. Mismatched nucleotides are indicated by bold lettering. The / symbol indicates that a nucleotide was absent from one sequence but identified in another.

```
BSS1AL1    1 GGTCTCAACA  AGAGAACTCC  TCGCAACCTT  AATATTAGTA  AACGCCCCCA   50
BSS1AL2    1 GGTCTCAACA  AGAGAACTCC  CCCCACGGGC  GAACAAACCG  GAGAGATCTA   50
BSS1AL3    1 GGTCTCAACA  AGAGAACTCC  CCCCACGGGC  GAACAAACCG  GAGAGATCTA   50
BSS1A

BSS1AL1   51 AGATATGAAT  CCTCGTATCC  GCAATCACAA  TCCTCGTCTT  CGTCGAGTTC  100
BSS1AL2   51 ACCCCGACGA  AAACGAAATC  AAATCAAACA  ATGGCCCTCT  TCTCCGCCGC  100
BSS1AL3   51 ACCCCGACGA  AAACGAAATC  AAATCAAACA  ATGGCCCTCT  TCTCCGCCGC  100
BSS1A

BSS1AL1  101 TCAATCGCAA  ACT/TCGTCT  TCCTCTCTGT  TTCCTCTCCG  TCTGTTAGCG  149
BSS1AL2  101 GCTTCCCCTT  CTCCTCCTCT  TCCTCTCTGT  TTCCTCTCCG  TCTGTTAGCG  150
BSS1AL3  101 GCTTCCCCTT  CTCCTCCTCT  TCCTCTCTGT  TTCCTCTCCG  TCTGTTAGCG  150
BSS1A

BSS1AL1  150 GAAACACTGC  GCAGTCCGTC  GAATGCTCCG  CCGTGATAAT  GACC/TTGAC  198
BSS1AL2  151 GAAACACTGC  GCAGTCCGTC  GAATGCTCCG  CCGTGATAAT  GACCAT/GAC  199
BSS1AL3  151 GAAACACTGC  GCAGTCCGTC  GAATGCTCCG  CCGTGATAAT  GACCAT/GAC  199
BSS1A

BSS1AL1  199 GAAGTCTTTG  CCTTTCGTGA  CGATCGGGAG  TCAAGTGGAG  AAGCCCGAG   248
BSS1AL2  200 GAAGTGTTTG  CCTTTCGTGA  CGATCGGTAG  TCACGTGGAG  AAACCCGAG   249
BSS1AL3  200 GAAGTGTTTG  CCTTTCGTGA  CGATCGGTAG  TCACGTGGAG  AAACCCGAG   249
BSS1A

BSS1AL1  249 ACCGCGTGTT  GCTCTGTTCT  CAAAACCGTG  CTCGA/CACG  GAAAGCTGAA  297
BSS1AL2  250 ACCGCGTGTT  GCTCTGTTAT  CAAAACCGTG  CTCGAACAC/  AAAAGCTGAA  298
BSS1AL3  250 ACCGCGTGTT  GCTCTGTTAT  CAAAACCGTG  CTCGAACAC/  AAAAGCTGAA  298
BSS1A

BSS1AL1  298 TGT/CTGTGC  GAA/GGGTT/  GAA/GAGCAG  TGCCGCCG/C  GGGGAATCAA  342
BSS1AL2  299 TGTTCTGTGC  GAAAGGGTTT  GAAGGAGCAG  TGCCGCCGGC  GGGGAATCAA  348
BSS1AL3  299 TGTTCTGTGC  GAAAGGGTTT  GAAAGAGCAG  TGCCGCCCGC  CGGGAATCAA  348
BSS1A
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BSS1AL1 343 TCTG/AAC/T  TCACTAAAGC  CGGTACTTTT  TCCCGATGCT  TG/CCAA/GT  388
BSS1AL2 349 TCTGGAACCT  TCACTAAAGC  CGGTACTCTC  T/CGGATGCT  /GGCCAAATT  396
BSS1AL3 349 TCTG/AACCT  /CACTAAAGC  CGGTACTCTC  T/CG/ATGCT  /GGCCAAATT  393
BSS1A

BSS1AL1 389 CAAAGCT/CC  TCCCATGCCT  GCTTGTGCAT  TGTTTG/CTA  AA/CCCCCT/  434
BSS1AL2 397 CAAAGCTTCT  TCCCATGCCT  GCTTGTGCAT  TGTTTGGCTA  AAACCCCCTT  446
BSS1AL3 394 CAAAGCTTCT  TCCCATGCCT  GCTTGTGCAT  TGTTTGGCTA  AAACCCC/TT  442
BSS1A

BSS1AL1 435 GCTAGTGCGC  CAGCTCCAGT  TCCTGCTGCT  AGACCACTAA  ATGGATCGGG  484
BSS1AL2 447 GCTAGTGCGC  CAGCTCCAGT  TCCTGCTGCT  AGACCACTAA  ATGGATCGGG  496
BSS1AL3 443 GCTAGTGCGC  CAGCTCCAGT  TCCTGCTGCT  AGACCACTAA  ATGGATCGGG  492
BSS1A

BSS1AL1 485 ACCGAGTTCA  AACTCAGCTC  CAGCTCCAAG  CCCTTCTCAT  AGTAACCATG  534
BSS1AL2 497 ACCAGGTTCA  AATTCTGCTC  CAGCTCCAAG  CCCTTCTCAT  AGTAACCATG  546
BSS1AL3 493 ACCAGGTTCA  AATTCTGCTC  CAGCTCCAAG  CCCTTCTCAT  AGTAACCATG  542
BSS1A     1                        CAGG  CCCTTCTCAT  AGTAACCATG   24

BSS1AL1 535 GATCTTCAAT  CTCTGTCTTG  TCACTTGCAA  TCAGCGGCGC  ATTGGTCATA  584
BSS1AL2 547 GATCTTCAAT  CTCTGTCTTG  TCACTTGCAA  TCAGCGGCGC  ATTGGTCATA  596
BSS1AL3 543 GATCTTCAAT  CTCTGTCTTG  TCACTTGCAA  TCAGCGGCGC  ATTGGTCATA  592
BSS1A    25 GATCTTCAAT  CTCTGTCTTG  TCACTTGCAA  TCAGCGGCGC  ATTGGTCATA   74

BSS1AL1 585 ATGTTCACCC  GTATCTAACA  CCCCTATCAT  TTTTAGTTAA  GAGTTTGCTT  634
BSS1AL2 597 ATGTTCACCC  GTATCTAACA  CCCCTATCAT  TTTTAGTTAA  GAGTTTGCTT  646
BSS1AL3 593 ATGTTCACCC  GTATCTAACA  CCCCTATCAT  TTTTAGTTAA  GAGTTTGCTT  642
BSS1A    75 ATGTTCACCC  GTATCTAACA  CCCCTATCAT  TTTTAGTTAA  GAGTTTGCTT  124

BSS1AL1 635 TTATCTTGTG  AGTAACTATG  GAGTTCTCTT  GTTGAGACCG  CTTATTTTTA  684
BSS1AL2 647 TTATCTTGTG  AGTAACTATG  GAGTTCTCTT  GTTGAGACCG  CTTATTTTTA  696
BSS1AL3 643 TTATCTTGTG  AGTAACTATG  GAGTTCTCTT  GTTGAGACCG  CTTATTTTTA  692
BSS1A   125 TTATCTTGTG  AGTAACTATG  GAGTTCTCTT  GTTGAGACCG  CTTATTTTTA  174

BSS1AL1 685 GTTATGACAT  TATAAAGACT  TGGTTAAAAA  AAAAAAA               720
BSS1AL2 697 GTTATGACAT  TATAAAGACT  TGGTTAAAAA  AAAAAAA               732
BSS1AL3 693 GTTATGACAT  TATAAAGACT  TGGTTAAAAA  AAAAAAA               728
BSS1A   175 GTTATGACAT  TATAAAGACT  TGGTTAAAAA  AAAAAAA               211
```

The PCR amplification of BSS11A produced a longer BSS11AL1 cDNA of about 450 nucleotides in length (Figure 95). The clone BSS11AL1 was sequenced once and revealed a 442 nucleotide fragment with a 3' end being 100 % identical to BSS11A (Figure 96). From the sequence it was determined that the decamer OPA4 (5'-AATCGGGCTG-3') had amplified the cDNA BSS11A by annealing to the complementary sequence 5'-CAGCCCGATG-3'. These sequences are 90 % homologous as the 5' A of the OPA4 primer mismatched to a G at the 3' end of the complementary sequence of BSS11A.

GenBank Homology Searches

The cDNA fragments BSS1A, BSS2C, BSS4A, BSS5B, BSS7B, BSS8A, BSS11A and BSS13A that represented mRNAs with enhanced expression in the *B. napus* cell suspension cultures in correlation with the increasing presence of sucrose were subjected to homology searches in GenBank (National Center for Biotechnology Information) as described in **MATERIALS AND METHODS**. The significance of alignments with nucleotide and protein sequences requires a critical evaluation of the quantification parameters. The score of an alignment is a value, based on the matrix used, representing the best ungapped local alignment "High Scoring Segment Pair" (HSP) between the sequence submitted and the ones present in the database. It has been reported that scores of ≥ 80, for the BLASTX (deduced amino acid sequence homology) programs, are worthy of further investigation (Pearson, 1991). It should be noted that a

Figure 95. Nucleotide sequence of differentially displayed cDNA BSS11AL1 (BSS1lA longer) with the 3' end of BSS11A added to the 3' end of BSS11AL1. The BSS11A sequence was identified by controlled ribonucleotide tailing of cDNA ends and is expressed as mRNA sequence. First strand cDNA, produced by reverse transcription with 5'-T_{12}AA-3', had the double stranded adaptor (5'-TAGTCCGAATTCAAGCAAGAGCC-3' annealed to 5'-CTCTTGCTTGAATTCGGACTA-3') ligated to the 3' end and then PCR was performed between the adaptor primer and a primer specific to BSS11A (double underlined). The sequence 5'-CATCGGGCTG-3', singly underlined was identified as being 90 % identical to OPA4 (5'-AATCGGGCTG-3') which provided a strong enough annealing of the decamer to allow the cDNA fragment BSS11A to be amplified by the differential display PCR reaction.

```
1    TAACAGAACA  AACCTCCTCC  CGCTCTCGCT  CCTTGCCATT  GCTCTCCTTC    50
51   CTCGCAAAGC  CCTAGCTTTC  TTCTCTTCCC  CCTCTCGCCG  TAAAGCTCGT   100
101  AAGTTGTTCC  TGCTGTTTTT  TTTCGCAGGT  TTCTGATGAA  AATTGAACAA   150
151  CGAAACAGTT  TCCATCGGGC  TGAAGAACGG  AACCATAGCT  CATGGAACCA   200
201  CCACTTCTAT  TCGGGTGTTT  AGGGTTTATT  CCTTTAAATC  TACGACTCTT   250
251  CATGTACCTT  GGAACCTGTC  TTAGCTTCTC  AGTTGAGTAT  TAACAAATTC   300
301  TTAGTTGTAA  TCTTGATTTA  TTTTATCTTT  GTCCTAGGAA  TGATTTGTGC   350
351  CTGAGTGAAT  ACTGGTTTAA  CAGTTAAGCT  TGTTTCTGTT  TATGAGTTTA   400
401  GTTTCAGCAG  TGGAGGAGGT  TTGTTCTGTT  AAAAAAAAAA  AA          442
```

Figure 96. Sequence alignment of BSS11A and BSS11AL1. The / symbol indicates that a nucleotide was absent from one sequence but identified in another. Mismatched nucleotides are indicated in bold.

```
BSS11AL1     1  TAACAGAACA  AACCTCCTCC  CGCTCTCGCT  CCTTGCCATT  GCTCTCCTTC   50
BSS11A

BSS11AL1    51  CTCGCAAAGC  CCTAGCTTTC  TTCTCTTCCC  CCTCTCGCCG  TAAAGCTCGT  100
BSS11A

BSS11AL1   101  AAGTTGTTCC  TGCTGTTTTT  TTTCGCAGGT  TTCTGATGAA  AATTGAACAA  150
BSS11A

BSS11AL1   151  CGAAACAGTT  TCCATCGGGC  TGAAGAACGG  /AACCATAGC  TCATGGAACC  199
BSS11A       1              AATCGGGC  TGAAGAACGG  GAACCATAGC  TCATGGAACC   38

BSS11AL1   200  ACCACTTCTA  TTCGGGTGTT  TAGGGTTTAT  TCCTTTAAAT  CTACGACTCT  249
BSS11A      39  ACCACTTCTA  TTCGG/TGTT  TAGGGTTTAT  TCCTTTAAAT  CTACGACTCT   88

BSS11AL1   250  TCATGTACCT  TGGAACCTGT  CTTAGCTTCT  CAGTTGAGTA  TTAACAAATT  299
BSS11ACA    89  TTTTGTACCT  TGGAACCTGT  CTTAGCTTCT  CAGTTGAGTA  TTAACAAATT  138

BSS11AL1   300  CTTAGTTGTA  ATCTTGATTT  ATTTTATCTT  TGTCCTAGGA  ATGATTTGTG  349
BSS11A     139  CTTAGTTGTA  ATCTTGATTT  ATTTTATCTT  TGTCCTAGGA  ATGATTTGTG  188

BSS11AL1   350  CCTGAGTGAA  TACTGGTTTA  ACAGTTAAGC  TTGTTTCTGT  TTATGAGTTT  399
BSS11A     189  CCTGAGTGAA  TACTGGTTTA  ACAGTTAAGC  TTGTTTCTGT  TTATGAGTTT  239

BSS11AL1   400  AGTTTCAGCA  GTGGAGGAGG  TTTGTTCTGT  TAAAAAAAAA  AAA        442
BSS11A     240  AGTTTCAGCA  GTGGAGGAGG  TTTGTTCTGT  TAAAAAAAAA  AAA        280
```

score of 80 or better is not an absolute guarantee that the sequences are related because the estimation of statistical significance for the BLASTX program is under the assumption that the equivalent of one entire reading frame in the submitted sequence codes for protein and that significant alignments will involve only coding regions. The P value for an alignment search quantifies the probability that the match could have occurred by chance and the number of HSPs observed against the database sequence with scores at least as high. The expect value is a quantification of the number of times a HSP of equal or greater score is expected to occur by chance alone during the database search. The expect and P values are dependent upon the following factors: the scoring scheme used, the residue composition of query sequences, an assumed residue composition for a typical database sequence, the length of the query submitted and the total length of the database. For BLASTN (nucleotide homology), a score of ≥ 140 was considered to be significant except in the cases where multiple alignments occurred within a given known sequence. When sequences were identified that had homology at multiple sites within the submitted sequence, the sum of the scores was added up, and if it was $\geq$ 140, then the homology was considered to be significant. For both BLASTN and BLASTX searches, sequence alignments were considered insignificant if a P value of $\geq$ 0.1 was obtained for the homology.

Fragment BSS1A was subjected to homology searches in GenBank after a longer sequence (BSS1AL1) was isolated for the fragment by controlled ribonucleotide tailing of cDNA ends (CRTC). The cDNA fragment BSS1AL1 was used to search, with the BLASTN program, for homologous sequences in the non-redundant databank and three

sequences displayed strong homology to BSS1AL1. The three sequences, *Saccharomyces cerevisiae* chromosome XV reading frame ORF YOR145c (SCYOR145C) (GenBank accession #Z75053) of 1967 nucleotides, *S. cerevisiae* cosmid pEOA477 from chromosome 15R (SCU55020) (GenBank accession #U55020) of 32,396 nucleotides and *S. cerevisiae* 130 kb DNA fragment from chromosome XV (SC130KBXV) (GenBank accession #X94335) of 129,528 nucleotides, displaying homology to BSS1AL1, were all identical sequences and therefore all three had the same degree of homology to the same region of BSS1AL1 (Figure 97). The gene SCYOR145C from nucleotide 882 - 1004 displayed a 64 % homology to nucleotides 8 - 130 of BSS1AL1 while the nucleotides 886 - 1008 of SCU55020 and nucleotides 128952 - 129074 of SC130KBXV shared the same degree of homology to the same region of BSS1AL1. The resulting score for all three alignments was 219, while the P and expect values were approximately 1×10^{-7} for all three sequences.

When BSS1AL1 was subjected to a homology search in the expressed sequence tag (EST) databank, only one clone of weak homology was found to be similar. The *A. thaliana* cDNA clone 164C1T7 (GenBank accession #R30484) of 379 nucleotides displayed weak homology to BSS1AL1. The region of 164C1T7 between nucleotides 293 and 359 displays a 67 % homology to the region of BSS1AL1 from nucleotide 261 to 327 (Figure 98). The score for this alignment was 145 with a P value of 0.092 and an expect value of 0.097. As with most sequences found in the EST database, the function of this sequence is presently unknown.

The fragment BSS1AL1 was used to search the protein non-redundant databanks and two proteins were detected to be similar to the -3 reading frame translation. The

Figure 97. Sequences displaying nucleotide homology to the fragment BSS1AL1 in GenBank when the non-redundant databanks were searched with the BLASTN program. The sequences exhibiting homology to BSS1AL1 were *Saccharomyces cerevisiae* chromosome XV reading frame ORFYOR145c (Yor145c), *S. cerevisiae* cosmid pE0A477 from chromosome 15R (pE0A477) and the S. cerevisiae 130 kb DNA fragment from chromosome XV (SC130). The nucleotide numbers are listed at the ends of the sequences. The nucleotides that are not identical to BSS1AL1 are in bold.

```
BSS1AL1:      8  AACAAGAGAACTCCTCGCAACCTTAATATTAGTAAACGCCCCCAAGATATGAATC      62
YOR145c:    882  AACCACAGATTCTCTTGCCATTCTGATGTGGGTGAACCCGCCCAAAATGTGGATT     936
pEOA477:    886  AACCACAGATTCTCTTGCCATTCTGATGTGGGTGAACCCGCCCAAAATGTGGATT     942
SC130: 128952  AACCACAGATTCTCTTGCCATTCTGATGTGGGTGAACCCGCCCAAAATGTGGATT  129006

1L1:         63  CTCGTATCCGCAATCACAATCCTCGTCTTCGTCGAGTTCTCAATCGCAAACTTCG      127
YOR145c:    937  TTAGAGTCAGCTAAGACAATTCTTGTTCTTGTAGCGTTTTCAATCGCAAATTTCG     991
pEOA477:    943  TTAGAGTCAGCTAAGACAATTCTTGTTCTTGTAGCGTTTTCAATCGCAAATTTCG     985
SC130: 129007  TTAGAGTCAGCTAAGACAATTCTTGTTCTTGTAGCGTTTTCAATCGCAAATTTCG  129061

1L1:        118  TCTTCCTCTCTGT                                              130
YOR145c:    992  TTTTACCGTCTTT                                             1004
pEOA477:    996  TTTTACCGTCTTT                                             1008
SC130: 129062  TTTTACCGTCTTT                                          129074
```

Figure 98. Sequences displaying nucleotide similarity to fragment BSS1AL1 in GenBank when the expressed sequence tag databanks were searched with the BLASTN program. The *Arabidopsis thaliana* cDNA clone 164C1T7 (164C1T7) was identified. The nucleotide numbers for both clones are listed at the ends of each line of sequence and the nucleotides that are different in 164C1T7 are in bold text.

```
BSS1AL1:   293 CTGAATGTCTGTGCGAAGGGTTGAAGAGCAGTGCCGCCGCGGGGAATCAATCTG   346
164C1T7:   261 CTCAGTGTCTATGTGAAGCGTTTAAGAGCAGTGCTTCTNNTTNGNGTTACTTTG   314


BSS1AL1:   347  AACTTCACTAAAG                                          359
164C1T7:   315  AATATCACTAAGG                                          327
```

proteins were encoded by the YOR3513c gene (GenBank accession #X94335) of 218 amino acids and the ORF YOR145c (GenBank accession #Z75053) of 274 amino acids genes both from *S. cerevisiae* and both being the identical to each other. The proteins YOR3513c and ORF YOR145c are potentially two copies of the same gene as they are both identical. The amino acids from 155 - 192 of YOR3513c and 211 - 248 of ORF YOR145c were 71 % identical and 94 % similar to the amino acids translation from nucleotide 121 - 8 of BSS1AL1 (Figure 99). The fact that YOR3513c and YOR145c had homology to both a translation of BSS1AL1 and to the nucleotide sequences should reinforce the homology identified. There is presently no assigned function to either YOR3513c or YOR145c

Both BLASTN and BLASTX analysis of the fragments BSS2C, BSS4A, BSS5B and BSS7B found no significant homology between these fragments and any other nucleotide sequence.

Comparison of BSS8A to the non-redundant database using BLASTN revealed one gene having significant homology to BSS8A. The sequences having homology to BSS8A was an *A. thaliana* acyl carrier protein isoform (ATHMTMACP) (GenBank accession #L23574). The ATHMTMACP was 562 nucleotides in length and displayed a strong homology to two separate regions of BSS8A. The nucleotides 6 - 87 and 140 - 190 of BSS8A were 91 % and 64 % homologous to nucleotides 314 - 395 and 476 - 526 of the ATHMTMACP (Figure 100). The score for this alignment was 347 and the P and expect values were both 4.3×10^{-24}.

Figure 99. Sequences displaying amino acid homology to a deduced amino acid sequence from the fragment BSS1AL1 when the GenBank non-redundant databanks were searched with the BLASTX program. The proteins YOR3513c and O3513p, both from *Saccharomyces cerevisiae,* were identified. The numbers for BSS1AL1 represent the nucleotides translated into amino acids while the numbers for the other sequences identify the amino acids of the protein. The + symbols identify amino acids that are different but structurally similar. Amino acid differences between BSS1AL1 and the homologous sequences are in bold text. The amino acids presented for BSS1AL1 are encoded by the -3 reading frame.

```
BSS1AL1:   121 KTKFAIENSTKTRIVIADTRIHILGAFTNIKVARSSLV     8
                     + +    +  ++        + ++    +
YOR3513c:  155 KTKFAIENATRTRIVLADSKIHILGGFTHIRMARESVV   192
                     + +    +  ++        + ++    +
O3513P:    211 KTKFAIENATRTRIVLADSKIHILGGFTHIRMARESVV   248
```

Figure 100. Sequences displaying nucleotide similarity to fragment BSS8A in GenBank when the non-redundant and expressed sequence tag databanks were searched with the BLASTN program. The sequences exhibiting homology to BSS8A were the *Arabidopsis thaliana* acyl carrier protein isoform (ATHMTMACP), the *A. thaliana* cDNA clone SBF3T7P and the *A. thaliana* transcribed sequence clone TAI217. The nucleotide numbers are listed at the ends of the sequences. The nucleotides that are not identical to BSS1AL1 are in bold text.

```
BSS8A:       6 CCTGACAAAGAAGCTGACAAGATCGACTCTTGCTCTCTTGCCATTGAATACGTTTTCAAT  65
ATHMTMACP:314 CCAGACAAAGAAGCTGACAAGATCGATTCTTGCTCTCTCGCCATTGAATACGTTTACAAT 373
SBF3T7P:  178 CCAGACAAAGAAGCTGACAAGATCGATTCTTGCTCTCTCGCCATTGAATACGTTTACAAT 237
TAI217:   191 CCAGACAAAGAAGCTGACAAGATCGATTCTTGCTCTCTCGCCATTGAATACGTTTACAAT 250


BSS8A:      66 CATCCTATGTCTAGCTAAACAC                                       87
ATHMTMACP:374 CATCCAATGTCTAGCTAATCGC                                      395
SBF3T7P:  238 CATCCAATGTCTAGCTAATCGC                                      259
TAI217:   251 CATCCAATGTCTAGCTAATCGC                                      272


BSS8A:    138 TTCCAAATAAATTCAGTTCTGTTTTGAGAGTCATTCTTGACTCTTTTAACAGTGTCC    194
ATHMTMACP:476   CAAAAAAATCACTTTTATTTGGAGAGCATTGTTATTGACTCTTTTAACGGT        526
SBF3T7P:  337 TGCAAAAAAATCACTTTTATTTGGAGAGCATTGTTATTGACTCTTTTAAACGGTTCC    393
```

BLASTN analysis of BSS8A to the EST databank revealed two additional genes having significant homology to BSS8A. The *A. thaliana* cDNA clone SBF3TP7 (GenBank accession #T04655) of 439 nucleotides and the *A. thaliana* transcribed sequence clone TAI217 (GenBank accession #Z26408) of 272 nucleotides, both exhibiting similarity to the ATHMTMACP and therefore had homology to the same regions of BSS8A (Figure 100). The clone SBF3TP7 from nucleotides 178 - 259 and 337 - 393 was 91 % and 61 % homologous to nucleotides 6 - 87 and 138 - 194 of BSS8A. The respective scores for this alignment are: 347 and 87, with P and expect values of 1.1×10^{-23}. The 3' end of TAI217 exhibited a 91 % homology between nucleotides 191 and 272 to nucleotides 6 - 87 of BSS8A. The score for this alignment is also 347 while the P and expect values are both 1.1×10^{-23}.

BLASTX analysis of BSS8A detected one sequence displaying strong homology. The *A. thaliana* mitochondrial ACP precursor NADH-Ubiquinone Oxidoreductase 9.6 KD subunit (MTACP-1) (GenBank accession # P53655) is 122 amino acids and exhibited strong sequence homology to the +3 frame translation of BSS8A. The last 25 amino acids of the MTACP-1 had 96 % identity and 100 % similarity to a translation of the nucleotides 6 - 80 from BSS8A (Figure 101). The score of this alignment is 130 with P and expect values of 1.6×10^{-11}. The *Neurospora crassa* mitochondrial acyl carrier protein precursor (NADH-Ubiquinone Oxidoreductase 9.6 KD subunit 12K chain EC 1.6.5.3) (ACPM_NEUCR) (accession # P11943) also had homology to the +3 frame translation of BSS1A (data not shown). The homology of the *N. crassa* ACP sequence was, however, much weaker, only producing a score of 65, a P value of 0.77 and an expect value of 1.5.

Figure 101. Sequences displaying amino acid homology to translations of the fragment BSS8A in GenBank when the non-redundant databanks were searched with the BLASTX program. The one clone identified is the *Arabidopsis thaliana* mitochondrial acyl carrier protein precursor (MTACP-1). Numbers for BSS8A represent the nucleotides translated into amino acids while the numbers for the other sequences identify the amino acids of the protein displaying homology to the +3 reading frame translation of BSS8A. The + symbols identify amino acids that are different but structurally similar.

```
BSS8A:          6   PDKEADKIDSCSLAIEYVFNHPMSS 80
                                    +
MTMACP-1:       98  PDKEADKIDSCSLAIEYVYNHPMSS 122
```

BLASTN analysis of the fragment BSS11A revealed two genes having relatively weak homology. The human autoantigen small nuclear ribonucleoprotein (Sm-D) (1633 nucleotides) and *Gallus gallus* genomic DNA repeat region clone 9C2 (293 nucleotides), have weak homology to two different regions of BSS11A. The region of Sm-D from nucleotide 175 - 239 had a 70 % homology to BSS11A from nucleotide 140 to 204 (Figure 102) and the region of clone 9C2 from 140 and 206 had 68 % homology to BSS11A from nucleotide 82 - 16 (Figure 103). The score of the alignment of BSS11A and Sm-D was 154, with P and expect values of 0.015, while the clone 9C2 had a score of 146, with a P value of 0.054 and an expect value of 0.056. The homology between BSS11A and clone 9C2 was questionable because it was in the opposite direction than predicted from the placement of the oligo(dT) and decamer primer sequences in this clone.

When the BSS11A cDNA fragment was compared to other genes in the EST databank with the BLASTN search tool, seven different cDNAs were identified that had a moderate homology and four were found with weak but significant homology (Figure 102). All the sequences identified had homology to all or part of a stretch of BSS11A between nucleotides 129 and 205. The sequences identified were: *A. thaliana* cDNA clone 139I4T7 (GenBank accession #T46269) of 546 nucleotides, six nearly identical clones from *Citrus unshiu*: a partial length cDNA (pcMFRI807.86) (GenBank accession #C22326) of 310 nucleotides, a partial length cDNA (pcMFRI729.30) (GenBank accession #C22188) of 228 nucleotides, a partial length cDNA (pcMFRI807.19) (GenBank accession #C22311) of 294 nucleotides, a partial length cDNA

Figure 102. Sequences displaying nucleotide similarity to the positive strand of fragment BSS11A in GenBank when the non-redundant and expressed sequence tag databanks were searched with the BLASTN program. The clones identified as exhibiting homology to BSS11A are the *Arabidopsis thaliana* cDNA clone 139I4T7, six very similar cDNA clones from citrus: pcMFRI807.86, pcMFRI729.30, pcMFRI807.19, pcMFRI804.106, pcMFRI719.135, pcMFRI807.15, the cDNA clone 73716 from *Homo sapiens*, the cDNA clone TgME49 from *Toxoplasma gondii*, the cDNA clone 37187 from *H. sapiens*, the cDNA clone 283193 from *H. sapiens* and the *H. sapiens* autoantigen small nuclear ribonucleoprotein Sm-D. The nucleotide numbers are listed at the ends of the sequences. The nucleotides that are not identical to BSS1AL1 are in bold text.

```
BSS11A:            55  CAAAGCCCTAGCTTTCTTCT                                74
pcMFRI807.86:      52  CAAAGCCCTAGCTTTCGTCT                                71
pcMFRI729.30:      54  CAAAGCCCTAGCTTTCGTCT                                73
pcMFRI807.19:      57  CAAAGCCCTAGCTTTCGTCT                                76
pcMFRI804.106:     54  CAAAGCCCTAGCTTTCGTCT                                73
pcMFRI719.135:     57  CAAAGCCCTAGCTTTCGTCT                                76


BSS11A:           129  GTTTCTGATGAAAATTGAACAACGAAACAGTTTCCATCGGGCTGAAGAAC 178
139I4T7:           66  GGTTTTTAATGAAATTNAACAACGAAACAGTTTCAATCGAGCTTAAGAAC 115
pcMFRI807.86:      96  GGTTCTTGATGAAGTTGAACAATGAGACCGTGTCGATTGAACTGAAGAAC 145
pcMFRI729.30:      98  GGTTCTTGATGAAGCTGAACAATGAGACCGTGTCGATTGAACTGAAGAAC 147
pcMFRI807.19:     101  GGTTCTTGATGAAGCTGAACAATGAGACCGTGTCGATTGAACTGAAGAAC 150
pcMFRI804.106:     98  GGTTCTTGATGAAGCTGAACAATGAGACCGTGTCGATTGAACTGAAGAAC 147
pcMFRI719.135:    101  GGTTCTTGATGAAGCTGAACAATGAGACCGTGTCGATTGAACTGAAGAAC 150
pcMFRI807.15:       8          GGAAGCTGAACAATGAGACCGTGTCGATTGAACTGAAGAAC  50
73716:            106            AAATTGAGTCATGAAACTGTAACCATTGAATTGAAGAAC 144
TgME49:           144            AAATTGAGTCATGAAACTGTAACCATTGAATTGAAGAAC 183
37187:            155            AAATTGAGTCATGAAACTGTAACCATTGAATTGAAGAAC 194
283193:            12            AAATTGAGTCATGAAACTGTAACCATTGAATTGNAGAAC  51
Sm-D :            175            AAATTGAGTCATGAAACTGTAACCATTGAATTGAAGAAC 213


BSS11A:           179  GGAACCATAGCTCATGGAACCACCACT                         205
139I4T7:          116  GGAACCATTGTTCATGGAACCATTACT                         142
pcMFRI807.86:     146  GGAACCATTGTTCACGGAACCATCAC                          171
pcMFRI729.30:     148  GGAACCATTGTTCACGGAACCATCAC                          173
pcMFRI807.19:     151  GGAACCATTGTTCACGGAACCATCAC                          176
pcMFRI804.106:    148  GGAACCATTGTTCACGGAACCATCAC                          173
pcMFRI719.135:    151  GGAACCATTGTTCACGGAACCATCAC                          176
pcMFRI807.15:      51  GGAACCATTGTTCACGGAACCATCAC                           74
73716:            145  GGAACACAGGTCCATGGAACAATCAC                          170
TgME49:           184  GGAACACAGGTCCATGGAACAATCAC                          208
37187:            195  GGAACACAGGTCCATGGAACAATCAC                          219
283193:            52  GGAACACAGGTCCATGGAACAATCAC                           71
Sm-D:             214  GGAACACAGGTCCATGGAACAATCAC                          239
```

Figure 103. The sequence displaying nucleotide similarity to the minus strand of fragment BSS11A in GenBank when the non-redundant and expressed sequence tag databanks were searched with the BLASTN program. The clone identified as exhibiting homology to BSS11A is the *Gallus gallus* genomic DNA repeat region from clone 9C2. The nucleotide numbers are listed at the ends of the sequences. The nucleotides that are not identical to BSS1AL1 are in bold text.

```
BSS11A:    82 GGGGGAAGAGAAGAAAGCTAGGGCTTTGCGAGGAAGGAGAGCAATGGCAAGGAGCGAGAG   23
9C2:      140 GGAGGAAGGGAGGGAAGGAAGGAGGGAGGGAGGAAGGAGAACTTTGGGAGGGAGGGACGG  199

BSS11A:    22 CGGGAGG                                                       16
9C2:      200 AGGGAGG                                                      206
```

(pcMFRI804.06) (GenBank accession #C22296) of 295 nucleotides, a partial length cDNA (pcMFRI719.135) (GenBank accession #C22132) of 306 nucleotides, a partial length cDNA (pcMFRI807.15) (GenBank accession #C22310) of 289 nucleotides, a *Homo sapiens* cDNA clone 73716 (GenBank accession # T54686) of 358 nucleotides, a cDNA fragment identified from *Toxoplasma gondii* but suspected to have originated from human contamination, clone TgMe49 (GenBank accession #AA012547) being 389 nucleotides in length, a *Homo sapiens* cDNA clone 37187 (GenBank accession #R35034) 501 nucleotides and a *Homo sapiens* cDNA clone 283193 (GenBank accession #N54730) of 449 nucleotides. The *A. thaliana* clone 139I4T7 from nucleotide 66 to 142 is 81 % homologous to BSS11A between nucleotides 129 and 205. The score of this alignment was 261, with a P value of 9×10^{-12} and an expect value of 9×10^{-12}. The Citrus cDNA clone pcMFRI807.86 from nucleotide 96 to 171 is 75 % homologous and from nucleotide 52 - 71 is 95 % homologous to BSS11A between nucleotides 129 and 204 and 55-74. The scores of this alignment were 209 and 91, with a P value of 6.1×10^{-10} and an expect value of 6.1×10^{-10}. The Citrus cDNA clone pcMFRI729.30 from nucleotide 98 to 173 is 73 % homologous and from nucleotide 54 - 73 is 95 % homologous to BSS11A between nucleotides 129 and 204 and 55-74. The scores of this alignment were 200 and 91, with a P value of 1.4×10^{-9} and an expect value of 1.4×10^{-9}. The Citrus cDNA clone pcMFRI807.19 from nucleotide 101 to 176 is 73 % homologous and from nucleotide 57 - 76 is 95 % homologous to BSS11A between nucleotides 129 and 204 and 55-74. The scores of this alignment were 200 and 91, with a P value of 3.1×10^{-9} and an expect value of 3.1×10^{-9}. The citrus cDNA clone pcMFRI804.106 from nucleotide 98 to 173 is

73 % homologous and from nucleotide 54 to 73 is 95 % homologous to BSS11A between nucleotides 129 and 204 and 55-74. The scores of this alignment were 200 and 91, with a P value of 3.1×10^{-9} and an expect value of 3.1×10^{-9}. The citrus cDNA clone pcMFRI719.135 from nucleotide 101 to 176 is 73 % homologous and from nucleotide 57 to 76 is 95 % homologous to BSS11A between nucleotides 129 and 204 and 55-74. The scores of this alignment were 200 and 91, with a P value of 3.4×10^{-9} and an expect value of 3.4×10^{-9}. The Citrus cDNA clone pcMFRI807.15 from nucleotide 8 to 74 is 77 % homologous to BSS11A between nucleotides 138 and 204. The score of this alignment was 200, with a P value of 1×10^{-6} and an expect value of 1×10^{-6}. The *Homo sapiens* cDNA clone 73716 from nucleotide 106 - 170 is 70 % homologous to BSS11A from nucleotide 140 to 204. The score of this alignment was 154, with a P value of 0.0089 and an expect value of 0.0089. The clone TgME49 from nucleotide 144 to 208 was 70 % homologous to BSS11A from nucleotides 140 to 204. The score of this alignment was 154, with a P value of 0.0091 and an expect value of 0.0091. The *Homo sapiens* clone 37187 from nucleotide 155 to 219 is 70 % homologous to BSS11A from nucleotide 140 - 204. The score of this alignment was 154, with a P value of 0.0096 and an expect value of 0.0097. The *Homo sapiens* clone 283193 from nucleotide 12 to 76 is 69 % homologous to BSS11A from nucleotide 140 - 204. The score of this alignment was 147, with a P value of 0.036 and an expect value of 0.037. All the sequences, in the EST data bank, displaying homology to BSS11A are presently of unknown function. The cDNAs from *C. unshiu* were isolated from a juice sac and pulp segment during a rapid developing stage.

The sequence of BSS11A was used to search for homologous sequences in the non-redundant databanks of GenBank using the BLASTX program and one sequence with weak homology was identified. A Na+/H+ antiporter-like protein from *Caenorhabditis elegans* (CAEEL) (609 amino acids) was identified as having homology to the amino acid translation of four different regions of BSS11A (Figure 104). The CAEEL fragment from amino acid 49 to 77 is 41 % identical and 55 % positive to the +3 frame translation of BSS11A from nucleotide 198 to 284, the amino acids from 109 - 125 of the CAEEL are 33 % identical and 60 % positive to the +2 frame translation from nucleotides 380 to 430, the amino acids from 291 to 305 are 33 % identical and 60% similar to the +3 frame translation from nucleotides 309 - 353 and the amino acids 18 to 34 of CAEEL are 41 % identical and 58 % similar to the +2 frame translation of BSS11A from nucleotide 158 to 208. The reading frame shift here may suggest that this alignment is not all that significant as the score and P values are quite low for this alignment. Alternatively the sequence of BSS11A may have errors in it because it was only sequenced once and these errors may be causing the frame shifts in the homologous regions, thereby reducing the homology between these sequences. The scores for these four regions of homology were 55, 34, 33 and 32 with P values of 0.3, 0.3, 0.77 and 0.3 and expect values of 0.35, 0.35, 1.5 and 0.35, respectively. Individually each of the homologous regions between BSS11A and the CAEEL fragment are insignificant but collectively this homology may be significant. The CAEEL protein has not been functionally characterized but it is suspected of being involved in the adjustment of cellular pH.

The sequence of BSS13A was subjected to a homology search in the non-

Figure 104. Sequences displaying homology to the fragment BSS11A in GenBank when the non-redundant databanks were searched with the BLASTX program. The protein sequence of a probable Na+/ H+ antiporter from *Caenorhabditis elegans* was identified as having homology to two stretches of each of the +2 and +3 reading frame translations of BSS11A. Numbers for BSS11A represent the nucleotides translated into amino acids while the numbers for the other sequences identify the amino acids of the protein. The + symbols identify amino acids that are different but structurally similar. The amino acids that are not similar or identical are in bold text.

```
BSS11A(Frame +2):      158 VSIGLKNGTIAHGTTTS                  208
                           + +           +
CAEEL:                 18  IIVGLALGWILHQTSLS                   34

BSS11A (Frame +2):     380 LFLFMSLVSAVEEVCSV                  430
                           + +  + +   +    +
CAEEL:                 109 ILVFSALISAVDPVAVI                  125

BSS11A (Frame +3):     198 PPLLFGCLGFIPLNLRLFMYLGTCLSFSV      284
                             ++     +           +
CAEEL:                 49  PPIIFGSSGYFMPNRALFENFDSVLVFSV       77

BSS11A (Frame +3):     309 NLDLFYLCPRNDLCL                    353
                           +   +++
CAEEL:                 291 HFDLYFICATLFFCL                    305
```

redundant databanks of GenBank using the BLASTN program and six sequences were identified that had strong homology (Figure 105). The identified sequences were: *A. thaliana* 5' adenylylphosphosulfate reductase (APR2) (GenBank accession #U56921) being 1450 nucleotides, *A. thaliana* 3'-phosphoadenosine-5'-phosphosulfate (PAPS) reductase homolog (PRH43) (GenBank accession #U53866) being 1738 nucleotides, *A. thaliana* PAPS reductase homolog (PRH26) (GenBank accession #U53865) being 1738 nucleotides, *A. thaliana* 5' adenylylphosphosulfate reductase (APR3) (GenBank accession #U56922) of 1482 nucleotides, *A. thaliana* PAPS reductase homolog (PRH19) (GenBank accession #U53864) being 1748 nucleotides and *A. thaliana* 3'-phosphoadenosine 5'-phosphosulfate reductase mRNA (ATU43412) (GenBank accession #U43412) being 1634 nucleotides.

The APR2 gene from nucleotides 1072 to 1293, 1389 to 1413 and 1274 to 1307 had 86 %, 80 % and 70 % homology to nucleotides 4 to 225, 296 to 320 and 205 to 238 of BSS13A. The alignment scores for these homologous regions were 840, 80 and 80 with P and expect values being 7.4×10^{-68}. The PRH43 gene from nucleotide 1315 to 1498, 1489 to 1539 and 1634 to 1658 was 88 %, 74 % and 80 % identical to BSS13A. The scores for the alignment of these homologous regions were 731, 138 and 80 with P and expect values of 2.2×10^{-63}. The PRH26 gene from nucleotide 1301 to 1465 is 73 % identical to nucleotides 3 to 167 of BSS13A. The alignment score for this region is 429 with P and expect values of 5.3×10^{-26}. The APR3 gene from nucleotide 1144 to 1331 is 69 % identical to nucleotides 14 to 201 of BSS13A. The score for this alignment is 427 with P and expect values of 7.7×10^{-26}. The PRH19 gene from nucleotide 1287 to 1449 is 72 % homologous to nucleotides 5 to 167 of BSS13A. The alignment score for this

Figure 105. Sequences displaying nucleotide similarity to fragment BSS13A in GenBank when the non-redundant databanks were searched with the BLASTN program. Six different clones: the a 5'-adenylylphosphosulfate reductase (APR2), a 3'-phosphoadenosine-5'-phosphosulfate (PAPS)-reductase homolog (PRH43), a PAPS reductase homolog (PRH26), a 5'-adenylylphosphosulfate reductase (APR3), a PAPS reductase homolog (PRH19) and a 3'-phosphoadenosine 5'-phosphosulfate reductase (U43412) all from *Arabidopsis thaliana*, were identified as exhibiting strong homology to BSS13A. The nucleotide numbers are listed at the ends of the sequences and the nucleotides that are not identical to BSS1AL1 are in bold text.

```
BSS13A:    4 CGGGCTGATGGTGACCAGAAGGAGTTTGCCAAGCAAGAGCTTCAATTAGGAAGCTTCCCG   63
APR2:   1072 CGAGCTGACGGTGAGCAGAAGGAGTTTGCTAAGCAAGAGCTTCAGTTAGGGAGCTTCCCG 1131
PRH43:  1315 CGAGCTGACGGTGAGCAGAAGGAGTTTGCTAAGCAAGAGCTTCAGTTAGGGAGCTTCCCG 1374
PRH26:  1302 AGAGCTGATGGTGACCAGAAGGACTTTGCCAAAAAGGAGTTGCAGCTTGGAAGCTTTCCG 1361
APR3:   1144           GTGACCAGAAGGACTTTGCCAAAAAGGAGTTGCAGCTTGGAAGCTTTCCA 1193
PRH19:  1287  GAGCAGATGGTGACCAGAAGGAGTTTGCTAAGCAGGAATTGCAGCTCGGTAGCTTCCCT 1345
U43412: 1245  GAGCAGATGGTGACCAGAAGGAGTTTGCTAAGCAGGAATTGCAGCTCGGTAGCTTCCCT 1303


BSS13A:   64 ACGATACTTCTCTTCCCGAAAAGCGCTCCGCGGGCAATTAAGTACCCGTCAGAGCATAGA  123
APR2:   1132 ACGATACTTCTCTTTCCGAAAAGAGCTCCACGGGCTATTAAGTACCCTTCAGAGCATAGA 1191
PRH43:  1375 ACGATACTTCTCTTTCCGAAAAGAGCTCCACGGGCTATTAAGTACCCTTCAGAGCATAGA 1434
PRH26:  1362 ACAATACTTGTGTTCCCAAAGAACTCTTCAATACCAATCAAGTATCCATCAGAGAAGAGA 1421
APR3:   1194 ACAATACTTGTGTTCCCAAAGAACTCTTCAAGACCAATCAAGTATCCATCAGAGAAGAGA 1253
PRH19:  1347 ACCATTCTGGTTTTCCCTAAGAACTCATCGAGACCGATCAAGTATCCGTCTGAGAAGAGA 1405
U43412: 1304 ACCATTCTGGTTTTCCCTAAGAACTCATCGAGACCGATCAAGTATCCGTCTGAGAAGAGA 1363


BSS13A:  124 GATGTGGATTCGCTTATGTCATTTGTGAATCTTCTCCGGTGAGTTGTGAGATTGGAAAGT  183
APR2:   1192 GATGTTGATTCACTCATGTCGTTTGTGAATCTTCTTCGGTGAGTTATGAGATAGAAAATA 1251
PRH43:  1435 GATGTTGATTCACTCATGTCGTTTGTGAATCTTCTTCGGTGAGTTATGAGATAAGAAAAT 1494
PRH26:  1422 GATGTTGATTCTTTGACATCTTTCTTGAATCTTGTTCGGTAAAT                  1465
APR3:   1254 GATGTTGATTCTTTGACATCTTTCTTGAATCTTGTTCGGTAAATCCGCAACTAGAACAGA 1313
PRH19:  1406 GATGTTGAGTCTTTGACTTCGTTCTTGAATCTTGTCCGATAAGT                  1449
U43412: 1364 G                                                         1365


BSS13A:  184 TAAACAGTGTGATGAAGAATGAAGAAAACTGAAGATGAAGGA                   225
APR2:   1252 AAAAGAGGGTGATGAAGAATGAAAGAGAATTGAGATGAAGGA                  1293
PRH43:  1495 AAAAGAGGGTGATGAAGAATGAAAGAGAATTGAGATGAAGGAGG                 1539
APR3:   1314 TGAAGAAGGTGAAGATGA                                          1331


BSS13A:  296 TATATAGACTCTTTTTCTGCCTTCT                                   320
APR2:   1389 TTTATAGACTCTTTTTGTTACCTCT                                  1413
PRH43:  1634 TTTATAGACTCTTTTTGTTACCTCT                                  1658


BSS13A:  205 AAGAAAACTGAAGATGAAGGATGATTTCATCTTG                          238
APR2:   1274 AAGAGAATTGAGATGAAGGAGTGATTTCATCTTG                         1307
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region is 410 with P and expect values of 2.3×10^{-24}. The ATU43412 gene from nucleotide 1245 to 1364 is 73 % identical to nucleotides 5 to 124 of BSS13A. The alignment score for this region is 312 with P and expect values of 4.9×10^{-16}.

The strong homology observed between BSS13A and these sequences suggested that BSS13A was a putatively a member of the thioredoxin superfamily. The APR26 clone from *A. thaliana* exhibited the strongest homology to BSS13A and it has been used to complement an *Escherichia coli* mutant defective in PAPS reductase activity. Furthermore the APR26 clone from *A. thaliana* also has a N terminal extension characteristic of a plastid transit peptide suggesting that BSS13A is putatively involved in sulfur metabolism in the plastid. These putative functions will have to be further verified by obtaining a full length clone for BSS13A and determining a function for the encoded protein.

BLASTX analysis of BSS13A detected six proteins which displayed strong homology to the +1 reading frame translation (Figure 106). The 5'-adenylylphosphosulfate reductase (accession #U56921), the PRH43 protein (accession #U53866), the PRH19 protein (accession #U53864), the PRH26 protein (accession #U53865), a 3'-phosphoadenosine-5'-phosphosulfate (PAPS)-reductase like protein (accession #U63784) and a 3'-phosphoadenosine 5'-phosphosulfate reductase (accession #U43412) all from *A. thaliana*, except for the PAPS reductase which was from *Catharanthus roseus*, exhibit strong homology to a translation of BSS13A. The amino acids 354 to 406 of U56921 had a 96 % identity and 98 % similarity to the +1 reading

Figure 106. Sequences displaying homology to BSS13A when the non-redundant databanks, of GenBank, were searched with the BLASTX program. Six different but similar clones: a 5'-adenylylphosphosulfate reductase from *Arabidopsis thaliana* (U56921), the cDNA clone PRH43 from *A. thaliana* (U53866), the cDNA clone PRH19 from *A. thaliana*, the PRH26 clone from *A. thaliana*, a 3'-phosphoadenosine-5'-phosphosulfate (PAPS) -reductase like protein from *Catharanthus roseus* (U63784) and the PAPS-reductase from *A. thaliana* (U43412) were identified that had homology to BSS13A. Numbers for BSS13A represent the nucleotides translated into amino acids while the numbers for the other sequences identify the amino acids of the protein. The + symbols identify amino acids that are different but are structurally similar. Amino acids present that are not identical or similar to BSS13A are in bold text.

```
BSS13A:     4 RADGDQKEFAKQELQLGSFPTILLFPKSAPRAIKYPSEHRDVDSLMSFVNLLR 162
                  +
U56921:   354 RADGEQKEFAKQELQLGSFPTILLFPKRAPRAIKYPSEHRDVDSLMSFVNLLR 406
                  +
U53866:   401 RADGEQKEFAKQELQLGSFPTILLFPKRAPRAIKYPSEHRDVDSLMSFVNLLR 453
                                      +   ++              +      + +
PRH19:    413 RADGDQKEFAKQELQLGSFPTILVFPKNSSRPIKYPSEKRDVESLTSFLNLVR 465
                        +   +-            +   ++                    + +
PRH26:    406 RADGDQKDFAKKELQLGSFPTILVFPKNSSIPIKYPSEKRDVDSLTSFLNLVR 458
              +         +                    + +                 +
U63784:   411 KADGDQKAFAQQELQLNSSPTILFFPKHSSKPIKYPSEKRDVDSLMAFVNALR 463
                                      +   ++
U43412:   412 RADGDQKEFAKQELQLGSFPTILVFPKNSSRPIKYPSEKR               451
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frame translation of BSS13A from nucleotide 4 to 162. This strong homology produced a score of 260, with P and expect values of 5.1×10^{-29}. The amino acids 401 to 453 of PRH43 were identical to amino acids 354 to 406 of U56921 and therefore exhibited the same homology to BSS13A. The amino acids 413 to 465 of PRH19 had an 81 % identity and 92 % similarity to the +1 frame translation of BSS13A from nucleotides 4 to 162. The amino acids 406 to 458 have a 77 % identity and 90 % homology to the +1 reading frame of BSS13A from nucleotide 4 to 162. The amino acids 411 to 463 of the PAPS-reductase like protein exhibit a 73 % identity and 83 % similarity to the +1 frame translation of BSS13A from nucleotides 4 to 162. The amino acids 412 to 451 of U43412 exhibited a 85 % identity and 92 % similarity to the +1 frame translation of BSS13A from nucleotide 4 to 123. The detection of these protein sequences displaying strong homology to a translation of BSS13A in combination with the observed homology to the nucleotide sequences suggests that BSS13A encodes part of a mRNA which is closely related to these genes.

DISCUSSION

This thesis has examined the effects of sucrose concentration on TAG biosynthesis and gene expression in an oil-forming MD cell suspension culture of oilseed rape. Zygotic and MD embryos of *B. napus* have been shown to accumulate TAG during a series of complex developmental processes (Pomeroy *et al.*, 1991; Taylor and Weber, 1994). Seed development in oilseed rape is comprised of a number of processes including TAG deposition, storage protein deposition, oleosin production and thickening of seed tissues. The developmental processes occurring in maturing seeds would likely complicate the identification of genes related to specific processes such as TAG accumulation. The cell suspension system, remains in a non-differentiated state therefore it is assumed that most of the gene expression associated with developmental processes does not occur (Orr *et al.*, 1986; Simmonds *et al.*, 1991). The cell suspension system, however, was induced to accumulate TAG by increasing the growth media sucrose concentration. Ross and Murphy (1983) have suggested that increasing the sucrose content or the carbon/nitrogen ratio of the growth medium of TAG forming plant cell cultures may direct more carbon flow from membrane lipid formation into TAG by induction of DGAT. In the current study, the increased TAG accumulation was also accompanied by increased DGAT activity. The elevated levels of DGAT activity may be associated with increased production of the corresponding mRNA. In the MD cell suspension system, the expression of the major oleosin transcript increased in response to the elevated levels of media sucrose. In studies with TAG-forming cultures of *Pimpinella anisum* L., an

association was observed between media sucrose content, TAG production and the abundance of an 18.4 kDa oleosin (Radetzky and Langheinrich, 1995). As well the expression of a 21.2 kDa oleosin isoform was reduced and delayed in a mutant of *A. thaliana* where TAG biosynthesis was impaired (Zou *et al.*, 1996).

The increased media sucrose concentration resulted in increased levels of the fatty acids 16:0, 16:1, 18:3 and 24:1 while the fatty acids 20:0, 20:1, 22:0 and 24:0 decreased in abundance. Studies involving other plant cell cultures have shown that sucrose and osmoticum can have an influence on fatty acid composition. Pence *et al.* (1981) have shown that increasing concentration of sucrose in the growth media of asexual embryos of *Theobroma cacao* L. cultured *in vitro* increased the proportion of monounsaturated fatty acids at the expense of polyunsaturates. In cultured zygotic embryos of *B. napus* L. cv Nugget increased osmoticum due to the addition of 0.69 M sorbitol resulted in enhanced accumulation of the 20:1 and 22:1 fatty acids (Finkelstein and Somerville, 1989).

Comparative studies of differential gene expression in MD cell suspension cultures of *Brassica napus* L. cv Jet Neuf at different sucrose concentrations may lead to identification of genes encoding TAG biosynthetic enzymes such as DGAT. Differential display was used to monitor gene expression in the oilseed rape cell suspension culture as a function of sucrose concentration in the growth medium. This method had previously been shown to be rapid and sensitive for the detection of genes for which expression levels were altered following different treatments (Torelli *et al.*, 1996; Benito et al., 1996). The method of mRNA DD was originally designed as a sensitive assay for

detection of altered gene expression in mammalian systems (Liang and Pardee, 1992). The isolation of cDNAs from the cell suspension system was part of a larger project aimed at the isolation and characterization of TAG biosynthetic enzymes. Eight cDNA fragments were identified, which appeared to be induced when the media sucrose concentration was increased from 2 % to 6 % to 14 %. These cDNAs were isolated and partially characterized.

The BSS8A fragment encoded a known component of lipid biosynthesis. A translation of BSS8A had a 96 % identity and 100 % homology to the last 25 amino acids of a mitochondrial acyl carrier protein (MTMACP) from *A. thaliana*. The BSS8A fragment also exhibited strong nucleotide homology to the MTMACP in the region between the stop codon and the poly A tail. This further substantiated the likely identity of BSS8A because this region of mRNA is usually less conserved than the coding region. The role of a mitochondrial ACP in fatty acid biosynthesis is not clear. Plastidial ACP isoforms are known to be essential protein cofactors for *de novo* fatty acid biosynthesis (Ohlrogge, 1978). Therefore, their expression may increase in the cell suspension system as sucrose concentration is increased. Plastidial ACPs have been characterized much more extensively than mitochondrial isoforms which have only recently been reported. A report by Shintani and Ohlrogge (1994) found that a mitochondrial ACP, from *A. thaliana*, could act as a cofactor for *in vitro* chloroplast fatty acid synthesis, suggesting that this protein plays a similar role in mitochondria. It has been reported that mitochondria of *Neurospora* are capable of *de novo* fatty acid synthesis (Mikolajczyk and Brody, 1990). Cytoplasmic fatty acid synthesis in animal and fungal systems, however,

has been found to be sufficient to maintain fatty acid metabolism in the cell (Shintani and Ohlrogge 1994). Acyl carrier proteins are predominantly localized in the plastids (Ohlrogge *et al.*, 1978), but the occurrence of ACPs in plant mitochondria suggests that it may be necessary to re-evaluate the compartmentalization of fatty acid synthesis and also possible alternative functions of ACP in plants. The apparent upregulation of a mRNA encoding a mitochondrial ACP in the oil-forming cultures suggested that a further investigation of mitochondrial involvement in fatty acid metabolism is warranted. The possibility of a larger contribution of mitochondria to lipid production in non-green tissues may also need to be examined.

The fragment BSS11A encoded a mRNA that was expressed at much higher levels in the cell suspension culture grown in the presence of 6 % and 14 % cultures versus those grown in 2 % sucrose cultures. BSS11A had strong homology to the *A. thaliana* cDNA clone 139I4T7 for which a putative function has not been identified. The homology of BSS11A to another clone verified that a non-artifactual cDNA fragment was cloned. Six other partial length clones from citrus also displayed homology to BSS11A. Their functions have not been reported. Weak, but significant homology, was also identified between BSS11A and a number of human sequences and a sequence from *Toxoplasma gondii*. Homology between the *G. gallus* DNA repeat region and BSS11A was likely a result of the C/T richness of these sequences.

Database searches indicated that the fragment BSS1AL1 had weak but significant homology to the cDNA clone 164C1T7 from *A. thaliana*, stronger homology to three very similar regions from *S. cerevisiae* and proteins encoded by two of the *S. cerevisiae*

regions. Neither the cDNA clones, nor the proteins, have been characterized and therefore are of unknown function. The cDNA clone 164C1T7 from *A. thaliana* should be further characterized in order to determine the extent of similarity to BSS1AL1. The alignment with the *A. thaliana* sequence was in a positive frame but the alignment with the proteins from *S. cerevisiae* was in a minus strand translation of BSS1AL1 which indicates that the alignment was not significant. Alternatively, the amplification of BSS1A may have occurred with the oligo(dT) primer annealing to an internal poly A site within the message. Amplification of cDNA during the DD procedure has been shown to occur between the oligo(dT) primer and a decamer where the primers are in the opposite orientation than expected (Benito *et al.*, 1996). This amplification, however, occurs much less frequently than those where the oligo(dT) primer is closer to the 3' end of the mRNA than the decamer.

The cDNA fragments BSS2C, BSS4A, BSS5B, and BSS7B revealed no significant homology to any known sequences at either the amino acid or nucleotide level. This suggested that these fragments have not been isolated before, making them ideal candidates to be investigated for a role in TAG biosynthesis. With the large sequencing projects underway for *A. thaliana* (Hofte *et al.*, 1993; Newman *et al.*, 1994), a close relative of *B. napus*, the detection of these novel cDNA sequences suggests that the corresponding mRNA sequences are expressed in relatively low abundance. The sequencing projects may not be able to detect the rare messages effectively. Clones are randomly picked, and those representing rare mRNAs are not very abundant and therefore picked less often. In contrast DD can easily detect rare sequences as PCR amplification

is involved.

The fragment BSS13A appeared to encode a protein involved in sulfur metabolism. The deduced amino acid sequence of BSS13A exhibited high sequence identity to other known proteins of the thioredoxin superfamily suggesting an involvement in the reductive assimilation of sulfur (reviewed in Schmidt *et al.*, 1992). The increased expression of enzymes involved in sulfur metabolism has previously been shown to occur rapidly upon sulfur starvation (Gutierrez-Marcos *et al.*, 1996). An increased demand for sulfur could be occurring in the cell suspension system in cells grown at higher sucrose concentrations. BSS13A was not further investigated because it probably does not encode a TAG biosynthetic enzyme.

None of the upregulated messages detected were similar to known sequences involved in cellular response to stress. For example, none of the cDNA fragments had homology to stress-inducible genes such as water channel proteins (Bohnert *et al.*, 1995) or heat shock proteins (Czarnecka *et al.*, 1984; Heikkila *et al.*, 1984). It is possible that the increased media sucrose concentrations did not induce the expression of osmotically related sequences in the mRNA subpopulation examined.

Overall, the results of the analysis of mRNA from the cell suspension system indicated that continued screening of the transcripts, coupled with characterization, should eventually reveal genes encoding TAG biosynthetic enzymes. In total, 22 primer combinations were used to examine gene expression from the cDNA fraction primed with the oligo(dT) primer 5'-T_{12}AA-3' and to identify the 8 upregulated fragments. Assuming that mRNAs are randomly distributed with respect to the bases adjacent to the polyA tail,

there are approximately 100 additional upregulated sequences to be identified in this system. Approximately 8 upregulated mRNAs should be identified in each of the other 11 fractions of mRNA.

A number of advantages were made apparent through the use of DD in analysis of gene expression in the cell suspension system. The technique was less time consuming than other methods for analysis of gene expression. Differential display encompasses RT, PCR, polyacrylamide gel electrophoresis and autoradiography. These are established molecular techniques which have been used for a number of years. The sensitivity level of DD was also relatively high. Fragments were detected that were not detectable by northern blot hybridization. Others have reported that the mRNA sequences detected by DD may represent rare messages (Benito *et al.*, 1996; Torelli *et al.*, 1996). Northern blot hybridizations have also been found to be inadequate to analyze the expression of those genes. The ability to compare cells grown under more than two sucrose concentrations was a distinct advantage because cDNAs were identified that were expressed maximally under growth in 6 % sucrose compared to culturing in 2 % and 14 % sucrose.

One disadvantage of DD in this study was the simultaneous amplification of a number of cDNA fragments of similar length. Extraction and cloning of multiple cDNAs of similar length from the DD gel led to a substantial amount of extra analysis to identify the sequence which actually represented upregulated mRNA. An extreme example of this problem was found in relation to the BSS1 cDNA which resulted in the cloning of 5 different cDNAs. BSS1B was an artifact and BSS1A was upregulated, BSS1C and BSS1E were unaffected and BSS1D was repressed by the increasing sucrose

concentrations in the growth medium. The difficulty of getting multiple messages of similar length may be due to the nature of the oligo(dT) primer that was employed. The use of a 3 base anchored primer may address this problem, but this additional base would mean that the number of cDNA fractions would increase to 48. This would substantially increase the amount of screening necessary to cover the full complement of mRNAs. Increasing the number of cDNA fractions up to 48 would, however, still be faster than examining a large number of fragments, by northern blots, to identify the ones that were actually upregulated.

A number of difficulties with the DD technique could be eliminated by alterations to the procedures following the isolation of cDNA fragments from the DD gel. Liang and Pardee (1994) suggested that cDNA fragments isolated from the DD gel could be used as probes for northern blots and cDNA libraries, as well as for subcloning and sequencing. Our experience with DD has led us to propose a revised protocol for analysis of cDNA fragments identified by the technique. With this modified approach, cDNA fragments are extracted from the DD gel, cloned and transformed into *Escherichia coli* competent cells. Following transformation, colony PCR is performed to determine which clones contain constructs corresponding in size to fragments of interest. For example, 10 clones with inserts of the appropriate size could be propagated and plasmid isolated from each. Following the isolation of these clones, they are dot blotted onto different membranes; individual membranes are used for each of the cellular conditions being analyzed. A reverse dot blot is performed by probing the membranes with labeled first strand cDNA from each of the cells analyzed by DD. This procedure should effectively eliminate

artifacts of the PCR reaction and the need to perform a large number of northern blot hybridizations. By reducing the number of northern blots to be carried out, the procedure would be much more efficient and would further reduce the need for isolated RNA. The reduced demand for RNA would be ideally suited to experiments performed with limited amounts of tissue. Furthermore, because dot blots could be performed using a multi-well vacuum plate, multiple clones could be analyzed simultaneously, thereby speeding up the procedure. This approach could prove to be a useful strategy for rapid identification of genes displaying altered mRNA expression levels.

Differential display may become a valuable tool for the identification of differentially regulated genes associated with oil accumulation. Other techniques of gene expression analysis such as two dimensional protein gels and differential or subtractive hybridization may be less useful for the identification of lipogenic enzymes. Difficulties with the two dimensional protein analysis stem from the low quantities of protein available for sequencing and the lack of enzyme activity following the denaturing polyacrylamide gel electrophoresis (Liang and Pardee, 1992). Differential hybridization may not be sensitive enough and too time consuming when searching for genes which are likely not of high abundance (Liang and Pardee, 1994). Subtractive hybridization has been reported to be much more sensitive but appears to more effectively detect newly transcribed sequences rather than upregulated ones. Direct purification of these enzymes is another approach, but difficulties have been experienced with maintaining activity during the purification process (Little *et al.*, 1994; Kocsis *et al.*, 1996). For example, the instability of DGAT during purification has proven to be a major obstacle in the

purification of this enzyme (Weselake *et al.*, 1993; Little *et al.*, 1994).

One approach commonly used in the examination of lipid production is mutagenesis (Somerville and Browse, 1991; Maluszynski et al., 1995; Gibson et al., 1994; Kinney, 1994). Deoxyribonucleic acid is randomly mutated and then the plants must be screened to determine if the mutations affect oil production and then further screened to identify the site of mutation. A mutation in DGAT may be difficult to detect if more than one isoform of the enzyme is present in developing seeds. Furthermore, the process of oil production could be altered at many points prior to the steps of the Kennedy pathway. Thus, a considerable amount of work may be required to identify the actual function lost by the mutation. If a plant was identified that was only defective in DGAT activity, then identification of the gene may be more direct than by a DD approach. Production of a DGAT mutant, however, may make the process less efficient than DD for the isolation of DGAT. Furthermore, there is presently not enough information regarding the regulation of TAG accumulation and DGAT to know if such a mutant could be produced. With the recent identification of the enzyme DGTA (Lehner and Kuksis, 1996) it is possible that there are multiple routes to form TAG and a mutation in one of these routes may not prevent oil production from occurring. If DGAT is a multimer, or if multiple isoforms are present, then possibly a mutation in one would not prevent oil production.

CONCLUSIONS

The use of DD to identify mRNAs that are upregulated in correlation with increased oil accumulation in the cell suspension culture of *B. napus* L. cv Jet Neuf has been shown to be rapid and sensitive. Furthermore, a number of changes have been suggested to improve upon the current DD methodology. Although the exact function of all of the upregulated cDNA fragments has yet to be determined, the similarity of BSS8A to an ACP, the expression of the major oleosin, the relatively low abundance of the mRNAs identified and the lack of identification of genes related to other processes suggests that our approach to identification of genes encoding TAG biosynthetic enzymes is reasonable. Furthermore the cell suspension system may also be a valuable tool for the identification of lipogenic enzymes.

FUTURE DIRECTIONS

Further investigations must be carried out to elucidate the role of the cDNAs identified by DD in relation to TAG biosynthesis. Initially, northern blots or RT-PCR should be performed with these fragments using RNA from developing seeds of *B. napus* that are actively accumulating oil. The fragments displaying upregulation during the active phase of oil accumulation could be used to screen a cDNA library to identify full length clones. The full length cDNAs could be expressed in a simple eukaryotic system, such as yeast, followed by analysis of lipid metabolism in order to assign a function to the gene. The yeast system would be much simpler and faster than expression in a plant

system but it may not produce a functional enzyme as the posttranslational modifications of yeast may be quite different than that of the plant system. Alternatively the full length cDNAs could be overexpressed or expressed in antisense orientation in *A. thaliana* and the plants could be examined for alterations of lipid metabolism. Expression of cDNAs in *A. thaliana* is more complicated than that of the yeast system however there is a greater chance of getting a functional enzyme from a plant system. Finally, the other eleven fractions of the mRNA pool from the cell suspension system should be examined to detect all the possible upregulated mRNAs. By screening all the mRNA subsets with DD, it should theoretically be possible to identify most of the mRNAs involved in TAG biosynthesis. Identification of all proteins associated with lipogenesis in *Brassica napus* should allow a more complete understanding of the mechanisms which facilitate seed oil production. This knowledge could then serve as a foundation for developing strategies for modification of seed oil content.

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Appendix 1. Reverse Transcription With Verification of Efficiency

		Final Concentration
RNA (200 ng/ µl)	1.0 µl (0.2 µg)	0.01 µg/µl
$T_{12}AA$ (20 µM)	1.0 µl (20 pmoles)	1.0 µM

Incubated at 65°C for 5 minutes to eliminate the RNA secondary structures such as hairpin loops, and cooled on ice

DTT (0.1 mM)	2.0 µl	10.0 µM
Buffer (5 X)	4.0 µl	1 X
H_2O	8.9 µl	
dNTPs (A,C,G and T) (250 µM of each)	1.6 µl	20.0 µM

Heated to 42°C for 10 minutes to equilibrate the reaction mixture.

RNase inhibitor (10 U/µl)	0.5 µl	0.25 µM
SuperScript II reverse transcriptase (400 U/µl)	1.0 µl	20.0 U/µl
	20 µl total volume	

removed 2.5 µl from each reaction and placed in a separate tube with 0.5 µl of [α-^{32}P]dCTP with a 3000 Ci/ mmol.

incubated at 42°C for 50 minutes to synthesize first strand cDNA.

incubated at 95°C for 2 minutes to quench the reaction by denaturing the enzyme.

Non radioactive fractions were maintained at -20°C until used for PCR amplification.

A 1 µl aliquot was taken from each labeled sample, placed on Whatman filter paper, placed on a vacuum filter and washed with 3 ml of 10 % TCA to remove the unincorporated nucleotides and dried by washing with 7 ml of 95 % ethanol. Each filter was then placed in a separate glass vial that had been previously counted to determine background and counted in a Beckman scintillation counter using an open channel.

The 5 X RT buffer consists of:
250 mM Tris-HCl (pH=8.3), 375 mM KCl, 15mM $MgCl_2$

Appendix 2. Differential display

- Performed according to Liang and Pardee, 1994

i) Reverse Transcription:

Performed according to appendix 1.

ii) PCR:

		Final concentration
Buffer (10 X)	2.0 µl	1 X
dNTPs (25 µM)	1.6 µl	2.0 µM
anchored oligo(dT) primer (20 µM)	1.0 µl	1.0 µM
decamer (2 µM)	2.0 µl	0.2 µM
[α-^{32}P]dCTP (3000 Ci/mmol)	0.2 µl	0.033 µM
Taq DNA polymerase(5 U/µl)	0.3 µl	0.075 U/µl
1st strand cDNA 2 fold dil'n	2.0 µl	
H_2O	10.9 µl	
	20 µl final volume	

overlay each reaction with 50 µl of light mineral oil (one drop)

40 cycles of: 94°C - 1 min, 40°C - 2 min, 72°C - 30 seconds
1 cycle of : 72°C - 10 minutes

The PCR reaction employed in DD is adapted somewhat from standard PCR protocols. The nucleotide concentration is only 2 µM while regular PCR uses 200 µM as larger amounts have been found to cause the production of multiple cDNA fragments where only one should be amplified (Liang and Pardee, 1992). The anchored oligo(dT) primer is present at 5 times the concentration of the decamer primer as the oligo(dT) should theoretically anneal to all the cDNA sequences in the reaction.

The 10 X PCR buffer consists of:
100 mM Tris-HCl (pH 8.3), 500 mM KCl, 15 mM $MgCl_2$ and 0.01 % gelatin.

iii) PAGE:

in a 0.6 ml microcentrifuge tube add:

3.5 µl from PCR reaction
2.0 µl of formamide loading buffer (95 % (w/v) formamide, 0.09 % (w/v) bromphenol blue, 0.09 % (w/v) xylene cyanol FF)
- heated to 95°C for 2 minutes, cool tubes to at least 60°C before moving them.
- spun tubes briefly in a microcentrifuge to collect vapors

Loaded 2 μl of the denatured PCR reaction and loading buffer mix to individual lanes on a 5.5 % polyacrylamide gel made to 1 X TBE. The running buffer was 1 X TBE (pH 8.0).

Ran gel until the xylene cyanol dye is roughly 80% through the gel and the bromphenol dye is well off the end of the gel.

Transferred gel to a sheet of Whatman 3 MM filter paper, with fluorescent rulers along the sides and covered with saran wrap

The counts of ^{32}P were determined with a hand held scanner and for counts between 100,000 and 10,000 autoradiography was performed at -80^{o}C for 1 - 48 hours respectively.

Appendix 3. Colony PCR

Colony PCR is performed by amplification from either side of the vector insert site. This provides information about the length of the insert that has been cloned into the vector.

PCR		**Final concentration**
Buffer (10 X)	5.0 µl	1 X
dNTPs (1.25 mM)	4.0 µl	200.0 µM
vector specific primer 1 (20 µM)	1.25 µl	1.0 µM
vector specific primer 2 (20 µM)	1.25 µl	1.0 µM
Taq DNA polymerase (5 U/µl)	0.3 µl	0.06 U/µl
H_2O	13.2 µl	
	25.0 µl final volume	

The PCR mixture was aliquoted into separate tubes and covered with 50 µl of sterile mineral oil. A sterile toothpick was touched to an individual bacterial colony, streaked on a master plate as a stock of the colony and rolled in the PCR reaction thereby transferring the plasmid DNA into the reaction. Care was taken not to transfer too many bacteria as this is inhibitory to the amplification. Generally if the bacteria can be detected on the end of the tooth pick then too many are being transfered.

The reaction tubes are then subjected to 35 cycles of:

94°C - 1 minute, 55°C - 1 minute, 72°C - 2 minute

followed by 72°C for 10 minutes

Following the PCR amplification approximately 2.5 µl of loading dye was mixed into each tube and 10 µl of each reaction was separated by agarose gel electrophoresis. The concentration of the agarose is determined by the expected size of the cloned insert plus the vector ends which are amplified by the vector specific primers. For example, an insert of 100 bp in a pGEM T vector will amplify 325 bp when the PUC primers are used as 225 bp of vector DNA is also amplified. Following electrophoresis the gels were stained with ethidium bromide for 20 minutes, destained for 10 minutes in water and visualized by placing the gel on UV transilluminator and photographed.

The 10 X PCR buffer:
100 mM Tris-HCl (pH 8.3), 500 mM KCl, 15 mM $MgCl_2$ and 0.01 % gelatin.

The Loading buffer consists of:
0.01 % (w/v) xylene cyanol and 40 % sucrose.

Appendix 4. Sequencing with the ABI PRISM Sequencing Kit

Part I. Sequencing reaction

0.5 μg of vector DNA with insert	5.0 μl
3.2 pmoles of M13 forward or reverse primer	1.0 μl
PCR mix (dNTPs, ddNTPs, taq DNA polymerase and buffer)	8.0 μl
H_2O	6.0 μl
	20.0 μl final volume

apply 50 μl of light mineral oil (one drop) and subject the reactions to:

25 cycles of: 96°C - 10 seconds, 50°C - 5 seconds, 60°C - 4 minutes

Part II. Purification of Products

Streak the PCR reactions out on parafilm to remove the oil and precipitated the DNA to allow removal of the unincorporated dye terminators.

To each tube add:

Sodium acetate (pH 4.6)	2.0 μl
ethanol (95 %)	50.0 μl

- vortex briefly and place on ice for 10 minutes
- centrifuge at 12,000 x g for 15 - 30 minutes.
- aspirate the ethanol supernatant
- rinse the pellet (usually invisible) with 250 μl of 70 % ethanol
- aspirate the ethanol
- dry pellet in a vacuum centrifuge.

The dried pellets can then be run through a sequencer to determine the DNA sequence.

Ideally the DNA being sequenced should be as clean as possible as contaminants present during the sequencing process will lead to a reduction in the accuracy and length of DNA that can be sequenced.

Appendix 5. Labelling a probe by PCR

- Performed according to instructions given in the PCR labelling kit (Life technologies).

		Final concentration
PCR buffer (10X)	5.0 µl	1X
dNTPs (A, G and T) (25 µM)	6.0 µl	3.0 µM
DNA 1 - 10 ng	1.0 µl	1 - 10 ng
primer 1 (10 µM)	1.3 µl	0.26 µM
primer 2 (10 µM)	1.3 µl	0.26 µM
Taq DNA polymerase (5 U/µl)	0.6 µl	0.06 U/µl
α^{32}P dCTP (3000 Ci/ mmol and 10Ci/l)	5.0 µl	0.33 µM
H_2O	29.8 µl	
	50.0 µl final volume	

overlay with 50 µl of light mineral oil

PCR is performed for 30 cycles with temperatures depending upon the primers being used and the time of the PCR reaction cycles being determined by the length of the template. For example, an 800 bp cDNA fragment that is to be amplified by primers with a Tm of 65°C would typically be amplified for 30 cycles with an annealing temperature of 60°C and an extension time of 1 - 2 minutes as the Taq DNA polymerase can extend approximately 1000 nucleotides per minute.
Non radioactive PCR amplifications are performed with the primers prior to the radioactive amplification so the optimum temperatures for the primers is already known.

After amplification the PCR reaction was run through a 1 ml disposable syringe containing Sephadex G-50 (Pharmacia) to remove unincorporated nucleotides. The probe was denatured by the addition 25 µl of 1 M NaOH and heated to 70°C for 2 minutes, neutralized by the addition of 25 µl of 1 M HCl and rapidly added directly to the hybridization buffer in the tube with the membrane being probed.

Appendix 6. Normalization of Blotted RNA

Normalization of northern blots is required to verify that an equal amount of RNA has been added to the denaturing gel and that equal amounts have been transferred to the membrane. Typically "house keeping" genes are used to probe the membrane as they are usually unaffected by changes in cellular conditions. Usually it is best to probe with a number of housekeeping genes as some can change in expression under different cellular conditions.

The sequence of the mitochondrial elongation factor TU from *Arabidopsis thaliana* was obtained from GenBank, accession #X89227, as it has been used to standardize northern blots by Wilkinson *et al.*, (1995). Primers were designed to amplify a 329 bp fragment, from nucleotide 1063 to 1392, of this nuclear encoded *A. thaliana* gene. The primers were designed using OLIGO 4.0 which enables the identification of primers which can efficiently amplify a region of interest and have a Tm of about 60°C. Ideally primers for this type of amplification should have a Tm of about 60°C so that annealing temperatures will be about 60°C but could be raised up to 72°C if other artifacts are also amplified. The primers were then used to amplify the fragment from *B. napus* first strand cDNA using the same amplification conditions as described in appendix 7.

Appendix 7. Reverse Transcription - Polymerase Chain Reactions (RT-PCR)

Step (I) Reverse Transcription With Verification of Efficiency.

			Final Concentration
total RNA	(200 ng/ µl)	1.0 µl	0.01 µg/µl
T_{15}	(20 µM)	1.0 µl	1.0 µM

- Heated to 65°C for 5 minutes to eliminate the RNA secondary structures such as hairpin loops.
- cooled on ice

DTT (0.1 mM)	2.0 µl	10.0 µM
Buffer (5 X)	4.0 µl	1 X
H_2O	8.9 µl	
dNTPs (A,C,G and T) (250 µM of each)	1.6 µl	20.0 µM of each

Heated to 42°C for 10 minutes to equilibrate the reaction mixture.

RNase inhibitor (10 U/µl)	0.5 µl	0.25 U/µl
SuperScript II reverse transcriptase (200 U/µl)	1.0 µl	20.0 U/µl
	20 µl	total volume

removed 2.5 µl from each reaction and placed in a separate tube with 0.5 µl of [α-^{32}P]dCTP with a 3000 Ci/ mmol.

incubated at 42°C for 50 minutes to synthesize first strand cDNA.

incubated at 95°C for 2 minutes to quench the reaction by denaturing the enzyme.

Non radioactive fractions were maintained at -20°C until used for PCR amplification.

A 1 µl aliquot was taken from each labeled sample, placed on Whatman filter paper, placed on a vacuum filter and washed with 3 ml of 10 % TCA to remove the unincorporated nucleotides and dried by washing with 7 ml of 95 % ethanol. Each filter was then placed in a separate glass vial that had been previously counted to determine background and counted in a Beckman scintillation counter using an open channel.

The RT buffer consists of:
250 mM Tris-HCl (pH 8.3), 375 mM KCl, 15 mM $MgCl_2$.

Step (II) PCR reactions

		Final concentration
Buffer (10 X)	2.5 µl	1 X
DNTPs (A,C,G,T, 1.25mM of each)	4.0 µl	200 µM of each
primer1 (20 µM)	1.25 µl	1.0 µM
primer2 (20 µM)	1.25 µl	1.0 µM
Taq DNA polymerase (5 U/µl)	0.3 µl	0.06 U/µl
DNA template	2.0 µl	
H_2O	13.7 µl	
	25.0 µl final volume	

overlay with 50 µl of mineral oil (one drop)

20 - 35 cycles of:

94°C 1 minute, 45 - 65°C 1 minute and 72°C 1 minute

One cycle of:

72°C 10 minutes

The number of cycles was varied so that the PCR products were just becoming visible when separated by agarose gel electrophoresis. The number of cycles was limited to prevent the PCR products from reaching a saturation level where they all will appear at equal intensity. Ideally one wants to be in the linear range of the PCR amplification which is typically before 30 cycles. The annealing temperature of the PCR depended on the Tm of the primers used to amplify the fragments.

The PCR reaction used for the RT-PCR analysis was a standard, non radioactive PCR protocol, so the nucleotide concentration was a lot higher (30 times) than that of the PCR reaction used for the differential display PCR amplification

The PCR buffer consists of
100 mM Tris-HCl (pH 8.3), 500 mM KCl, 15 mM $MgCl_2$ and 0.01 % gelatin.

Appendix 8. Controlled Ribonucleotide Tailing of cDNA Ends (CRTC)

i) Reverse Transcription:

		Final Concentration
poly A mRNA (1.0 µg/µl)	1.0 µl	0.025 µg/µl
5'-T_{12}AA-3' or gene specific primer (1.0 µg/µl)	1.0 µl	0.025 µg/µl
H_2O	7.0 µl	

Incubated at 65°C for 5 minutes to remove RNA secondary structure, cool on ice

RT buffer (5 X)	8.0 µl	1 X	
0.1 M DTT	4.0 µl	0.01	M
dNTPs (1.25mM of each of A,C,G and T)	16.0 µl	0.5	mM

Incubated at 42°C for 10 minutes to equilibrate the reaction

SuperScript II reverse transcriptase (200 U/µl)	2.0 µl	10	U/µl
RNase inhibitor (10 U/µl)	1.0 µl	0.4	U/µl
	40.0 µl final volume		

> 42°C for 60 minutes to allow the assembly of a DNA strand complementary to the mRNA

- Check RT efficiency as described in Appendix 1.

- Labelled first strand cDNA was run through a Sephadex G-50 column, to remove unincorporated labelled dNTPs and separated out on 1.5 % agarose gel to verify that efficient RT has been performed on the the longer mRNAs. Following electrophoresis, autoradiography was performed to visualize the separated cDNAs and it is expected that most will appear between 500 and 2000 nucleotides as most mRNAs are of that size.

The RT buffer consists of:
250 mM Tris-HCl (pH 8.3), 375 mM KCl, 15 mM $MgCl_2$.

ii) Removal of RNA from the first strand cDNA

Following the verification that RT was performed successfully, 20 µl of the first strand cDNA was added to 80 µl of Na_2CO_3/Na_2HCO_3 (pH 9.5).

Incubated at 90°C for 30 minutes

The temperature in combination with the basic pH will effectively hydrolyze RNA while leaving the cDNA intact.

The reaction was incubated at 37°C for 60 minutes to allow the phosphorylation of the primer. Then the reaction was then incubated at 65°C for 20 minutes to denature the enzyme.

Add:

primer A_1 (1 µg/µl) 5.0 µl

Incubate at 45°C for 10 minutes to anneal the primers and thereby assemble the adaptor.

vi) Addition of Adaptor to cDNA:

		Final concentration
adaptor from step v	2.0 µl	0.27 µg/µl
T_4 DNA ligase (1 unit/µl)	3.0 µl	0.3 U/µl
T_4 DNA ligase buffer (5 X)	6.0 µl	1 X
ATP (10 mM)	3.0 µl	1.0 mM
H_2O	16.0 µl	
	30.0 µl final volume	

Incubate at 15°C for 12 hours to allow ligation of the adaptor to the cDNA.

vii) Second strand synthesis:

The following reagents were added to 5.0 µl of the cDNA from step vi.

		Final Concentration
PCR buffer (10 X)	2.5 µl	1 X
dNTPs (1.25 mM of each of A,C,G and T)	4.0 µl	200 µM of each
H_2O	13.0 µl	
Taq DNA polymerase (5 U/µl)	0.5 µl	0.1 U/µl
	25.0 µl final volume	

overlay each tube with 50 µl of mineral oil (one drop)

The tube was incubated at 72°C for 40 minutes to allow the extension of the second strand of the cDNA.

viii) PCR to amplify out specific cDNAs

		Final concentration
Buffer (10 X)	2.5 µl	1 X
dNTPs (A,C,G,T, 1.25 mM of each)	4.0 µl	200 µM of each
primer1 (20 µM)	1.25 µl	1.0 µM
primer2 (20 µM)	1.25 µl	1.0 µM
Taq DNA polymerase (5 U/µl)	0.3 µl	0.075 U/µl
template DNA	2.0 µl	
H_2O	13.7 µl	
	25.0 µl final volume	

overlay each reaction with 50 µl of mineral oil (one drop)

20 - 35 cycles of:

94°C 1 minute, 45 - 65°C 1 minute, 72°C 1 minute

One cycle of:

72°C 10 minutes

Following the PCR amplification half of the reaction is placed in a separate tube with 1.25 µl of loading dye, mixed and the subjected to electrophoresis in a 1.5 % agarose gel. If not products are visible then a second PCR amplification may be performed on a dilutions of the first PCR amplification. Typically 1 µl from each of 10, 100, 500, 1000, 2000, 5000, and 10000 fold dilutions of the first PCR reaction are subjected to further amplifications. Following the second PCR amplification products are separated on an agarose gel as for the first amplification.

Alternatively the first amplification may be performed with only the gene specific primer in which case 1.25 µl more water is added to maintain the final volume at 25 µl. If only the gene specific primer is used for the first reaction a second PCR is performed employing both the adaptor and gene specific primer. The second PCR is performed on dilutions of the first as shown in the standard method and then agarose gel electrophoresis is performed as listed in the standard method.

This procedure is technically challenging especially for mRNAs which are not expressed at very high levels. Typically smearing results from the use of too much template and a lack of specificity in the PCR reaction. Sometimes specificity can be increased by increased annealing temperatures and by the use of a nested primer designed to anneal upstream from the first gene specific primer.

cDNA was then purified using the Glass Max purification system (Canadian Life Technologies)

iii) Tailing Reaction:

		Final Concentration
cDNA eluted from Glass Max column	25.0 µl	
H_2O	8.5 µl	
5 X Tdt buffer	10.0 µl	1 X
$CoCl_2$ (25 mM)	3.0 µl	1.5 mM
rGTP (100 µM)	2.5 µl	5.0 µM
Tdt (25 U/µl)	1.0 µl	0.5 U/µl
	50.0 µl	

iv) Precipitation

The following reagents were added to the reaction from step iii:

125 µl 100 % ethanol
2.5 µl 5 mg/ml glycogen
5 µl 3M NaOAC (pH 5.2)
Incubate for 30 minutes at -80°C (do not let it freeze) and centrifuge for 20 minutes at 12,000 x g and 4°C.
remove supernatant
wash with 200 µl of 70 % ethanol, remove supernatant without disturbing pellet, dry in a vacuum centrifuge.

v) Synthesis of Adaptor:

made primers using an Oligo 1000 DNA synthesizer:

A1 5'-TAGTCCGAATTCAAGCAAGAGCC-3'
A2 5'-CTCTTGCTTGAATTCGGACTA-3'

Addition of a phosphate group to the 5' end of A_2

		Final volume
primer A_2 (1.0 µg/µl)	5.0 µl	5.0 µg/µl
ATP (10 mM)	2.5 µl	1.0 mM
polynucleotide kinase buffer (10 X)	2.5 µl	1 X
T4 Polynucleotide kinse (10 U/µl)	0.5 µl	0.2 U/µl
H_2O	14.5 µl	
	25.0 µl final volume	