

2001

Molecular strategies for increasing seed oil content in canola

Wiehler, William B.

Lethbridge, Alta. : University of Lethbridge, Faculty of Arts and Science, 2001

<http://hdl.handle.net/10133/126>

Downloaded from University of Lethbridge Research Repository, OPUS

**MOLECULAR STRATEGIES FOR INCREASING SEED
OIL CONTENT IN CANOLA**

William B. Wiehler

(Bachelor of Science, University of Lethbridge, 1998)

A Thesis

**Submitted to the School of Graduate Studies Council
of the University of Lethbridge
in Partial Fulfillment of the
Requirements for the Degree**

MASTER OF SCIENCE

UNIVERSITY OF LETHBRIDGE

LETHBRIDGE, ALBERTA, CANADA

Copyright William B. Wiehler, 2001

May 22, 2001

**TO MY FAMILY: BRENDA, HECTOR, TIM, MICHELLE, BRAD AND
MACKINLEY**

**AND TO SHAHINA, MY FIANCÉE AND BEST FRIEND IN THE WHOLE
WORLD**

ABSTRACT

Previous research has shown that microsomal DGAT activity from cultures of *Brassica napus* was stimulated by human acylation stimulating protein (ASP) and bovine serum albumin (BSA). Genetic constructs were engineered to facilitate the expression of ASP or BSA in developing seeds of *B. napus*. Genetic constructs have also been engineered to facilitate the expression of ASP or BSA in the ER of developing seeds to test the stimulatory effect of these proteins at the site of TAG formation. As well, genetic constructs were designed to produce a truncation of the BSA polypeptide in an attempt to localize the portion of the macromolecule responsible for stimulation of DGAT activity. An oleosin promoter was used for seed specific expression and to express the polypeptides at a precisely coordinated time when oil was accumulating in the developing seeds. Lipid analysis was performed on the seeds of transgenic plants designed to cytosolically express these mammalian proteins and the seeds of control plants. The first generation data revealed that the total lipid within the mature seeds of ASP and BSA plants was not significantly different from the total lipid of negative control plants using both gravimetric and low resolution-nuclear magnetic resonance methods of analysis. The seeds from ASP 8 and ASP 10 plants, however, did produce significantly more lipid on a per seed basis as compared to negative control plants. The levels of the fatty acid composition for the total acyl lipids were measured in these first generation transgenic plants. ASP 3 had significantly lower levels of linoleic acid, ASP 14 had significantly lower levels of α -linolenic acid and BSA 11 had significantly higher levels of both of these fatty acids in comparison to negative control plants.

ACKNOWLEDGMENTS

I would like to thank the following individuals:

My graduate committee members, Drs. Kevin Smith, Barry Micallef and Brent Selinger for their guidance and suggestions.

My co-supervisor, Dr. André Laroche for his expertise and support with all of the challenges that I encountered.

My supervisor, Dr. Randy Weselake, for approaching me to do a graduate program with him, always making time to help when I was having trouble, and being a mentor in every sense of the word.

Dr. Maurice Moloney for allowing me to work in his lab and interact with the many experts that he has working for him.

Dr. Nii Patterson for introducing me to several new molecular techniques and always making himself available as a scientist and a friend.

Joemel Alcantara for his expertise in plasmid drawing.

Tara Furukawa-Stoffer, Chris Kazala, Mandy Bondaruk, Dr. Cory Nykiforuk and Dr. Fred Lozeman for always providing insight and making the lab a fun place to conduct research.

Carol Anderson for helping me with the growth of transgenic plants and countless amounts of valuable advice.

Parveen Boora and Rafael Bustamante for their hard work with the bacterial expression systems.

My fiancée, Shahina, for always believing in me and being an important part of my life.

Finally, I would like to thank the Alberta Agricultural Research Institute (Farming for the Future) and the Natural Sciences and Engineering Research Council of Canada for supporting this research.

TABLE OF CONTENTS

Title Page	i
Signature Page	ii
Dedication	iii
Abstract	iv
Acknowledgments	v
Table of Contents	vi
List of Tables	xi
List of Figures	xii
Abbreviations	xvii
1. INTRODUCTION	1
2. LITERATURE REVIEW	4
2.1. FA Biosynthesis	4
2.2. TAG Bioassembly	9
2.3. Genetic Manipulations Affecting TAG Production	11
2.4. Oleosins	13
2.4.1. Structure of Oleosins	14
2.4.2. Function of Oleosins	15
2.4.3. Incorporation of Oleosins into Oil Bodies	16
2.4.4. Oil Body Degradation and Oleosins	16
2.5. Plant Transformation	17

2.6.	<i>In vivo</i> Formation of ASP	19
2.7.	Link Between Obesity and ASP Levels	20
2.8.	Effect of ASP on TAG Biosynthesis	21
2.9.	Properties of Bovine Serum albumin (BSA)	23
2.10.	<i>In vivo</i> Formation of Native Serum Albumin.	25
2.11.	Effect of BSA on DGAT Activity	25
2.12.	Bacterial Protein Expression	26
3.	MATERIALS AND METHODS	30
3.1.	Chemicals	30
3.2.	Isolation and Purification of Plasmid DNA	30
3.2.1.	Miniscreen for Sequencing Purity Level DNA	30
3.2.2.	Quick Alkaline Lysis	31
3.2.3.	Kit Method	31
3.3.	Restriction and Ligation of DNA	32
3.4.	Polymerase Chain Reaction	32
3.5.	DNA Purification: Glass Powder Elution of DNA (Gene- Cleaning)	32
3.6.	DNA sequencing	33
3.7.	Preparation of Competent <i>E. coli</i> Cells	33
3.8.	Transformation of Competent <i>E. coli</i> .	34
3.9.	Preparation of Electrocompetent <i>A. tumefaciens</i> Cells	34
3.10.	Electro-Transformation of Competent <i>A. tumefaciens</i> Cells	35
3.11.	Transformation of <i>B. napus</i>	36

3.12.	Plant Material and Growth Conditions	. . .	36
3.13.	Neomycin Phosphotransferase Detection in Leaf Tissue	. . .	36
3.14.	Total DNA Extraction From Leaf Tissue	. . .	37
3.15.	Total RNA Extraction From Developing Seeds	. . .	38
3.16.	Protein Extraction From Mature Seeds	. . .	39
3.17.	Generation of ³² P-Labelled Probes	40
3.18.	Southern and Northern Blotting	40
3.19.	Western Blotting	41
3.20.	RT-PCR	41
3.21.	Oil Analysis Using Low Resolution NMR	42
3.22.	Lipid Extraction With Hexane-Isopropanol	43
3.23.	Analysis of FA Composition of Seed Acyl Lipids Using GLC	44
3.24.	Statistics	44
4.	RESULTS AND DISCUSSION	46
4.1.	Towards Expression of ASP or BSA in the Cytoplasm of Seeds	46
4.1.1.	Genetic Constructs for Cytosolic Expression of ASP	47
4.1.2.	Insertion of the <i>ASP02</i> Gene Into the Binary Vector, pCGN1559	61
4.1.3.	Identification of Transgenic Plants: NptII Assay	71
4.1.4.	Integration of ASP cDNA into the Plant Genome	78
4.1.5.	Production of ASP Transcripts	82

4.1.6.	Production of ASP Protein	85
4.1.7.	Genetic Constructs for Cytosolic Expression of BSA	88
4.1.8.	Insertion of the <i>BSA02</i> Gene into the Binary Vector, pCGN1559	107
4.1.9.	Identification of Transgenic Plants: NptII Assay	116
4.1.10.	Integration of BSA cDNA into the Plant Genome	116
4.1.11.	Production of BSA Transcripts	121
4.1.12.	Production of BSA Protein	124
4.2.	Characteristics of Mature Seeds From Transgenic Plants	129
4.2.1.	Total Lipid for Negative Control Plants	130
4.2.2.	Total Lipid for ASP Plants	132
4.2.3.	Total Lipid for BSA Plants	132
4.2.4.	Correlation Between Gravimetric and NMR Analysis for Total Lipid From Mature Seeds	135
4.2.5.	Seed Weight and Amount of Lipid Per Seed For Negative Control Plants	138
4.2.6.	Seed Weight and Amount of Lipid Per Seed For ASP Plants	138
4.2.7.	Seed Weight and Amount of Lipid Per Seed For BSA Plants	142
4.2.8.	FA Composition of the Total Acyl Lipids From Negative Control Plants	142
4.2.9.	FA Composition of the Total Acyl Lipids From ASP Plants	147

4.2.10.	FA Composition of the Total Acyl Lipids From BSA Plants	149
4.3.	Towards Expression of ASP and BSA in the ER	152
4.3.1.	Genetic Constructs to Facilitate Expression of ASP in the ER.	152
4.3.2.	Genetic Constructs to Facilitate Expression of BSA in the ER.	168
4.4.	Bacterial Expression of BSA and a Truncation of the Protein	193
4.4.1.	Genetic Construct to Facilitate Bacterial Expression of Mature BSA	197
4.4.2.	Genetic Construct to Facilitate Bacterial Expression of a Truncation of BSA	215
5.	SUMMARY	227
6.	FUTURE DIRECTIONS	228
6.1.	Transgenic Plants Carrying ASP or BSA for Cytosolic Expression	228
6.2.	Genetic Constructs to Facilitate Expression of ASP or BSA in the ER	228
6.3.	Crossing ASP Plants With BSA Plants	229
6.4.	Bacterial Expression of Mature BSA and a Truncation of BSA	229
6.5.	Biophysical Studies With ASP or BSA and Recombinant DGAT	229
6.6.	Expression of Truncated BSA in Developing Seeds	230
7.	LITERATURE CITED	231

LIST OF TABLES

Table 1.	Results of NptII dot blot assay of extracts of leaves from putative transformed canola plants.	79
Table 2.	Results of NptII dot blot assay of extracts of leaves from putative transformed canola plants.	120
Table 3.	Total lipid expressed as percent in mature seeds of transgenic negative control plants analyzed gravimetrically and with low-resolution NMR.	131
Table 4.	Total lipid expressed as percent in mature seeds of transgenic ASP plants analyzed gravimetrically and with low-resolution NMR.	133
Table 5.	Total lipid expressed as percent in mature seeds of transgenic BSA plants analyzed gravimetrically and with low-resolution NMR.	134
Table 6.	Seed weight and amount of oil per seed measured gravimetrically for mature seeds of negative control plants.	139
Table 7.	Seed weight and amount of oil per seed measured gravimetrically for the mature seeds of transgenic ASP plants.	141
Table 8.	Seed weight and amount of oil per seed measured gravimetrically for the mature seeds of transgenic BSA plants.	143
Table 9.	Fatty acid composition of total acyl lipids expressed as percent in mature seeds of transgenic negative control plants.	146
Table 10.	Fatty acid composition of total acyl lipids expressed as percent in mature seeds of transgenic ASP plants.	148
Table 11.	Fatty acid composition of total acyl lipids expressed as percent in mature seeds of transgenic BSA plants.	150

LIST OF FIGURES

Figure 1.	Generalized representation of fatty acid (FA) and triacylglycerol (TAG) biosynthesis in developing oilseeds.	5
Figure 2.	Primers used to amplify the DNA sequence encoding ASP for incorporation into pOlePN3'.	48
Figure 3.	Plasmid map for pOlePN3'.	50
Figure 4.	Agarose gel electrophoresis analysis of PCR amplification of ASP cDNA using <i>Pfu</i> polymerase and primers ASP742 and ASP743.	53
Figure 5.	Restriction analysis using <i>Bam</i> HI and <i>Pst</i> I on a putative clone containing pBW(ASP01).	55
Figure 6.	Sequence alignment for a putative <i>ASP01</i> gene in colony 1 against the known DNA sequence encoding ASP.	57
Figure 7.	Plasmid map for pBW(ASP01).	59
Figure 8.	Restriction analysis using <i>Bam</i> HI and <i>Pst</i> I on plasmid DNA from putative pBW(ASP02) clones.	62
Figure 9.	Plasmid map for pBW(ASP02).	64
Figure 10.	Plasmid map for the binary vector, pCGN1559.	67
Figure 11.	Restriction analysis using <i>Kpn</i> I on pBW(ASP02) and pCGN1559.	69
Figure 12.	Orientation analysis using <i>Bam</i> HI on plasmid DNA from a putative pBW(Bin-ASP03) clone.	72
Figure 13.	Sequence alignment for a putative <i>ASP03</i> gene in colony 1 against the known DNA sequence encoding oleosin promoter, ASP and ubiquitin terminator.	74
Figure 14.	Plasmid map for pBW(Bin-ASP03).	76

Figure 15.	Southern blot analysis of <i>KpnI</i> digests of 30 µg of genomic DNA isolated from transformed <i>B. napus</i> plants.	80
Figure 16.	Northern blot analysis of 30 µg of total RNA extracted from developing seeds of transformed <i>B. napus</i> plants.	83
Figure 17.	Western blot analysis using monoclonal antibodies against ASP of negative control mature seed protein extract spiked with purified ASP polypeptide.	86
Figure 18.	Primers used to amplify the mature BSA cDNA sequence for incorporation into pOlePN3'.	89
Figure 19.	Agarose gel electrophoresis analysis of PCR of BSA cDNA using <i>Pfu</i> polymerase and primers BSA736 and BSA747.	91
Figure 20.	Restriction analysis using <i>BamHI</i> and <i>PstI</i> for putative pBW(BSA01) clones.	94
Figure 21.	Sequence alignment for a putative <i>BSA01</i> gene in colony 2 against the known BSA cDNA sequence.	96
Figure 22.	Plasmid map for pBW(BSA01).	99
Figure 23.	Restriction analysis using <i>BamHI</i> and <i>PstI</i> on plasmid DNA from putative pBW(BSA02) clones.	102
Figure 24.	Plasmid map for pBW(BSA02).	104
Figure 25.	Restriction analysis using <i>KpnI</i> on pCGN1559 and pBW(BSA02).	108
Figure 26.	Restriction analysis using <i>KpnI</i> on plasmid DNA from a putative pBW(Bin-BSA03) clone.	110
Figure 27.	Orientation analysis using <i>BamHI</i> on plasmid DNA from putative pBW(Bin-BSA03) clones.	112
Figure 28.	Sequence alignment for a putative <i>BSA03</i> gene in colony 3 against the known DNA sequence encoding oleosin promoter, BSA and ubiquitin terminator.	114
Figure 29.	Plasmid map for pBW(Bin-BSA03).	117

Figure 30.	Southern blot analysis of <i>KpnI</i> digests of 30 µg of genomic DNA isolated from transformed <i>B. napus</i> plants.	122
Figure 31.	Agarose gel electrophoresis of an internal DGAT cDNA fragment that was amplified from total RNA extractions of developing seeds of transformed <i>B. napus</i> using RT-PCR.	125
Figure 32.	Agarose gel electrophoresis of BSA cDNA that was amplified from total RNA extractions of developing seeds of transformed <i>B. napus</i> using RT-PCR.	127
Figure 33.	Relationship between lipid percentage obtained gravimetrically and lipid percentage obtained using LR-NMR from mature seeds of all transgenic plants.	136
Figure 34.	Primers used to amplify the DNA sequence encoding ASP for insertion into pSBS2050.	153
Figure 35.	Integration of DNA sequence encoding ASP into pSBS2050 by disrupting the stop codon in the DNA sequence encoding oleosin protein.	156
Figure 36.	Agarose gel electrophoresis analysis of PCR amplification of pASP using <i>Pfu</i> polymerase and primers ASP871 and ASP872.	158
Figure 37.	Electrophoretic analysis of DNA following colony PCR using <i>Taq</i> polymerase and primers ASP871 and ASP872.	160
Figure 38.	Restriction analysis using <i>EcoRV</i> on putative pBW(ASP04) clones.	162
Figure 39.	Sequence alignment for a putative <i>ASP04</i> gene in colony 1 against the known DNA sequence encoding ASP.	164
Figure 40.	Plasmid map for pBW(ASP04).	166
Figure 41.	Primers used to amplify the mature BSA cDNA for insertion into pSBS2050.	169
Figure 42.	Plasmid map for pSBS2050.	171
Figure 43.	Integration of BSA cDNA into pSBS2050 by disrupting the stop codon in the DNA sequence encoding oleosin protein.	174

Figure 44.	Agarose gel electrophoresis analysis of PCR amplification of pBSA using <i>Pfu</i> polymerase and primers BSA861 and BSA862.	176
Figure 45.	Electrophoretic analysis of DNA following colony PCR using <i>Taq</i> polymerase and primers BSA861 and BSA862.	178
Figure 46.	Restriction analysis using <i>EcoRV</i> on plasmid DNA from colony 1, which was presumed to contain the <i>BSA05</i> gene.	181
Figure 47.	Sequence alignment for a putative <i>BSA05</i> gene in colony 1 against the known BSA cDNA sequence.	183
Figure 48.	Plasmid map for pBW(BSA05).	186
Figure 49.	Restriction analysis using <i>PstI</i> on plasmid DNA from putative pBW(BSA06) clones.	189
Figure 50.	Orientation analysis using <i>PstI</i> and <i>ScaI</i> on plasmid DNA from putative pBW(BSA06) clones.	191
Figure 51.	Plasmid map for pBW(BSA06).	194
Figure 52.	Primers used to amplify the mature BSA cDNA for insertion into pET26b(+).	198
Figure 53.	Agarose gel electrophoresis analysis of PCR amplification of BSA cDNA using <i>Pfu</i> polymerase and primers BSA975 and BSA976.	200
Figure 54.	Plasmid map for the bacterial expression vector, pET26b(+).	202
Figure 55.	Electrophoretic analysis of DNA following colony PCR using <i>Taq</i> polymerase and primers BSA975 and BSA976.	205
Figure 56.	Restriction analysis using <i>BamHI</i> and <i>XhoI</i> on plasmid DNA from putative pPB(Ex-BSA07) clones.	207
Figure 57.	Sequence alignment for a putative <i>BSA07</i> gene in colony 1 against the known BSA cDNA sequence.	209
Figure 58.	Plasmid map for pPB(Ex-BSA07).	212
Figure 59.	Primers used to amplify a truncation of the BSA cDNA for insertion into pET26b(+).	216

Figure 60.	Agarose gel electrophoresis analysis of PCR amplification of BSA cDNA using <i>Pfu</i> polymerase and primers BSA842 and BSA843.	218
Figure 61.	Electrophoretic analysis of DNA following colony PCR using <i>Taq</i> polymerase and primers BSA842 and BSA843.	220
Figure 62.	Sequence alignment for a putative <i>BSA04</i> gene in colony 10 against the known BSA cDNA sequence.	223
Figure 63.	Plasmid map for pRB(Ex-BSA04).	225

LIST OF ABBREVIATIONS

ACCase	acetyl-CoA carboxylase
ACP	acyl carrier protein
ASP	acylation stimulating protein
<i>A. tumefaciens</i>	<i>Agrobacterium tumefaciens</i>
<i>B. napus</i>	<i>Brassica napus</i>
BSA	bovine serum albumin
cDNA	complementary deoxyribonucleic acid
CoA	coenzyme A
DAG	<i>sn</i> -1,2-diacylglycerol
DEPC	diethyl pyrocarbonate
DGAT	diacylglycerol acyltransferase
DNA	deoxyribonucleic acid
DTT	dithiothreitol
<i>E. coli</i>	<i>Escherichia coli</i>
ER	endoplasmic reticulum
FA	fatty acid
FAB	fatty acid biosynthesis
FAMES	fatty acid methyl esters
FAS	fatty acid synthesis
G3P	<i>sn</i> -glycerol-3-phosphate

GLC	gas liquid chromatography
GPAAT	glycerol-3-phosphate acyltransferase
HIP	hexane-isopropanol
HSA	human serum albumin
IAA	isoamyl alcohol
IMAC	immobilized metal-ion affinity chromatography columns
KAS	β-ketoacyl-ACP synthase
kb	kilobase
kDa	kilodalton
LPA	lysophosphatidic acid
LPAAT	lysophosphatidate acyltransferase
LPCAT	lysophosphatidylcholine acyltransferase
LR	low resolution
mRNA	messenger ribonucleic acid
NADH	nicotiamide-adenine dinucleotide
NADPH	nicotiamide-adenine dinucleotide phosphate
NMR	nuclear magnetic resonance
NptII	neomycin phosphotransferase
PA	phosphatidic acid
PC	phosphatidyl choline
PCR	polymerase chain reaction
PDH	pyruvate dehydrogenase

PL	phospholipid
RT	reverse transcription
SDS	sodium dodecyl sulfate
<i>sn</i>	stereochemical numbering
TAG	triacylglycerol
Ti	tumor-inducing
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactoside

1. INTRODUCTION

Canada is internationally recognized for its high quality canola oil and Alberta is one of the largest producers of this oilseed crop. In Canada, one quarter of crop production is from oilseeds (Scarth *et al.*, 1992) and canola represents approximately 63% of vegetable oil production (McDonald, 1990). As such, the development of crops with improved characteristics is of constant concern for Canada to remain agriculturally competitive. Many genetic manipulations have been performed with *Brassica napus* to improve oil characteristics and many of these manipulations involve the expression of lipid biosynthetic enzymes that may be under different regulation than the native counterparts (Brough *et al.*, 1996; Knutzon *et al.*, 1999; Pollard *et al.*, 1991; Rousler *et al.*, 1997; Sun *et al.*, 1988). There appear to be no reports from other research groups, however, on expression of these proteins in canola.

Triacylglycerol (TAG), the primary storage lipid in oilseeds, consists of a glycerol backbone with three fatty acids (FAs) esterified to each of the *sn*-positions. A major route for TAG biosynthesis is the Kennedy pathway (Kennedy, 1961). The pathway involves sequential acylation of the *sn*-glycerol-3-phosphate to ultimately generate TAG. The phosphate group is removed prior to the final acyl attachment. This final acylation step is catalyzed by diacylglycerol acyltransferase (DGAT; EC 2.3.1.20). The enzyme catalyzes the committed reaction in the process and may be rate-limiting in TAG production (Bouvier-Navé *et al.*, 2000; Perry and Harwood, 1993; Perry *et al.*, 1999).

Plant DGAT activity has been shown to be stimulated by human acylation stimulating protein (ASP) and bovine serum albumin (BSA). Human ASP is a small

(MW 14,000), basic (pI 9.0) protein (Cianflone *et al.*, 1989). It is produced by the interaction of three proteins: the third component of complement (C3), factor B and adipsin (Baldo *et al.*, 1993). There is no natural gene encoding ASP, rather it is formed through several post-translational modifications as described above (Baldo *et al.*, 1993; Cianflone and Maslowska, 1995). ASP is the most potent stimulator of TAG synthesis in human adipocytes (Cianflone *et al.*, 1989). Weselake *et al.* (2000a) used microsomes from microspore-derived cell suspension cultures of *B. napus* L. cv Jet Neuf to study the stimulatory effect of ASP on TAG biosynthesis. DGAT activity was stimulated by 50% at 100 µg ASP/mL and over 100% at 400 µg ASP/mL. BSA, on the other hand, is produced almost exclusively in the liver (Judah *et al.*, 1973; Powell *et al.*, 1984) and is further processed in the Golgi complex to form a mature peptide without a signal sequence (Brennan *et al.*, 1984). Microsomal DGAT from microspore-derived embryos has been shown to be stimulated by BSA (Little *et al.*, 1994). DGAT activity in the 1500-100 000 g particulate fraction was stimulated 4- to 5-fold by 3 to 4 mg of BSA/mL of reaction mixture.

The goal of this thesis was to engineer genetic constructs to facilitate the expression of ASP or BSA in developing seeds of canola. This was to be followed by lipid analysis on the seeds of these transgenic plants as compared to control plants in order to investigate the possible effects of these proteins on plant lipid biosynthesis. An oleosin promoter was used for seed specific expression and to express the polypeptides at a precisely coordinated time when oil was accumulating in the developing seeds (Tzen *et al.*, 1993; van Rooijen *et al.*, 1992). Through expression of ASP or BSA in developing

seeds at the time when oil is accumulating, they would theoretically stimulate the DGAT enzyme and increase the total lipid within those seeds. The first set of genetic constructs were designed to facilitate expression of these mammalian proteins in the cytosol of developing seeds and the first generation transgenic seeds were obtained and analyzed. A second strategy involved oleosin protein fusion constructs to facilitate expression of these mammalian proteins as lipid body-protein fusions in the ER.

Genetic constructs were also engineered to facilitate the bacterial expression of recombinant BSA and a truncation of the recombinant BSA having high acyl-CoA binding capacity. Since native BSA has a stimulatory effect on DGAT activity *in vitro* (Little *et al.*, 1994), testing the stimulatory nature of synthetic mature BSA and a truncation of synthetic BSA was desirable. Vectors were engineered such that recombinant BSA or a truncated form of BSA could be expressed in *E. coli* to provide protein for future *in vitro* studies on stimulation of microsomal DGAT.

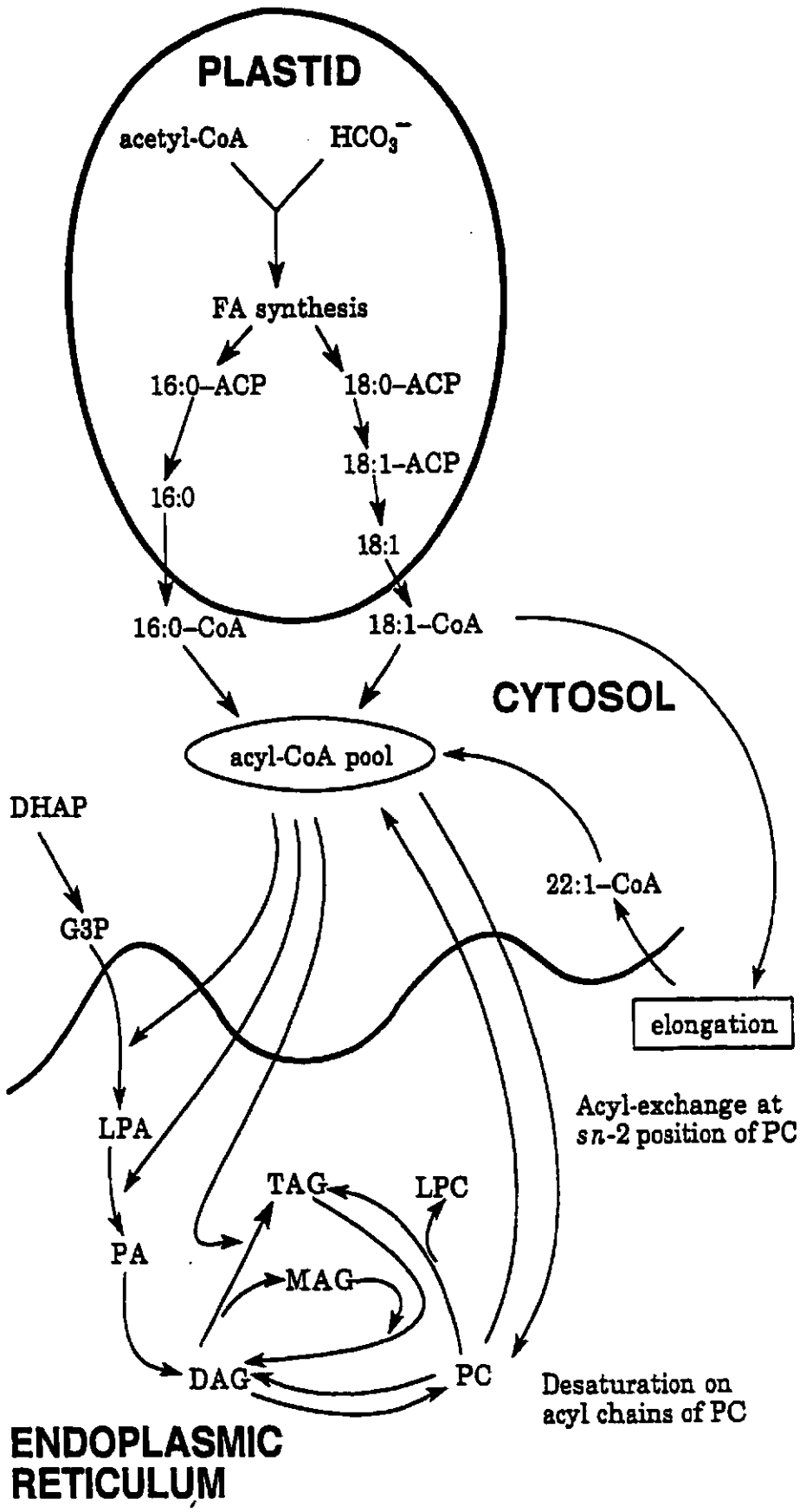
2. LITERATURE REVIEW

The primary storage compound within oilseeds is triacylglycerol (TAG). There are several reactions that occur in the plastid, cytosol and endoplasmic reticulum (ER) that are involved in forming TAG. A generalized scheme for this process is shown in Figure 1. TAGs do not perform a structural role, but rather serve as a stored carbon form. They consist of a glycerol backbone with three FAs (acyl groups) esterified to each of the three hydroxy groups.

2.1. FA Biosynthesis

To form FAs, a source of carbon must be acquired. In plants, the most common form of transportable carbon that is imported into seeds is sucrose produced from photosynthesis in the leaf (Champ and Randall, 1985; Rijven and Gifford, 1983; Schussler *et al.*, 1984). Through glycolysis, some of this sucrose can be converted into acetyl-coenzyme A (CoA). The pool of acetyl-CoA present in the plastid ranges from 30 to 50 μM (Post-Beittenmiller *et al.*, 1992) and is the source of carbon atoms found in a FA. The concentration of acetyl-CoA within this pool seems to remain constant. *In vitro* experimentation was performed to try to account for these constant levels. One suggestion is that acetyl-CoA produced by mitochondrial pyruvate dehydrogenase (PDH) is followed by movement of acetate or acetylcarnitine to the plastid. Once in the plastid, it can be converted to acetyl-CoA by acetyl-CoA synthetase, an enzyme having higher

Figure 1. Generalized representation of fatty acid (FA) and triacylglycerol (TAG) biosynthesis in developing oilseeds (Weselake, 2000). ACP, acyl carrier protein; DAG, diacylglycerol; DHAP, dihydroxyacetone phosphate; G3P, glycerol-3-phosphate; LPA, lysophosphatidate; LPC, lysophosphatidylcholine; MAG, monoacylglycerol; PA, phosphatidate; PC, phosphatidylcholine; TAG, triacylglycerol.



activity than the *in vivo* rate of FA synthesis (Roughan and Ohlrogge, 1994). It has also been proposed that cytosolic malate and glucose-6-phosphate act as precursors to the plastid acetyl-CoA pool (Kang and Rawsthorne, 1994; Smith *et al.*, 1992).

The first committed step of FA biosynthesis is a two-stage reaction catalyzed by a single enzyme complex (Kim *et al.*, 1989). In the first stage, biotin carboxylase of acetyl-CoA carboxylase (ACCase, EC 6.4.1.2) catalyzes the transfer of CO₂ (from HCO₃⁻) to nitrogen of a biotin prosthetic group attached to the ε-amino group of a lysine residue. This stage of the reaction requires ATP. In the second stage of the reaction, malonyl-CoA is formed by transferring the CO₂ from biotin to acetyl-CoA (Harwood, 1988). This second stage is catalyzed by the enzyme on the other portion of the ACCase enzyme complex, carboxyltransferase. The aforementioned two-staged reaction is a regulatory step that controls the rate of FA biosynthesis. *In vitro* experimentation has found that when ACCase was rapidly extracted from light-incubated chloroplasts it was two- to four-fold more active than from control chloroplasts incubated in dark conditions (Ohlrogge *et al.*, 1993). This suggested that light levels regulate ACCase activity and hence also control the rate of FA biosynthesis (Post-Beittenmiller *et al.*, 1991).

Further regulation of FA biosynthesis is achieved by condensing enzymes. The malonyl group of malonyl-CoA is transferred from CoA to an acyl carrier protein (ACP) prior to entering the FA biosynthesis pathway. Growing acyl chains attach to a phosphopantethein prosthetic group of ACP as a thioester. Acyl-ACPs enter into a series of condensation reactions with the malonyl-thioester that results in the formation of a carbon-carbon bond and the release of CO₂ that was added by ACCase. An elongated FA

requires three separate condensing enzymes called 3-ketoacyl-ACP synthases (KAS). A four-carbon product is initially produced when acetyl-CoA and malonyl-ACP are condensed. This condensation reaction is catalyzed by KAS III (Jaworski *et al.*, 1989). Chain lengths ranging from 6 to 16 carbons are formed via the condensing enzyme KAS I. Elongation beyond 16 carbons is achieved through the enzyme KAS II. After each condensation reaction, the first product that is produced is 3-ketoacyl-ACP. A saturated FA is produced following three additional reactions. These three subsequent reactions involve reduction of the carbonyl group by 3-ketoacyl-ACP reductase, dehydration of the molecule by hydroxyacyl-ACP dehydratase, and reduction of the *trans*-2 double bond by enoyl-ACP reductase to form a saturated FA. The first and third reactions involved in the production of a saturated FA use NADPH as an electron donor. Taken together, the four reactions discussed result in the addition of two carbons to the precursor FA while still attached to ACP as a thioester.

Although the FAs are saturated when they are elongated, double bonds can be introduced by desaturases. In fact, the majority of FAs in plant tissues are unsaturated. The first double bond that is introduced into saturated FAs is catalyzed by stearoyl-ACP desaturase (EC 1.14.99.6) (Shanklin and Cahoon, 1998). This enzyme is a homodimer where each monomer has an active site consisting of a diiron-oxo cluster. During the reaction, the reduced iron center binds oxygen, and has been postulated that the iron-oxygen complex removes hydrogen from the C-H bond (Fox *et al.*, 1993).

The elongation reaction is terminated when the acyl group is removed from ACP. This can happen in two ways. The first type of termination occurs when an acyl-ACP

thioesterase catalyzes the hydrolysis of acyl-ACP, causing the release of the FA. The second type of termination occurs when one of two acyltransferases, one that prefers oleoyl-ACP as a substrate and the other that prefers palmitoyl-ACP as a substrate, catalyzes the transfer of the FA from acyl-ACP to glycerol-3-phosphate (G3P) or to monoacylglycerol-3-phosphate. Of the two options, the first is the most predominant. Presumably, plant thioesterases involved in acyl-ACP hydrolysis are specific for C16:0 and C18:1. Because not all C18:0 undergoes desaturation reactions, the FAs that are synthesized in the plastid are C16:0, C18:0 and C18:1. The FA products are transported through the plastid envelope and are converted to acyl-CoA thioesters on the outer envelope by acyl-CoA synthetase (EC 6.2.1.3) (Pollard and Ohlrogge, 1999). These FAs are made available to the ER for use in TAG biosynthesis and for the production of C18-unsaturated FAs. In addition, 2-carbon fragments can be added to oleoyl-CoA to form erucoyl-CoA (Fehling and Mukherjee, 1991; Fuhrmann *et al.*, 1994).

2.2. TAG Bioassembly

TAGs consist of a glycerol backbone with three FAs (acyl groups) esterified to each of the three hydroxy groups. The attachment of acyl groups on the glycerol backbone of TAG is not random. In some plants, certain FAs are excluded from specific *sn*-positions on the glycerol backbone (Griffiths *et al.*, 1988a; Gunstone, 1986). It was proposed that this non-random attachment of acyl groups is the result of substrate selectivity of acyltransferases in the lipid biosynthetic pathway (Oo and Huang, 1989; Sun *et al.*, 1988).

A major route for TAG biosynthesis is the Kennedy pathway (Kennedy, 1961). The process begins by FAs being transferred sequentially from CoA to G3P. The first two FAs are attached to *sn*-1 and *sn*-2 positions of G3P forming phosphatidic acid (PA). The attachment of FAs to G3P is a two-stage process. In the first stage, the enzyme glycerophosphate acyltransferase (EC 2.3.1.15) catalyzes the acylation of a FA to the *sn*-1 position of G3P to form lysophosphatidic acid (LPA). In the second stage, the enzyme lysophosphatidate acyltransferase (LPAAT, EC 2.3.1.51) acylates a FA to the *sn*-2 position of LPA to form PA. PA is dephosphorylated via the catalytic action of PA phosphatase (EC 3.1.3.4) to form diacylglycerol (DAG). In the third and final step of lipid biosynthesis, a FA is transferred to the *sn*-3 position of DAG to form TAG. This final step is catalyzed by diacylglycerol acyltransferase (DGAT; EC 2.3.1.20) of the Kennedy pathway. The enzyme catalyzes the committed reaction in the process and may be rate-limiting in TAG production (Bouvier-Navé *et al.*, 2000; Perry and Harwood, 1993; Perry *et al.*, 1999).

Further desaturation can occur at the level of phosphatidylcholine (PC) (Tocher *et al.*, 1998). PC is formed from DAG by choline phosphotransferase (EC 2.7.8.2). These desaturated FAs enter the pathway via an acyl exchange when a FA attached to CoA trades places with a FA on PC. Oleoyl-CoA, for example, can be transferred to the *sn*-2 position of PC by the action of lysophosphatidylcholine acyltransferase (LPCAT, EC 2.3.1.23). The reaction is reversible to allow for linoleate and linolenates formed at the *sn*-2 position of PC to enter into the acyl-CoA pool where they can be acylated to the

glycerol backbone (Stymne and Glad, 1981). Alternatively, PC can participate in TAG synthesis by donation of its entire DAG portion.

2.3. Genetic Manipulations Affecting TAG Production

Genetic engineering holds great promise for modification of seed FA composition and seed oil content. One attempt to increase seed oil content was to increase FA biosynthesis. Rousler *et al.* (1997) used a promoter and signal peptide to direct cytosolic ACCase to the plastid. In doing this, the engineered plants displayed a 5% increase in seed oil content.

Experimentation has also been performed in an attempt to obtain canola having high levels of lauric acid. The enzyme acyl-ACP hydrolase, found in *Umbellularia californica* is specific for medium chain ACP-thioesters (Pollard *et al.*, 1991). The cDNA encoding this enzyme was expressed in *A. thaliana* and resulted in the accumulation of medium chain FAs in the transgenic seeds (Voelker *et al.*, 1992). A canola line was subsequently developed that expressed this acyl-ACP hydrolase, and the seeds of this line contained up to 50% of the seed FA as C12:0 (Voelker *et al.*, 1996). Levels of this FA at the *sn*-2 position of the glycerol backbone were very low, indicating that LPAAT discriminated against lauryl-CoA (Voelker *et al.*, 1996).

In an attempt to change the attachment site of C12:0 on the glycerol backbone, the canola expressing the cDNA encoding *U. californica* acyl-ACP hydrolase was crossed with canola expressing the cDNA encoding LPAAT from coconut endosperm (Knutzon *et al.*, 1999). This was done because previous experimentation revealed that the coconut

LPAAT was specific for medium chain FAs (Davies *et al.*, 1995). These crossed plants did incorporate C12:0 at the *sn*-2 position on the glycerol backbone.

Native canola LPAAT is incapable of utilizing erucoyl-CoA as a substrate (Bernert and Frentzen, 1990). To overcome this obstacle, the cDNA encoding LPAAT from developing meadowfoam seeds was incorporated into oilseed rape (Brough *et al.*, 1996). The reason this was done is because LPAAT from meadowfoam seeds has been shown to incorporate C22:1 on the *sn*-2 position of LPA (Laurent and Huang, 1992). This resulted in increased levels of TAGs having C22:1 at the *sn*-2 position (Brough *et al.*, 1996).

Another research group was able to increase seed oil content in canola by expressing a mutated yeast *sn*-2 acyltransferase gene (*SLC1-1*) (Zou *et al.*, 1997). The *SLC1-1* gene has been shown to encode a LPAAT capable of accepting a range of acyl-CoAs including erucoyl-CoA. As a result, there was also an increase in the levels of very long chain FAs at the *sn*-2 position of TAG.

Desaturation experiments have also been performed. A rat liver Δ -9 desaturase was successfully transformed into soybeans (Liu *et al.*, 1996). This resulted in the levels of C16:0 in transformed embryos dropping from 25% to approximately 5% of total FAs. Similarly, when a yeast Δ -9 desaturase, under the control of a CaMV 35S promoter, was expressed in *Petunia hybrida* there were significantly increased levels of palmitoleic acid (Choudhary *et al.*, 1994). A similar effect was observed when the yeast Δ -9 desaturase was expressed in tobacco with a CaMV 35S promoter. A 10-, 11- and 6-fold increase was found for C16:1 in the leaf, stem and root tissues, respectively. The seeds showed a

2-3 fold increase in the levels of C16:1. Yadav *et al.* (1993) expressed an *Arabidopsis* microsomal Δ -12 desaturase gene in transgenic plant tissues such as carrot hairy roots and the level of C18:3 was significantly increased. They found that when this desaturase gene was expressed with a seed specific promoter, the level of C18:3 was increased 12-fold in second generation transgenic *Arabidopsis* plants. When the rapeseed version of this gene was expressed in *Arabidopsis*, the roots showed a 1.6-fold increase in C18:3 (Arondel *et al.*, 1992).

2.4. Oleosins

Oleosins are seed specific proteins found on the surface of storage lipid bodies in plants (Murphy, 1993; Vance and Huang, 1987; van Rooijen *et al.*, 1992). The expression of oleosins is regulated by abscisic acid (Hatzopoulos *et al.*, 1990; Holbrook *et al.*, 1991). It has been suggested that oleosins function to stabilize TAG molecules, provide a large surface area for the oil body and to act as a recognition signal for lipase attack during germination (Tzen and Huang, 1992). Unlike lipoproteins in mammals, oil bodies are intracellular and located in the cytoplasm of the cell (Hills *et al.*, 1993). Oil bodies consist of a TAG matrix, a "half-unit" membrane of phospholipids (PLs), and embedded proteins (Qu and Huang, 1990). These spherical oil droplets range in diameter from 0.2 to 2.5 μm depending on the species and environmental factors (Huang, 1992). Oleosins make up 1-4% of the total mass of the oil body (Ting *et al.*, 1997) and are the most abundant protein in oil bodies (Huang, 1996).

2.4.1. Structure of Oleosins

About 22 oleosin molecules have been sequenced and characterized (Murphy, 1993) and all range from about 15 to 26 kDa in size (Huang, 1996). An oleosin protein has three structural domains that are categorized as the amphipathic N-terminus, the hydrophobic central domain and the amphipathic α -helical domain located near the C-terminus.

The amphipathic N-terminus consists of about 40-60 amino acids. This region is characterized as being highly variable in its amino acid sequence. Although there are no apparent secondary structures, it has been suggested that this region may associate with the oil body surface (Huang, 1994).

The hydrophobic or central domain is about 11 nm long and consists of 68-74 amino acids that are highly conserved (Huang, 1992). Due to the distribution of amino acids, the central hydrophobic region is comprised of two antiparallel β -strands (Tzen *et al.*, 1992). These two antiparallel β -strands act as a hydrophobic anchor into the TAG matrix (Tzen *et al.*, 1992). At the end of the anchor lies a proline knot. This 12-residue region is highly conserved and consists of three proline residues and one serine residue. This region provides a mechanism for the β -strand to change directions and allows the protein to resurface on the oil body (Huang, 1992). The proline knot is not required for ER integration or for the determination of topology, but is required for oil body targeting (Abell *et al.*, 1997). Although highly conserved, the proline knot's functionality has yet to be completely characterized.

The amphipathic α -helical domain located near the C-terminus is between 33-40 amino acids long and has a highly variable amino acid sequence. The α -helix secondary structure, however, is highly conserved (Huang, 1996).

2.4.2. Function of Oleosins

The structure of the oleosin protein plays a major role in function. The N-terminal and C-terminal segments both display charge alignment with the negative PL heads of the monolayer. The positively charged amino acids align to face the negative PL heads, resulting in the negatively charged amino acids being exposed to the cytosol (Huang, 1996). The negatively charged oil body creates an electric repulsion between other oil bodies at physiological pH, thereby stabilizing the oil body and preventing coalescing of the TAG molecules.

Trypsin can catalyze the hydrolysis of oleosin (Tzen and Huang, 1992). In the presence of phospholipase A2 or phospholipase C, however, there was no effect on the oil bodies. By first treating the oil bodies with trypsin and then with phospholipase, oil bodies coalesced. The studies suggested that oleosins acted as an umbrella, shielding the PLs from phospholipase attack (Tzen and Huang, 1992).

Oleosins provide the oil body with a large surface to volume ratio. This is desirable because a large number of small spherical oil bodies accelerate oil mobilization and metabolism during germination, and more so than a single oil droplet would. In addition to stabilizing TAG molecules in the cell, oleosins also maintain the integrity of oil bodies during seed desiccation under conditions where oil bodies are known to be

pressed against each other for long periods of time (Cummins *et al.*, 1993). Even when the pH of the cell drops to the oleosins isoelectric point of 6.2, oil bodies aggregate but do not coalesce (Huang, 1994).

2.4.3. Incorporation of Oleosins into Oil Bodies

The formation of oil bodies and the method by which oleosins are incorporated into oil bodies are issues still under debate (Huang, 1992; Murphy, 1993; Napier *et al.*, 1996). Expression of oleosins is precisely coordinated with oil accumulation in developing seeds of oilseed rape (Tzen *et al.*, 1993; van Rooijen *et al.*, 1995). The ER budding model, also termed the signal recognition particle dependent pathway, has become a widely accepted explanation for how oleosins incorporate into oil bodies (Galili *et al.*, 1998). This model theorizes that newly synthesized TAG molecules move between the phospholipid bilayer of the ER membrane due to its hydrophobicity. Synthesized PLs are incorporated into the “half-unit” membrane being formed. At the same time, oleosin mRNA is translated into proteins on polyribosomes bound to the ER membrane (Beaudoin *et al.*, 1999). The oleosins are thought to be targeted and co-translationally inserted into the membrane by the signal recognition particle pathway (Napier *et al.*, 1996). Once the budding particle pinches off, a mature oil body is formed.

2.4.4. Oil Body Degradation and Oleosins

Oil body degradation is an area with many unanswered questions. During germination a large amount of energy is needed to support vigorous growth and the

packaged TAG molecules must be used to supply this energy. Lipase enzymes, synthesized *de novo* on free polyribosomes, bind directly to the oil bodies. This binding is thought to be via recognition of the oleosins on the oil body surface by lipase (Wang and Huang, 1987; Huang, 1996). The oil body membrane fuses with a vacuole where the PLs and the lipase are salvaged and incorporated into the vacuole membrane. The oleosins are known to be completely degraded during oil mobilization. The pathways for oleosin degradation are unknown in addition to the reasons why this occurs. Nykiforuk and Johnson-Flanagan (1999) found that the breakdown of oleosin provides an indirect measure of lipid mobilization. The investigators found that levels of oleosin began to decrease after germination when the radicle had elongated to 5 mm and was still present when the radicle was 30 mm. This suggested that the complete mobilization of storage lipids were far from completion (Nykiforuk and Johnson-Flanagan, 1999).

2.5. Plant Transformation

Modifications of crop attributes via genetic engineering are dependent on effective plant transformation techniques. Several techniques have been used to transform plants with foreign genes including vacuum infiltration with *Agrobacterium tumefaciens* (Poirier *et al.*, 2000; Tjokrokusumo *et al.*, 2000; Ye *et al.*, 1999), floral dip/spray with *A. tumefaciens* (Chung *et al.*, 2000; Clough and Bent, 1998), bombardment with DNA-coated projectiles (Barandiaran *et al.*, 1998; Hagio *et al.*, 1991) and tissue culture with *A. tumefaciens* (Moloney *et al.*, 1989; Radke *et al.*, 1988). Tissue culture with *A. tumefaciens* has been used extensively by a number of research groups

and transformation of canola using this technique has been very well characterized. Genes can be inserted into plants by taking advantage of a natural soil phytopathogen, *A. tumefaciens*. *A. tumefaciens* causes crown gall tumors at the soil-air junction, the so-called crown of the plant (Hooykaas and Beijersbergen, 1994). In the pathogenic process, the DNA contained within the tumor-inducing (Ti) plasmid is transferred to the plant cell nucleus resulting in neoplastic growths on the host. The transferred DNA (T-DNA) carries genes that cause the synthesis of plant growth opines. The tumors are formed as a result of these synthesized growth hormones. The tumors synthesize opines, a major carbon and nitrogen source for *Agrobacterium* (Sheng and Citovsky, 1996). Binary vectors and non-oncogenic *A. tumefaciens* strains were developed to increase the chance of regenerating transgenic plants with normal phenotypes (Guerche *et al.*, 1987; Hoekema *et al.*, 1983; Ooms *et al.*, 1985).

Maurice Moloney has pioneered a novel way of expressing and purifying foreign proteins in plant systems by taking advantage of oleosins. He recognized that because oleosins in oilseeds made up between 8 and 20% of total seed proteins (Huang, 1996), the oleosin genes were quite strongly transcribed during seed development. Since oleosins are embedded in oil bodies purification becomes far easier when foreign proteins are fused to the oleosins. Performing extraction in aqueous solvents forms a three-phase mixture of insoluble material, an aqueous extract and an emulsion of oil bodies (van Rooijen and Moloney, 1995). Upon low speed centrifugation, the oil bodies float to the surface of the aqueous solvent. This caused almost all other seed proteins to be absent, and thus the oleosin fraction (fused with a foreign protein) was highly enriched (van

Rooijen and Moloney, 1995). The foreign protein could be extracted from the oil bodies because of an endopeptidase cleavage site that was engineered between oleosin and the recombinant protein. In doing this, the plant essentially acts as a factory that mass produces recombinant proteins within the seeds (Ward *et al.*, 1999; Chaudhary *et al.*, 1998). Several recombinant proteins have been successfully expressed with this novel method including an insulin-like growth factor from goldfish (Kermouni *et al.*, 1998), xylanase from the rumen fungus *Neocallimastix patriciarum* (Liu *et al.*, 1998) and hirudin which is a blood anti-coagulant naturally found in the head of leeches (Parmenter *et al.*, 1995).

2.6. *In vivo* Formation of Acylation Stimulating Protein (ASP)

ASP is a small (MW 14,000), basic (pI 9.0) protein (Cianflone *et al.*, 1989). It is produced by the interaction of three proteins: the third component of complement (C3), factor B and adipsin (Baldo *et al.*, 1993). These three proteins are synthesized and secreted by human adipocytes relatively late in differentiation, but before the marked increase in the capacity of adipocytes to synthesize TAG (Cianflone and Maslowska, 1995). The three proteins interact such that a seventy-seven amino acid fragment is generated that is the N-terminal fragment of C3a. The C-terminal arginine of this fragment, however, is removed by carboxypeptidases to produce a seventy-six amino acid peptide, C3a-desArg or ASP (Baldo *et al.*, 1993). ASP is nondialyzable and is trypsin sensitive (Cianflone *et al.*, 1987; Cianflone *et al.*, 1989). Murine and human adipocytes have been shown to generate ASP and differentiated adipocytes are more

responsive to ASP than preadipocytes (Cianflone *et al.*, 1994; Choy *et al.*, 1992). As adipocytes differentiate, not only do they become more responsive to ASP, but they generate more ASP (Cianflone *et al.*, 1994). There is no single natural gene encoding ASP. Rather, the protein is formed through several post-translational modifications as described above (Baldo *et al.*, 1993; Cianflone and Maslowska, 1995). A synthetic DNA sequence was required as a result.

2.7. Link Between Obesity and ASP Levels

ASP has been shown to play a role in TAG clearance from plasma and FA storage in adipose tissue (Saleh *et al.*, 1998; Sniderman *et al.*, 1997; Sniderman *et al.*, 2000). Obese individuals were studied with respect to their levels of ASP before and after weight loss as compared to an age- and sex-matched lean control group (Sniderman *et al.*, 1991). The study revealed that obese people had ASP levels four times greater than the lean group. With weight loss, however, plasma ASP levels decreased significantly, but were still approximately three times greater than the lean control group (Sniderman *et al.*, 1991). When adipocytes of morbidly obese versus normal weight patients were measured for oleate incorporation into TAG against varying ASP concentrations, a significantly increased incorporation was observed in obese individuals (Walsh *et al.*, 1989). In addition, TAG clearance and FA uptake by subcutaneous adipose tissue were significantly greater in the obese group compared with the lean group (Kalant *et al.*, 2000). This was correlated with greater ASP release in the postprandial period (0-90 minutes) in the obese group. This was further verified when mice lacking ASP showed

postprandial TAG clearance that was significantly delayed compared to wildtype mice (Murray *et al.*, 1999b). The data suggests that increased levels of ASP cause increased FA trapping that may be a feature of some forms of obesity (Kalant *et al.*, 2000). Studies performed to define the functionally active domains of ASP have revealed that: (a) the N-terminal region plays little role in ASP receptor binding and TAG synthesis stimulation; (b) the native C-terminal region had no activity; (c) the intact disulfide-linked core region was essential for TAG synthesis stimulation activity but not for receptor interaction (Murray *et al.*, 1999a).

2.8. Effect of ASP on TAG Biosynthesis

ASP is the most potent stimulator of TAG synthesis in human adipocytes (Cianflone *et al.*, 1989). In fact, ASP is six-fold more potent in its effect on TAG synthesis than insulin (Walsh *et al.*, 1989). These results suggested that ASP levels are correlated with TAG levels in people, but the stimulatory effect of ASP on TAG formation remains the same between obese and lean individuals. Weselake *et al.* (2000a) used microsomes from microspore-derived cell suspension cultures of *B. napus* L. cv Jet Neuf to study the effect of ASP on TAG biosynthesis. DGAT activity was stimulated by 50% at 100 µg ASP/mL and over 100% at 400 µg ASP/mL. In addition, the investigators found that optimal DGAT stimulation by ASP ranged from pH 6 to 9 with the highest stimulation occurring at neutral pH in the presence of HEPES-NaOH buffer. The effect of ionic strength was also studied and the results suggested that the interaction between ASP and its binding site in the microsome involved charge-charge interactions because

increasing ionic concentrations resulted in the decreased ASP-dependent stimulation of DGAT activity. It was also suggested that the stimulatory nature of ASP is not the result of improving substrate delivery to the active site of DGAT because the degree of stimulation by human ASP remained constant over a range of acyl-CoA concentrations. Yasruel *et al.* (1991) found that ASP doubled the activity of DGAT from human adipose tissue at saturating concentrations of both oleate and diolein, suggesting that the intrinsic DGAT activity was increased rather than improving substrate delivery.

ASP may also act on DGAT indirectly via the protein kinase C pathway. In earlier studies with human adipose tissue, ASP appeared to exert a number of effects on TAG biosynthesis. In addition to activation of DGAT by possible dephosphorylation of the enzyme (Baldo *et al.*, 1996), protein kinase C activation is also accompanied by a number of intracellular changes, including increased levels of DAG (Baldo *et al.*, 1995). Studies with fibroblasts, adipocytes and other cells verified that DAG levels increase when the protein kinase C pathway is activated (Ishizuka *et al.*, 1989; Etscheid, *et al.*, 1991).

ASP increases the rate of entry of glucose into the cells by promoting the translocation of glucose transporters (Germinario *et al.*, 1993; Tao *et al.*, 1997). The increase in specific membrane glucose transport occurs in a time and concentration dependent manner with ASP (Maslowska *et al.*, 1997). Maslowska *et al.* (1997) found that the effect was maximal between 1 and 4 hours and was downregulated after 24 hours of exposure to ASP. The translocation of glucose transporters occurs from an intracellular pool to the plasma membrane (Germinario *et al.*, 1993). The resultant

increase in intracellular glucose availability acts as a precursor for G3P, the backbone of the TAG molecule.

2.9. Properties of Bovine Serum Albumin (BSA)

BSA is known to interact with a number of molecules including FAs involved in TAG biosynthesis. BSA is the bovine form of a group of serum proteins that is highly conserved among mammals and is the most abundant non-glycosylated plasma protein (Carter and Ho, 1994). The primary function of these proteins is to bind a variety of compounds and act as carriers in the serum. Most notably, BSA binds FAs and MAG and is the main carrier of such in circulating plasma of cattle (Carter and Ho, 1994; Cistola *et al.*, 1987; Parks *et al.*, 1983; Thumser *et al.*, 1998). FAs and MAGs are the primary products of serum TAG hydrolysis by lipoprotein lipase. BSA has a molecular weight of 66,300 and is thought to play a role in the cellular uptake of FAs (Trigatti and Gerber, 1995). The protein is characterized by a low content of tryptophan and methionine, and a high content of cystine and charged amino acids such as aspartic acid, glutamic acid, lysine and arginine (Giancola *et al.*, 1997). BSA is highly soluble at neutral pH as a result of its total charge resulting from 185 ionized groups per molecule (Giancola *et al.*, 1997).

¹³C NMR (Hamilton *et al.*, 1991; Cistola *et al.*, 1987) and ¹⁴C-palmitic acid affinity labeling studies have shown BSA to be a 3-domain protein (Reed, 1986) with two strong FA binding sites near the C-terminus, domain I, and one near the N-terminus, domain III (Hamilton *et al.*, 1991). Two weaker, secondary binding sites, are located in

the middle domain of the protein. Acyl-CoA may bind to the same sites as FA (Richards *et al.*, 1990). Results obtained by Reed (1986) indicated that palmitic acid binds to lysine residues located at positions 116, 349, and 473 of the BSA amino acid sequence, suggesting that the regions surrounding these residues may be responsible for FA binding. The sequence of amino acid residues at the presumed opening of the regions where FA bind to BSA were identified (Cistola *et al.*, 1987). The three binding sites consisted of two clusters of His-Arg-Arg (amino acids 143-145 and 334-336), and one of Lys-His-Lys (amino acids 532-534). Parks *et al.* (1983) also indicated that the amino acid residue tyrosine is located at or near FA binding sites on BSA. This observation was made after noting that the ionization of tyrosine residues was hindered by the binding of FA.

A separate binding site for MAG is also present on the albumin protein (Thumser *et al.*, 1998). This binding site was shown to be located in subdomain IIIA by competitive assays with dansylsarcosine, a ligand known to bind to a site in subdomain IIIA (Carter and Ho, 1994). The dansylsarcosine was displaced from serum albumin by monooleoylglycerol in a competitive manner with a calculated K_d of approximately 2.5 μM (Thumser *et al.*, 1998).

Through attenuated total reflection and Fourier transform infrared spectroscopy, it was determined that BSA is conformationally different at pH 5.0 and pH 9.0 (Qing *et al.*, 1996). This was due to denaturation intermediates that were formed with differing pH conditions that could be mimicked using urea-induced denaturation (Giancola *et al.*, 1997; Ahmad and Qasim, 1995). Ahmad and Qasim (1995) studied the conformation of

free BSA and BSA interacting with FAs at varying urea concentrations. Binding of FAs prevented the denaturation of BSA by urea. The investigators suggested that domain III was responsible for the increased stability of the BSA-FA complex because domain III unfolded before the other two domains (Khan *et al.*, 1987).

2.10. *In vivo* Formation of Native Serum Albumin

Natural serum albumins are produced almost exclusively in the liver (Judah *et al.*, 1973; Powell *et al.*, 1984). Serum albumin is initially synthesized as an immature protein termed prepro-albumin and is released from the ER. The N-terminal prepeptide is removed and the resultant protein is termed pro-albumin (Judah *et al.*, 1973). Pro-albumin is further processed in the Golgi complex by removing the residues of the propeptide by a serine proteinase in the Golgi complex (Brennan *et al.*, 1984). The mature polypeptide is subsequently secreted from the cell into the serum. Upon exiting the cell, the mature polypeptide folds properly in the reducing environment of the serum.

2.11. Effect of BSA on DGAT Activity

BSA has been shown to stimulate the *in vitro* activity of acyltransferases involved in TAG production (Hershenson and Ernst-Fonberg, 1983; Stobart and Stymne, 1990; Oo and Chew, 1992). Microsomal DGAT from microspore-derived embryos has been shown to be stimulated by BSA (Little *et al.*, 1994). DGAT activity in the 1,500 - 100,000 x g particulate fraction was stimulated 4-5 fold by 3 to 4 mg of BSA/mL of reaction mixture. A similar result was obtained by Oo and Chew (1992) who found that

at BSA levels of 2 mg/mL DGAT from oil palm mesocarp was stimulated by about 3-fold. It is unclear how BSA stimulates acyltransferases, but it is theorized that BSA binds acyl-CoAs, making them better substrates for the enzyme (Hershenson and Ernst-Fonberg, 1983). This would also prevent the formation of acyl-CoA micelles that are thought to inhibit acyltransferase activity (Stobart and Stymne, 1990). This emulsifying property of BSA was studied *in vitro* with a variety of vegetable oils using conductivity techniques (Al-Malah *et al.*, 2000). They found that as BSA concentration increased, the stability of a given oil emulsion also increased.

2.12. Bacterial Protein Expression

Bacterial expression provides a means to produce sufficient amounts of protein for *in vitro* studies. *Escherichia coli* are ideal for such protein expression work because of their rapid growth to high densities on inexpensive substrates and their well-characterized genetics (Baneyx, 1999). The bacterial pET vectors are also well suited for expression work because of their ability to accumulate recombinant proteins to levels of about 40-50% of total cell protein after only a few hours following induction as described in the pET system manual (Novagen, Madison, WI). The pET26b(+) bacterial expression system has a number of desirable traits: kanamycin resistance, a multiple cloning site situated within a *lac* operon, a T7 promoter, a ribosomal binding site, a *pelB* leader sequence and a 6 X His tag sequence.

The kanamycin resistance gene allows for screening of putative clones, as does the *lac* operon. Putative clones containing the pET26b(+) plasmid would grow on agar

plates containing kanamycin based on the resistance gene within the plasmid. In addition, by also incorporating 5-bromo-4-chloro-3-indolyl- β -D-galactoside (X-gal), putative clones could be identified using α -complementation. Since the multiple cloning site of the pET26b(+) plasmid contains regulatory sequences and the coding information for the N-terminal amino acids of the β -galactosidase gene (*lacZ*), disrupting this multiple cloning site by inserting a DNA sequence, for example, results in the production of an N-terminal fragment that is not capable of α -complementation. Using this technique, putative clones containing genetic inserts in the multiple cloning site grow white whereas clones containing the pET26b(+) vector simply ligated back together grow blue.

The T7 promoter is a strong promoter that requires T7 RNA polymerase in order for transcription to take place as described in the pET system manual (Novagen, Madison, WI). This can be exploited by performing cloning work in the *E. coli* strain DH5 α , a strain lacking T7 RNA polymerase, causing the genes cloned into the pET vector to remain silent until translation is induced (Dubendorff and Studier, 1991). Once the cloning work is completed, however, the vector can be transferred into the strain BL21 (DE3), which carries an isopropyl- β -D-thiogalactopyranoside inducible chromosomal copy of T7 RNA polymerase (Studier and Moffat, 1986).

The ribosomal binding site produces a marked stimulation of expression even when located downstream from the initiation codon (Olins and Rangwala, 1989). Studies involving the *lacZ* gene reported a translation efficiency of up to 110-fold, indicating that the ribosomal binding site acts as a translational enhancer (Olins and Rangwala, 1989).

The *pelB* leader and 6 X His tag sequences assist in the purification of recombinant proteins. Once translated, the *pelB* leader instructs the cell to localize the attached protein in the periplasmic space of the host (Matthey *et al.*, 1999). The leader sequence is then theoretically cleaved off from the rest of the protein as described in the pET system manual (Novagen, Madison, WI). Only 4% of the total cellular protein (approximately 100 proteins) are present in the periplasmic space (Pugsley and Schwartz, 1985). If the periplasmic proteins are released by osmotic shock (Nossal and Heppel, 1966), it is much easier to purify the recombinant protein from this smaller background milieu of proteins. The 6 X His tag is used during the purification of the recombinant protein from the background proteins. The translated 6 X His sequence places a “tag” of 6 histidine residues on the recombinant protein. The imidazole ring in histidine has a high affinity for nickel ions. Histidine tags facilitate the purification of cloned proteins by Ni(II) immobilized metal-ion affinity chromatography columns (IMAC) (Smith *et al.*, 1988). His-tagged proteins will bind very strongly to nickel resins such as nickel-nitrilotriacetic acid while untagged proteins will not. The immobilized metal ions are bound by the chelating peptide, which then can be eluted either by decreasing the pH or by introducing a ligand with a higher affinity for the metal ions to which the recombinant protein is bound. One such example is eluting with a higher imidazole concentration (Porath *et al.*, 1975; Smith *et al.*, 1988; Janknecht *et al.*, 1991). Because it is highly unlikely that a 6 X His tag would occur naturally, this is a very powerful tool in purifying recombinant proteins. Hang *et al.* (1999) found that the bioactivity of proteins containing a hexa-his tag was indistinguishable from those same proteins without tags attached.

Although this may not be the case for all proteins, it does give promising results for a means of protein purification having minimal effects on protein activity.

There are concerns that eukaryotic proteins may not be post-translationally modified correctly in prokaryotic protein expression systems. This may cause a protein to be formed that is not folded properly or is completely inactive. Latta *et al.* (1987), however, expressed human serum albumin (HSA) in *E. coli* and were able to get proper protein folding, even after denaturing the protein in extraction buffer (6 M guanidine HCl, 100 mM β -mercaptoethanol). After denaturing the protein, the investigators slowly diluted it 100-fold in a renaturation solution (100 mM NaCl, 50 mM Tris-HCl, pH 8.5, 1 mM EDTA). They were not able to distinguish the renatured protein from the native polypeptide. Of particular interest was that the recombinant HSA had reformed its disulfide bonds and regained full antigenic activity.

3. MATERIALS AND METHODS

3.1. Chemicals

Gas liquid chromatography grade solvents were obtained from VWR Canlab (Mississauga, ON). All ^{32}P -labeled compounds used for Northern blotting, Southern blotting and neomycin phosphotransferase (NptII) assays were from Amersham Canada Ltd. (Oakville, ON). All other biochemicals used were of the highest purity available and were obtained from Sigma-Aldrich Canada Ltd. (Oakville, ON).

3.2. Isolation and Purification of Plasmid DNA

3.2.1. Miniscreen for Sequencing Purity Level DNA

Bacteria that had grown overnight in 5 mL of LB medium containing antibiotic selection at 37°C with shaking at 200 rpm were collected by centrifugation at 13,000 x g (4°C) for 1 minute and resuspended in 350 μL of Tris-sucrose buffer (25% sucrose, 50 mM Tris-HCl pH 8.0, 10 mM EDTA). Lysis was initiated by incubating the resuspended bacteria for 5 minutes at room temperature with 50 μL of lysozyme (10 mg/mL) and completed by adding 400 μL of Triton-X lysis buffer (0.2% Triton X-100, 70 mM Tris-HCl pH 8.0, 70 mM EDTA, 70 mM EGTA), and incubating for an additional 10 minutes at room temperature. The slurry was centrifuged at 13,000 x g for 10 minutes and the supernatant was transferred to a fresh tube and 2 μL of diethyl pyrocarbonate (DEPC) were added. After incubation at 68°C for 15 minutes, the tubes were placed on ice for 15

minutes and subsequently centrifuged at 13,000 x g (4°C) for 5 minutes. The sticky pellet that resulted was removed using 2 sterile toothpicks. Filling the tubes with 95% ethanol and centrifuging at 13,000 x g (4°C) for 30 minutes precipitated the DNA. The pellet was resuspended in 50 µL of double distilled water (d²H₂O).

3.2.2. Quick Alkaline Lysis

An aliquot of 1.5 mL of a bacterial culture that had grown overnight in 5 mL of LB medium containing antibiotic selection at 37°C with shaking at 200 rpm was collected by centrifugation at 13,000 x g (4°C) for 1 minute. The pellet was resuspended in 100 µL GET buffer (0.9% glucose, 25 mM Tris-HCl pH 7.5, 10 mM EDTA pH 8.0) followed by the addition of 200 µL of lysis mixture (200 mM NaOH, 1% SDS). After sitting for 5 minutes at room temperature, the solution was neutralized by adding 150 µL of 5 M potassium acetate (pH 5.2) and the cellular debris was pelleted by centrifugation at 13,000 x g (4°C) for 1 minute. The supernatant was transferred to a new microfuge tube and adding 1 mL of 95% ethanol precipitated the DNA. DNA was collected by centrifugation at 13,000 x g (4°C) for 1 minute and subsequently washed with 70% ethanol. After drying the pellet, the DNA was resuspended in 100 µL of d²H₂O.

3.2.3. Kit Method

Plasmid DNA was extracted and purified using the Wizard Plus Miniprep Kit (Promega Corporation, Madison, WI) according to the protocol supplied by the manufacturer.

3.3. Restriction and Ligation of DNA

New England BioLabs (Beverly, MA) supplied all restriction and ligation enzymes. These reactions were carried out according to the protocol supplied by the manufacturer.

3.4. Polymerase Chain Reaction (PCR)

A PCR reaction mixture was made with the following components: 2.5 μL 10xPCR buffer, 4.0 μL dNTP mix (1.25 mM), 1.25 μL forward primer (20 μM), 1.25 μL reverse primer (20 μM), 0.3 μL *Taq* polymerase (~1.7 Units) or 2.0 μL *Pfu* polymerase (2.5 Units/ μL), 13.7 μL OPTIMA H₂O (or 12.0 μL if *Pfu* polymerase was used in place of *Taq* polymerase) and 2.0 μL DNA template (0.05 $\mu\text{g}/\mu\text{L}$). PCR Amplification conditions were 94°C for 1 minute (hot start) and 35 subsequent PCR cycles at 94°C for 45 seconds, 52°C-55°C (depending on the primers) for 1 minute and 72°C for 1 minute. Following the PCR cycles, a final extension of 72°C for 10 minutes was performed. The amplified DNA was subsequently electrophoresed on a 0.8% (w/v) agarose gel.

3.5. DNA Purification: Glass Powder Elution of DNA (Gene-Cleaning)

Gene-cleaning was performed from a modified version of the protocol by Vogelstein *et al.* (1979). All surfaces and instruments used for the excision of agarose gel slabs were lint-free and sterilized with 95% ethanol. Restricted DNA was run on a 0.8% (w/v) agarose gel for 60 minutes at 85 V. Under UV light (260 nm), the desired DNA fragment(s) were cut out with a sterile scalpel blade and placed in a microfuge tube.

To each gel slab, 800 μL of sodium iodide (saturated) were added followed by incubation at 55°C for 10 minutes. A 6 μL aliquot of resuspended glass milk was added to the reaction tube and it was incubated on ice for 15 minutes with intermittent mixing. The glass milk then underwent three washes where it was centrifuged at 13,000 $\times g$ (4°C) for 1 minute, resuspended with 600 μL of New Wash Solution (49% ethanol, 10 mM Tris-HCl pH 7.4, 0.5 mM EDTA, 50 mM NaCl) and recentrifuged. After the three washes, the remaining ethanol was allowed to evaporate off and the pellet was resuspended with 6 μL of $\text{d}^2\text{H}_2\text{O}$. The tube was incubated at 55°C for 5 minutes and centrifuged at 13,000 $\times g$ for 1 minute. The supernatant was placed in a fresh tube being careful not to carry over any glass milk.

3.6. DNA Sequencing

Plasmid DNA was sent to University Core DNA Services (Calgary, AB) for sequencing to be performed.

3.7. Preparation of Competent *E. coli* Cells

A 250 mL flask containing LB medium (0.5% yeast extract, 1% tryptone, 1% NaCl, pH 7.0 with 1 M NaOH) was inoculated with a single colony of a desired strain of *E. coli*. The cells were grown at room temperature overnight with constant shaking at 160 rpm. The following day the optical density was read at 600 nm. When the optical density was between 0.3 and 0.4, cells were harvested by spinning at 13,000 $\times g$ for 10 minutes (4°C). The cells were subsequently resuspended in 30 mL of TB buffer (10 mM

PIPES-HCl, pH 6.7, 15 mM CaCl₂, 250 mM KCl, 55 mM MnCl₂), and incubated on ice for 10 minutes. The cells were again harvested at 13,000 x g for 10 minutes (4°C), after which they were resuspended in 9.3 mL of ice cold TB buffer. While the resuspended cells were gently shaking on ice, 700 µL of dimethyl sulfoxide was added in a drop-wise fashion. Cells were kept on ice overnight and were partitioned into 100 µL aliquots the following morning, flash frozen in liquid nitrogen and stored at -80°C until required.

3.8. Transformation of Competent *E. coli*

The entire volume of a ligation reaction or 5 µL of plasmid DNA was gently mixed with a 100 µL aliquot of competent *E. coli* thawed on ice. The mixture was incubated on ice for 1 hour and was subjected to a 45 second heat shock at 42°C followed by a cold shock on ice for 2 minutes. The cells were incubated at 37°C for 1 hour following the addition of 1000 µL of LB medium pre-warmed to 37°C. The cells were concentrated by centrifugation at 13,000 x g (4°C) and subsequent resuspension in 200 µL of LB medium.

3.9. Preparation of Electrocompetent *Agrobacterium tumefaciens* Cells

A flask containing 100 mL of LB medium was inoculated with 1 mL of a fresh overnight culture of *A. tumefaciens* EHA 101. The cells were grown at 28°C and vigorous shaking until an optical density of 0.5 to 1.0 at 600 nm was achieved and they were subsequently harvested by centrifugation for 15 minutes at 4000 x g (4°C). The

cells were resuspended in 100 mL of cold sterile water, recentrifuged, resuspended in 50 mL of cold sterile water and recentrifuged again. After decanting the supernatant, the cells were resuspended in 5 mL of cold 10% glycerol and centrifuged as before. The cells were resuspended to a final volume of 1.5 mL using cold 10% glycerol, dispensed into 40 μ L aliquots, flash frozen in liquid nitrogen and stored at -80°C until required.

3.10. Electro-Transformation of Competent *Agrobacterium tumefaciens* Cells

A 40 μ L aliquot of competent *A. tumefaciens* cells was thawed on ice and 25 ng of DNA in water were added to the thawed cells. The mixture was kept on ice for 2 minutes while a Gene Pulser was set to 25 μ F capacitance and 2.5 kV and the corresponding Pulse Controller was set to 200 Ω . The mixture of cells was transferred to a 0.2 cm electroporation cuvette and subsequently slid between the contacts of the reaction chamber allowing an electrical pulse to be initiated. The cuvette was immediately removed from the chamber and 1 mL of SOC broth (2% Bacto tryptone, 0.5% Bacto yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl₂, 10 mM MgSO₄, 20 mM glucose) was added. The cell suspension was transferred to a sterile test tube and incubated at 28°C for 2 hours at 100 rpm. A 200 μ L aliquot of the cell suspension was added to AB plates (Watson *et al.*, 1975) containing 25 μ g gentamycin/mL and 50 μ g kanamycin/mL. The plates were incubated at 28°C for 2 days.

3.11. Transformation of *Brassica napus*

The binary vector pCGN1559 was modified to carry the genetic inserts of interest between the right and left borders. Following electroporation of the modified binary vector into *A. tumefaciens*, tissue culture was carried out according to Moloney *et al.* (1989). Seeds of *B. napus* L.cv Westar that were surface sterilized in 1% sodium hypochlorite for 20 minutes and washed with sterile distilled water 3 times were used for tissue culture and transformation.

3.12. Plant Material and Growth Conditions

Following transformation and antibiotic selection, plantlets that developed a small root mass were transferred to potting mix (Redi-Earth) supplemented with osmocote fertilizer granules. After 2-3 weeks in a misting chamber at 24°C (75% relative humidity) with a 16 hour light/8 hour dark photoperiod, leaf samples were collected for NptII assays. Positive plants were transferred to a greenhouse where they were allowed to flower and set seed.

3.13. Neomycin Phosphotransferase (NptII) Detection in Leaf Tissue

A leaf disk with a diameter of 1 cm was collected from fresh leaf tissue and placed in a microcentrifuge tube. An aliquot of 100 µL of NptII extraction buffer was added (50 mM Na₂HPO₄ pH 7.0, 0.07% β-mercaptoethanol, 10 mM Na₂EDTA pH 8.0, 0.1% Sarcosyl, 0.1% Triton X-100) and the leaf tissue was ground using a teflon bit sterilized in 95% ethanol. The homogenized sample was centrifuged at 13,000 x g (4°C)

for 20 minutes and the supernatant was tested for NptII activity by assaying one negative control tube containing 20 μL supernatant, 10 μL reaction buffer (67 mM Tris-maleate pH 7.1, 42 mM MgCl_2 , 400 mM NH_4Cl , 1.7 mM DTT) and 10 μL [$\gamma\text{-}^{32}\text{P}$] ATP mix (15 μM cold ATP, 4 μM [$\gamma\text{-}^{32}\text{P}$] ATP (3000 Ci/mmol) in reaction buffer) and a test sample containing 20 μL supernatant, 10 μL reaction buffer containing 0.4 mg kanamycin/mL and 10 μL [$\gamma\text{-}^{32}\text{P}$] ATP mix. After incubation at 37°C for 60 minutes, the reaction mixtures were spotted on Whatman P81 paper, which was cleansed two times in a wash solution (10mM Na_2HPO_4 , 1% SDS) for 30 minutes at 65°C. After protecting the paper in plastic wrap, X-ray film was exposed at -80°C for two days and subsequently developed.

3.14. Total DNA Extraction From Leaf Tissue

Fresh leaf material was collected, flash frozen in liquid nitrogen and freeze dried. A mortar and pestle was used to crush 0.2 g of freeze dried leaf tissue under liquid nitrogen. An aliquot of 5.0 mL of Kirby solution (1% sodium tri-isopropyl naphthalene sulphonate, 6% sodium 4-amino salicylate, 50 mM Tris-HCl, pH 8.0, 6% Tris saturated phenol) was added after a powder had formed. The Kirby solution was mixed thoroughly with the leaf powder and the slurry was incubated at 65°C for 10 minutes followed by gentle rocking at 30 rpm for 1 hour in a Falcon tube. An equal volume of phenol:chloroform:isoamyl alcohol (IAA) (25:24:1) was mixed into the slurry and phase separation was achieved through centrifugation at 3,000 x g for 10 minutes. Two more phenol chloroform IAA washes were performed on the upper phase followed by

overnight precipitation using 0.1 volume of 3 M sodium acetate (pH 5.2) and 0.6 volumes of isopropanol. The following day the precipitate was collected by centrifugation at 3,000 x g for 10 minutes and resuspended in 1.0 mL of TE containing 40 µg RNase/mL. Pronase was then added to a final concentration of 20 µg/mL and the solution incubated for 1 hour at 37°C. An equal volume of phenol:chloroform:IAA (25:24:1) was mixed into the solution and was subsequently centrifuged at 3,000 x g for 10 minutes. The upper phase was subsequently precipitated overnight as described above and the following day centrifugation at 3,000 x g for 10 minutes was performed. The pellet was resuspended in 250 µL of TE.

3.15. Total RNA Extraction From Developing Seeds

All pipette tips, microfuge tubes, mortars and pestles were double autoclaved (30 minutes each time) and then baked at 80°C for 48 hours just prior to performing the protocol. Liquid nitrogen was used when grinding 20 immature (mid cot) seeds in a pre-cooled mortar and pestle. Once the seeds appeared to be a fine powder, 1450 µL extraction buffer (25 mM Tris-HCl pH 8.0, 25 mM EDTA, 75 mM NaCl, 1% SDS), 950 µL Tris-saturated phenol and 500 µL chloroform were added and the slurry was ground for an additional 2 minutes. The samples were collected in 2 microfuge tubes and centrifuged at 13,000 x g (4°C) for 20 minutes and the upper phase underwent two 25:24 (v/v) phenol/chloroform and one chloroform extraction. The upper phase was mixed with one volume of 4 M LiCl and incubated at -20°C for 2 hours to facilitate precipitation. The precipitate was collected by centrifugation at 13,000 x g (4°C) for 15

minutes. The pellet was washed with 2 M LiCl and resuspended in 200 μ L diethyl pyrocarbonate (DEPC)-treated water. The solution was placed at -80°C for 1 hour following the addition of 0.1 volume 3 M NaOAc (pH 5.2) and 2 volumes of 95% ethanol to facilitate precipitation. The precipitate was collected by centrifugation at $13,000 \times g$ (4°C) for 15 minutes and was washed with 70% ethanol. After drying the pellet at 55°C for 20 minutes, it was resuspended in 100 μ L of DEPC-treated water and subsequently quantified. RNA extractions were stored at -80°C .

3.16. Protein Extraction from Mature Seeds

Mature seeds were removed from their siliques. Twenty seeds were placed in a mortar and ground under liquid nitrogen with a pestle until a fine powder formed. An aliquot of 400 μ L of extraction buffer containing 4 mM 2-mercaptoethanol and 20 μ g phenylmethylsulfonyl fluoride/mL was added to the seeds and they were ground until a homogenous slurry was formed. Several extraction buffers were attempted including buffer 1 (50 mM potassium phosphate, pH 7.0), buffer 2 (130 mM NaCl, 10 mM $\text{Na}_2\text{HPO}_4/\text{NaH}_2\text{PO}_4$, pH 7.2), buffer 3 (100 mM Tris-HCl, 10 mM EDTA, pH 6.8) and buffer 4 (125 mM Tris-HCl, 4% SDS). The slurry was added to a microfuge tube and centrifuged at $13,000 \times g$ (4°C) for 15 minutes. The supernatant below the lipid layer was collected and the protein content was quantified using the BCA Protein Assay (Pierce Chemical Co., Rockford, IL) with BSA as a standard.

3.17. Generation of ³²P-Labelled Probes

After 1 µg of DNA was isolated via gel extraction and gene-cleaning, it was diluted to a total volume of 31 µL and heat denatured for 10 minutes followed by a cold shock on ice of 2 minutes. To the 31 µL of DNA was added 10 µL OLB buffer (random hexamers and dNTPs), 2 µL BSA (10 mg/mL), 2 µL Klenow enzyme (2 U/µL) and 5 µL [α -³²P]-dCTP (3000 Ci/mmol). After a 4 hour incubation at room temperature, the reaction was terminated by adding 50 µL of stop dye (1% dextran blue, 0.33% bromophenol blue, 13.3% glycerol, 40 mM EDTA pH 7.6). The reaction was added to a Biogel P-60 column (bed volume = 2.0 cm³) that was suspended in 20 x TES buffer (2 mM EDTA, 4% SDS, containing 200 mM Tris-HCl buffer, pH 7.6). The lower phase was collected in a microfuge tube and heat denatured for 8 minutes followed by a cold shock as before.

3.18. Southern and Northern Blotting

A mass of 30 µg of DNA was restricted overnight and subsequently electrophoresed on an agarose gel as a precursor to Southern blotting. Also, 30 µg of RNA was run on a denaturing agarose gel as a precursor to Northern blotting. The DNA or RNA was transferred to Hybond-N+ positively charged nylon membrane (Version 2.0) (Amersham Canada Ltd, Oakville, ON). Southern and Northern blotting was performed according to the protocol provided by the supplier.

3.19. Western Blotting

Aliquots of the protein extracts were reacted with SDS-PAGE disruption buffer and subjected to SDS-PAGE according to Laemmli (1970). The proteins were transferred to Millipore Immobilon-P membrane (VWR Canlab, Mississauga, ON) according to the protocol supplied by the manufacturer. The membrane was blocked overnight in 1xTBS-Tween (200 mM Tris-HCl, pH 7.5, 9% NaCl, 10% Tween-20) containing 10% (w/v) skim milk or 10% (w/v) gelatin from cold water fish skin for putative BSA extractions. The membrane was washed with 1xTBS-Tween followed by incubation at room temperature for 2 hours in a primary antibody solution [ASP/BSA antibody diluted 2000-fold in 1xTBS-Tween containing 0.05% (w/v) BSA or 0.05% (w/v) gelatin from cold water fish skin for putative BSA extractions]. After washing the membrane three times for 10 minutes each in 1xTBS-Tween, the membrane was incubated at room temperature for 1 hour in a secondary antibody solution [Anti-ASP/BSA antibody conjugated with alkaline phosphatase diluted 2900-fold in 1xTBS-Tween containing 0.05% (w/v) BSA or 0.05% (v/v) cold water fish skin for putative BSA extractions]. The membrane was washed twice in 1xTBS-Tween for 10 minutes each and color development was performed using the Alkaline Phosphatase Conjugate Substrate Kit (Bio-Rad Laboratories Ltd., Mississauga, ON).

3.20. Reverse Transcription - Polymerase Chain Reaction (RT-PCR)

Total RNA from developing seeds was reverse transcribed at 42°C using SuperScript II (Life Technologies, Burlington, ON) with Oligo (dT) according to the

protocol supplied by the manufacturer. A PCR reaction mixture was made with the following components: 2.5 μL 10xPCR buffer, 4.0 μL dNTP mix (1.25 mM), 1.25 μL forward primer (20 μM), 1.25 μL reverse primer (20 μM), 0.3 μL *Taq* polymerase (~1.7 units), 13.7 μL OPTIMA H₂O and 2.0 μL DNA template from RT reaction. PCR Amplification conditions were 94°C for 1 minute (hot start) and 35 subsequent PCR cycles at 94°C for 1 minute, 52°C-55°C (depending on the primers) for 1 minute and 72°C for 1 minute. Following the 35 cycles, a final extension of 72°C for 10 minutes was performed. The amplified DNA was subsequently electrophoresed on a 0.8% (w/v) agarose gel.

3.21. Oil Analysis Using Low Resolution Nuclear Magnetic Resonance (NMR)

Seed oil analysis was performed on an Oxford Newport Mark III NMR. The RF current level was set to 100 μamps , the gate width was set to 1.5 gauss and an analysis time of 32 second was used. Bulk seeds were added to a NMR tube. The mass was measured using an analytical balance accurate to three decimal places. These masses were all approximately 1 g (~200-300 seeds). The NMR tube was placed in the instrument and analysis was commenced. After 32 seconds a lipid percentage was obtained. Having a tube of known mass and known lipid percentage allowed the percent lipid in the samples to be corrected for precise masses. The analysis was performed in duplicate for seeds of each plant.

3.22. Lipid Extraction with Hexane-Isopropanol

Only glass pipettes and glass vials were used during these extractions and all pieces were thoroughly washed and then rinsed with hexane. Approximately 0.1 g of seeds (~20-30 seeds) were weighed (precise measurement made with an analytical balance), counted and placed in a glass homogenizer containing 1 mL of isopropanol (IP). After 10 minutes of boiling at 80°C, the homogenizer containing the seeds was placed on ice for 2 minutes. The seeds were crushed following the addition of 1 mL of hexane and 1 mL of 3:2 (v/v) hexane:isopropanol (HIP). The solution containing the oil was transferred to a clean glass vial. The homogenizer was rinsed with 1 mL 3:2 (v/v) HIP and this rinse was also transferred to the glass vial. The volume was taken to 6 mL with 3:2 (v/v) HIP and 2 mL of 3.3% (w/v) Na₂SO₄ were added. The solution was mixed and the upper phase was transferred to a new vial upon settling. One more extraction was performed on the first glass vial using 4 mL of 7:2 (v/v) HIP, transferring the upper phase to the second vial upon settling. The contents of the second glass vial were dried down at 40°C under nitrogen gas. The oil was transferred to a pre-weighed glass vial using three washes of 2:1 (v/v) chloroform:methanol and the vial was dried at 40°C under nitrogen gas. Once the weight of the vial did not change (just oil left with no solvents), the final weight was recorded and used to calculate a percent oil based on the mass and number of seeds used. The oil was resuspended in 1 mL of hexane.

3.23. Analysis of Fatty Acid Composition of Seed Acyl Lipids Using Gas Liquid Chromatography (GLC)

To determine the fatty acid composition of the total acyl lipids within the mature seeds, 50 μL of the original extract in hexane were added to a glass vial and 50 μL of 2 mg pentadecanoic acid/mL in hexane were added to all tubes as an internal standard. Methylation was carried out using methanolic HCl in sealed tubes at 50°C for 24 hours as described by Christie (1992). Upon cooling, 0.25 mL of water was added to each tube, the fatty acid methyl esters (FAMEs) were extracted two times with 2.5 mL of hexane. The tubes were dried at 40°C under nitrogen gas, resuspended in 0.5 mL of hexane, transferred to a GLC vial, flushed with nitrogen gas and sealed.

Separation of FAMEs was performed on a flame ionization gas chromatograph (model 5890, Hewlett Packard, Mississauga, ON) equipped with a J&W Scientific 30-m DB-23 narrowbore column (Chromatographic Specialties, Brockville, ON) with helium as the carrier gas at a flow rate of 12 mL/min. Initial temperature was 180°C for 5 minutes, increased to 230°C at a rate of 2°C/min. Peaks were assigned by comparing retention times to those of FAME standards from Nu-Chek-Prep (Elysian, MN) and relative proportions were determined as percentages of summed peak areas.

3.24. Statistics

A derivative of the *t*-test that allows the comparison of a single observation with the mean of a sample (Sokal and Rohlf, 1995) was used to test for differences in the oil content of seeds from genetically engineered plants to those of control plants.

Correlation analysis between different methods of total lipid percentage on mature seeds was performed using JMP IN statistical software (version 3.2.1, Duxbury Press, Toronto, ON) according to procedures outlined by Sall and Lehman (1996). Spearman Rho correlation coefficients were generated for data sets of total percent lipid from NMR, gravimetric and GLC analysis.

4. RESULTS AND DISCUSSION

4.1. Towards Expression of ASP or BSA in the Cytoplasm of Seeds

Previous studies on the *in vitro* stimulating effects of ASP and BSA were conducted with soluble microsomal DGAT in an environment that mimics the cytoplasm of seeds. ASP and BSA were both shown to be stimulatory to DGAT as compared to other control proteins (Little *et al.*, 1994; Weselake *et al.*, 2000a). Therefore, initial constructs were designed for expression of ASP or BSA in the cytosol of developing seeds. The proteins had to be expressed in the correct location within the plant and at the correct time for optimal stimulatory impact. Expression of these mammalian proteins within the cytoplasm of developing seeds was essential because most oil of *B. napus* is found within the seeds. To accomplish this timed localization, an oleosin promoter was selected because expression of oleosins is precisely coordinated with oil accumulation in developing seeds of oilseed rape (Tzen *et al.*, 1993; van Rooijen *et al.*, 1992). There is no single gene encoding ASP, rather it is formed through several post-translational modifications (Baldo *et al.*, 1993; Cianflone and Maslowska, 1995). The cDNA encoding ASP used for this work was engineered using synthetic nucleotides (Murray *et al.*, 1997). Also, the genetic code is universal between mammalian and plant systems. As a result, translational differences should not be a problem when expressing these mammalian DNA sequences in plants. The steps taken to engineer the genetic constructs to facilitate the cytosolic expression of these proteins within developing seeds are described below.

4.1.1. Genetic Constructs for Cytosolic Expression of ASP

PCR was performed using *Pfu* polymerase on the original vector carrying the ASP cDNA. The primers ASP742 and ASP743 (Figure 2) were selected so that the DNA sequence encoding ASP would be amplified with the appropriate restriction sites to be compatible with pOlePN3' (Figure 3). *Bam*HI and *Pst*I restriction sites were engineered on the 5' and 3' ends of the ASP cDNA sequence, respectively, for compatibility with the multiple cloning site of pOlePN3'. The amplified product was analyzed by agarose gel electrophoresis (Figure 4). A DNA fragment of ~250 bp was produced, corresponding to the size of the DNA sequence encoding ASP.

The blunt-ended PCR fragment was ligated into a pUC19 plasmid that was previously linearized with *Sma*I. Using agar plates containing X-gal, putative clones were identified using α -complementation. A white colony was inoculated into selective broth and restriction analysis was performed on the plasmid DNA, using linearized pUC19 as a control (Figure 5). A DNA fragment of ~250 bp was produced in the restricted plasmid DNA from the putative clone. This DNA fragment corresponded to the size of the DNA sequence encoding ASP. The plasmid DNA from this colony was positive for the restriction analysis so it was sent for sequencing. The sequence for this plasmid was correct when compared to the known sequence for ASP cDNA and the engineered restriction sites were also intact (Figure 6). The pUC19 plasmid carrying this ASP DNA sequence with engineered restriction sites was designated pBW(ASP01) (Figure 7).

Figure 2. Primers developed to amplify the DNA sequence encoding ASP for incorporation into pOlePN3'. A *Bam*HI restriction site was incorporated into the ASP742 primer and a *Pst*I restriction site was incorporated into the ASP743 primer so that these sites would be present at the 5' and 3' ends of the sequence for compatibility with the multiple cloning site of pOlePN3'.

Forward Primer: ASP742**5' - GGATCCATGTCCGTTTCAGCTGACT - 3'**

| |
*Bam*HI Restriction Site

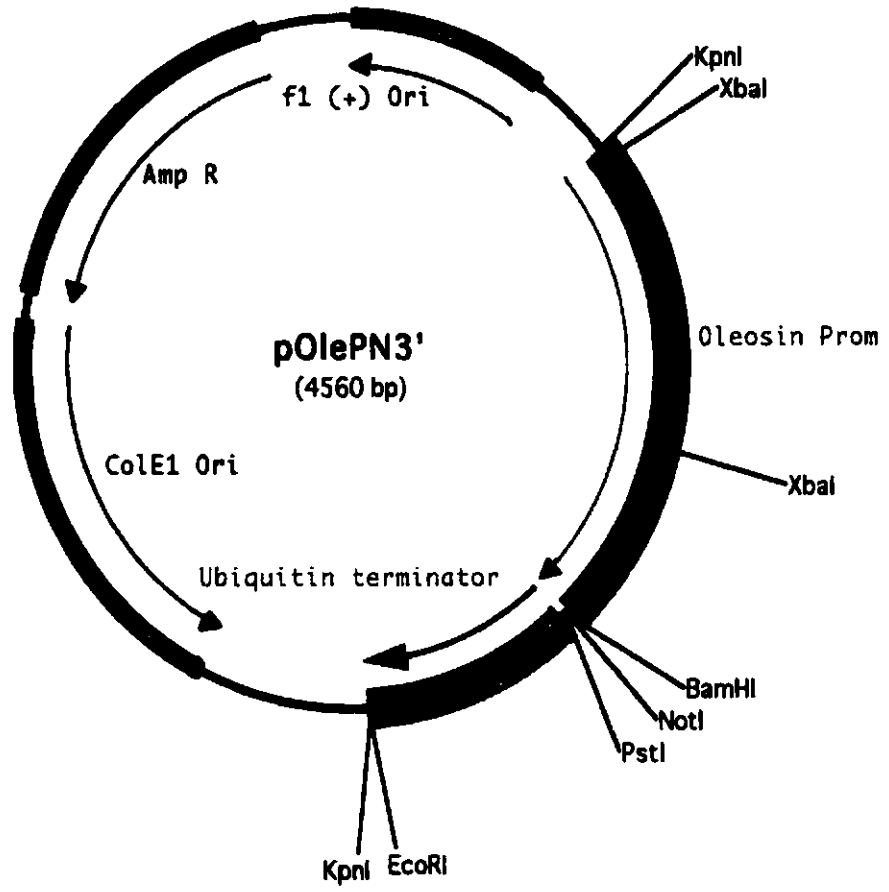
|
Bases Complementary to Lagging Strand of DNA Sequence
Encoding ASP

Reverse Primer: ASP743**5' - CTGCAGTCATTAAGCCAGACCCAG - 3'**

| |
*Pst*I Restriction Site

|
Bases Complementary to Leading Strand of DNA Sequence
Encoding ASP

Figure 3. Plasmid map for pOlePN3'. The vector contains a 1.1 Kb *Arabidopsis* oleosin promoter (blue arrow), multiple cloning site (green), ubiquitin terminator (pink arrow) and ampicillin resistance gene (solid black arrow). The page following the plasmid map contains the detailed sequence of the oleosin promoter, multiple cloning site and ubiquitin terminator.



KpnI **XbaI**
 GGTACCCAAATACGATCTGATACTGATAACGTCTAGATTTTTAGGGTTAAAGCAATCAATCACCTG
 ACGATTCAAGGTGGTTGGATCATGACGATTCCAGAAAACATCAAGCAAGCTCTCAAAGCTACACTC
 TTTGGGATCATACTGAACTCTAACAACTCGTTATGTCCCGTAGTGCCAGTACAGACATCCTCGTA
 ACTCGGATTATGCACGATGCCATGGCTATACCCAACCTCGGTCTTGGTCACACCAGGAACTCTCTG
 GTAAGCTAGCTCCACTCCCAGAAAACAACGGCGCCAAATTGCCGGAATTGCTGACCTGAAGACGG
 AACATCATCGTCCGGTCTTGGGCGATTGCGGCGGAAGATGGGTCAGCTTGGGCTTGAGGACGAGA
 CCCGAATCGAGTCTGTTGAAAGGTTGTTCAATTGGGATTTGTATACGGAGATTGGTCGTCGAGAGGT
 TTGAGGAAAGGACAAATGGGTTTGCTCTGGAGAAAGAGAGTGGGCTTTAGAGAGAGAATTGAG
 AGGTTTAGAGAGAGATGCGGCGGCGATGACGGGAGGAGAGACGACGAGGACCTGCATTATCAAAGC
 AGTGACGTGGTGAATTTGGAACTTTTAAGAGGCAGATAGATTTATTATTTGTATCCATTTTCTTC

XbaI
 ATTGTTCTAGAATGTCGCGGAACAAATTTTAAACTAAATCCTAAATTTTTCTAATTTGTTGCCA
 ATAGTGGATATGTGGCCGTATAGAAGGAATCTATTGAAGGCCCAAACCCATACTGACGAGCCCAA
 AGGTTTCGTTTTGCGTTTTATGTTTCGGTTCGATGCCAACGCCACATTCTGAGCTAGGCAAAAAACA
 AACGTGTCTTTGAATAGACTCCTCTCGTTAACACATGCAGCGGCTGCATGGTGACGCCATTAACAC
 GTGGCCTACAATTGCATGATGTCTCCATTGACACGTGACTTCTCGTCTCCTTTCTAATATATCTA
 ACAAACACTCCTACCTCTTCCAAAATATATACACATCTTTTTGATCAATCTCTCATTCAAATCTC

BamHI **NotI** **PstI**
 ATTCTCTCTAGTAAACAGGATCCCCCTCGCGCCGCGAGGGCTGCAGAATGAGTTCCAAGATGGTTT
 GTGACGAAGTTAGTTGGTTGTTTTATGGAACCTTGTTTAAGCTTGTAATGTGGAAGAACGTGTG
 GCTTTGTGGTTTTTAAATGTTGGTGAATAAAGATGTTTCCTTTGGATTAAGTAGTATTTTCTAT
 TGGTTTCATGGTTTTAGCACACAACATTTTAAATATGCTGTTAGATGATATGCTGCCTGCTTTATT
 ATTTACTTACCCCTCACCTTCAGTTTCAAAGTTGTTGCAATGACTCTGTGTAGTTAAGATCGAGT
 GAAAGTAGATTTTGTCTATATTTATTAGGGGATTTGATATGCTAATGGTAAACATGGTTTATGAC
 AGCGTACTTTTTTGGTTATGGTGTGACGTTTCCTTTAAACATTATAGTAGCGTCTTGGTCTGT

EcoRI
 GTTCATTGGTTGAACAAAGGCACACTCACTTGGAGATGCCGTCTCCACTGATATTTGAACAAAGAA

KpnI
 TTCGGTACC



Figure 4. Agarose gel electrophoresis analysis of PCR amplification of ASP cDNA using *Pfu* polymerase and primers ASP742 and ASP743. The reaction conditions were 94°C for 1 minute (hot start) and 35 subsequent PCR cycles at 94°C for 45 seconds, 55°C for 1 minute and 72°C for 1 minute. Following the 35 cycles, a final extension of 72°C for 5 minutes was performed. The amplified DNA was run on a 0.8% (w/v) agarose gel. Lane 1 contains λ DNA/ *EcoRI*+*HindIII* Ladder (MBI Fermentas, Flamborough, ON) and lanes 2 and 3 contain 5 μ L of the PCR reaction from two independent trials.

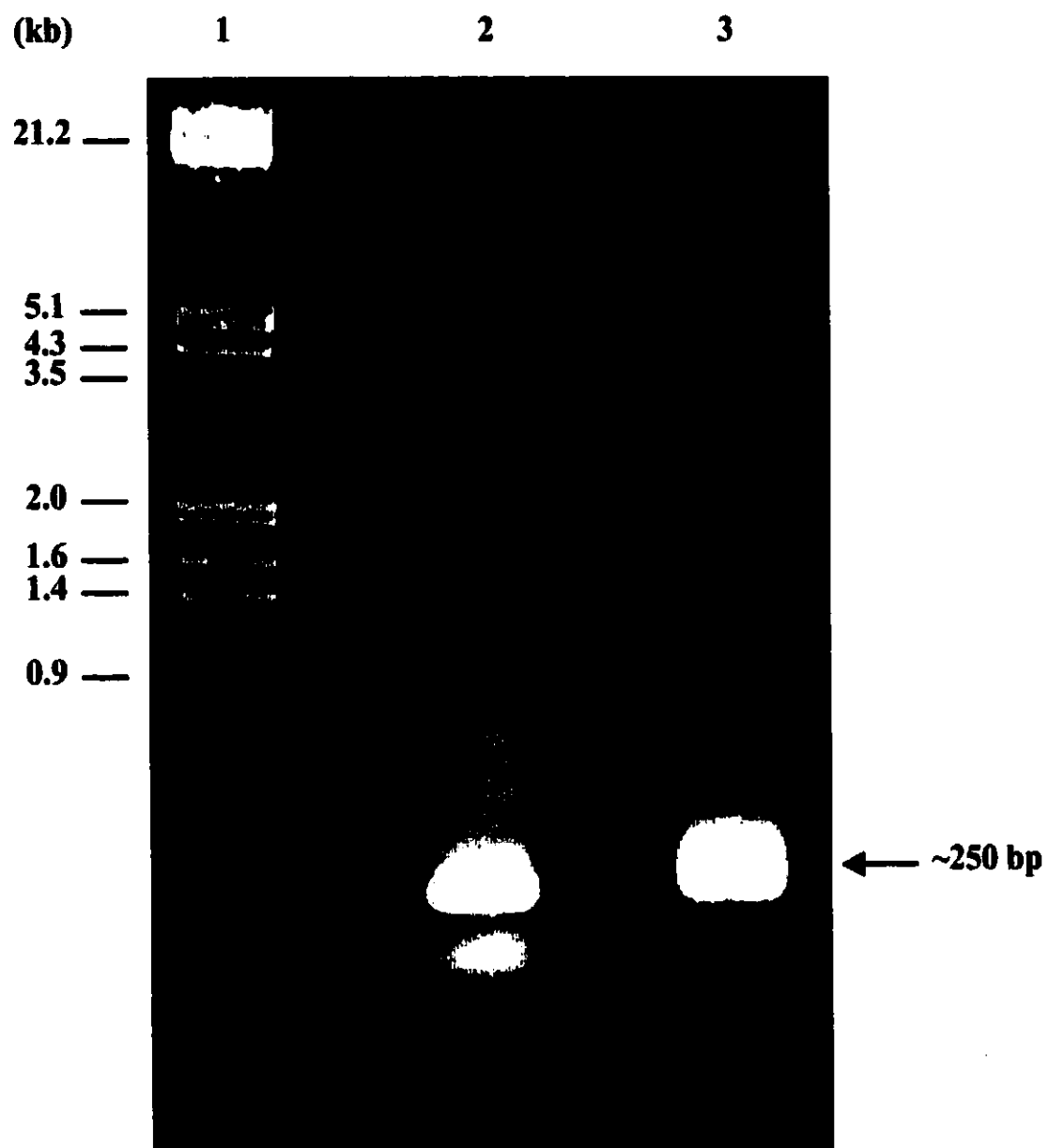


Figure 5. Restriction analysis using *Bam*HI and *Pst*I on a putative clone containing pBW(ASP01). DNA (1.5 µg) was restricted for 3 hours at 37°C and run on a 0.8% (w/v) agarose gel. The contents of each lane are as follows: 1, λ DNA/ *Eco*RI+*Hind*III Ladder (MBI Fermentas, Flamborough, ON); 2, uncut colony 1 plasmid DNA; 3, restricted colony 1 plasmid DNA; 4, uncut pUC19; 5, restricted pUC19.

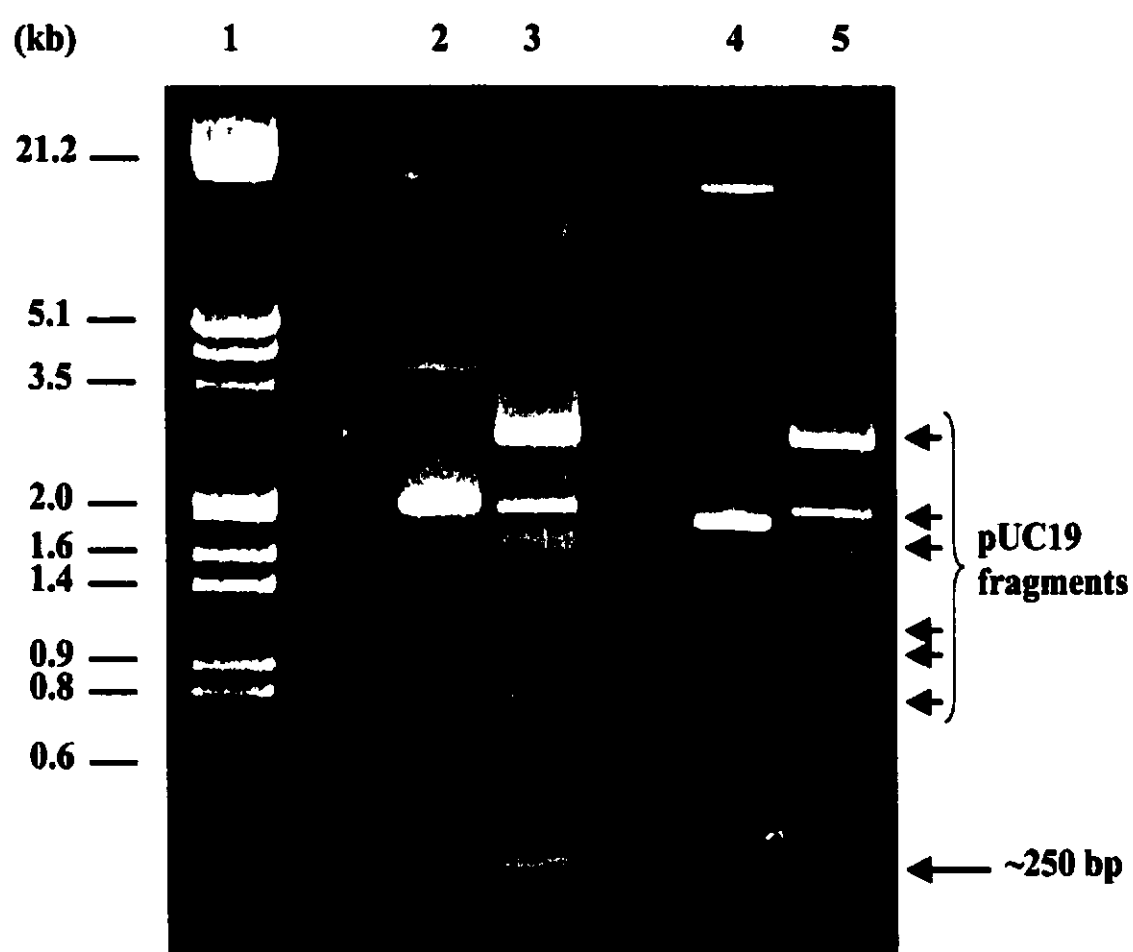
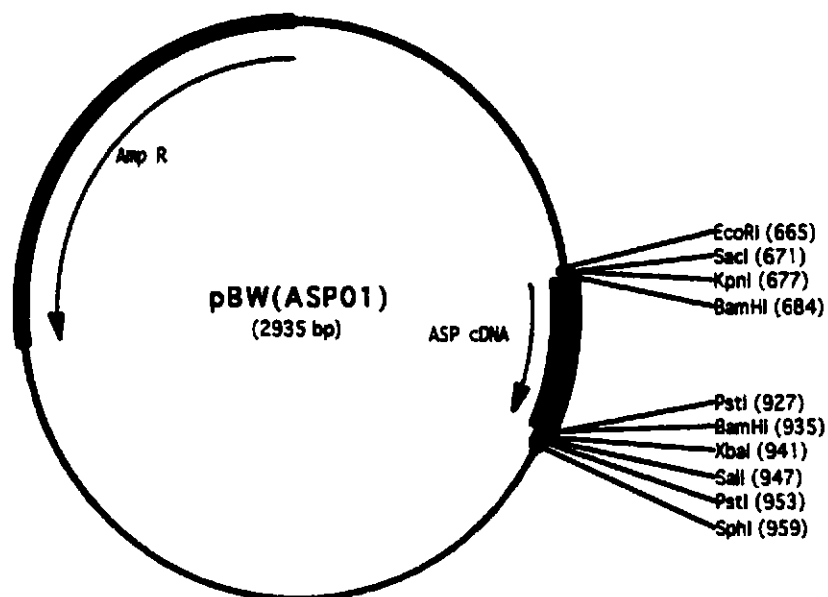


Figure 6. Sequence alignment for a putative *ASP01* gene in colony 1 against the known DNA sequence encoding ASP. Identical nucleotides are highlighted in yellow. The engineered *Bam*HI (5' end) and *Pst*I (3' end) restriction sites are shown in blue.

PUTATIVE ASP01 ASP cDNA	GGATCCATGTCCGTTTCAGCTGACTGAAAAACGTATGGATAAAGTTGGTAAGTACC ATGTCCGTTTCAGCTGACTGAAAAACGTATGGATAAAGTTGGTAAGTACC	55 49
PUTATIVE ASP01 ASP cDNA	CGAAGGAACTGCGTAAATGTTGCGAAGACGGGATGCGTGAAAACCCGATGCGTTT CGAAGGAACTGCGTAAATGTTGCGAAGACGGGATGCGTGAAAACCCGATGCGTTT	110 104
PUTATIVE ASP01 ASP cDNA	CTCTTGCCAAACGTCGTACCCGCTTCATCTCCCTGGGCGAAGCTTGCAAAAAGGTT CTCTTGCCAAACGTCGTACCCGCTTCATCTCCCTGGGCGAAGCTTGCAAAAAGGTT	165 159
PUTATIVE ASP01 ASP cDNA	TTCCTGGACTGCTGCAACTACATCACTGAACTGGTCTGTCAGCACGCTCGTGCTT TTCCTGGACTGCTGCAACTACATCACTGAACTGGTCTGTCAGCACGCTCGTGCTT	220 214
PUTATIVE ASP01 ASP cDNA	CTCACCTGGGTCTGGCTTAATGACTGCAG CTCACCTGGGTCTGGCTTAATGA	249 237

Figure 7. Plasmid map for pBW(ASP01). The vector contains an ampicillin resistance gene (black arrow) as well as the ASP cDNA (purple arrow) that was inserted into the multiple cloning site of pUC19 (green) via the blunt-ended *Sma*I restriction site. The detailed sequence of the insert is shown as well as some flanking sequences.



SacI (671) BamHI (684)
 EcoRI (665) KpnI (677)
 GAATTCGAGCTCGGTACCCGGATCCATGTCGGTTCAGCTGACTGAAAAACGTATGGATAAAGTTGGTAAGTACCCGAAGGAACTGC
 GTAAATGTTGCGAAGACGGGATGCGTGAAAACCCGATGCGTTTCTCTTGCCAACGTCGTACCCGCTCATCTCCCTGGGCGAAGCT
 TGCAAAAAGGTTTTCTGGACTGCTGCAACTACATCACTGAACTGCGTCGTCAGCAGGCTCGTGCTTCTCACCTGGGTCTGGCTTA
 XbaI (941) PstI (953)
 PstI (927) BamHI (935) Sall (947) SphI (959)
 ATGACTGCAGGGGATCCTCTAGAGTCGACCTGCAGGCATGCAA
 →

The DNA fragment encoding ASP was extracted from pBW(ASP01) by restricting with *Bam*HI and *Pst*I restriction enzymes and the fragment was subsequently gel extracted and gene-cleaned. This fragment was ligated into gel purified pOlePN3' that had been previously restricted with *Bam*HI and *Pst*I. The ligation reaction was used to transform *E. coli* the following day. Several colonies were inoculated into broth and restriction analysis was performed (Figure 8). In addition to a DNA fragment of ~4600 bp, corresponding to the pOlePN3' parent plasmid, a fragment of ~250 bp was also produced for each of the clones following restriction with *Bam*HI and *Pst*I. This fragment of ~250 bp was the *ASP01* gene. The new plasmid containing the *Arabidopsis* oleosin promoter, ASP cDNA and ubiquitin terminator was designated pBW(ASP02) (Figure 9).

4.1.2. Insertion of the *ASP02* Gene into the Binary Vector, pCGN1559

The *ASP02* gene, containing the *Arabidopsis* oleosin promoter, DNA sequence encoding ASP and ubiquitin terminator, was extracted from pBW(ASP02) by restricting the plasmid with *Kpn*I for compatibility with the multiple cloning site of pCGN1559 (Figure 10). This restriction as well as pCGN1559 linearized with *Kpn*I were electrophoresed on an agarose gel (Figure 11). The restricted pBW(ASP02) revealed DNA fragments of ~1800 bp and ~3000 bp, corresponding to the *ASP02* gene and the remainder of the parent pOlePN3' plasmid. In addition, the restricted pCGN1559 was ~14,400 bp. The *ASP02* gene and linearized pCGN1559 were excised from the agarose gel and subsequently gel extracted and gene-cleaned. The two pieces were ligated and

Figure 8. Restriction analysis using *Bam*HI and *Pst*I on plasmid DNA from putative pBW(ASP02) clones. DNA (1.5 μ g) was restricted for 3 hours at 37°C and run on a 0.8% (w/v) agarose gel. The contents of each lane are as follows: 1, λ DNA/*Hind*III Ladder; 2, restricted colony 1 plasmid DNA; 3, restricted colony 2 plasmid DNA; 4, restricted colony 3 plasmid DNA; 5, restricted colony 4 plasmid DNA; 6, restricted colony 5 plasmid DNA; 7, restricted colony 6 plasmid DNA; 8, restricted colony 7 plasmid DNA; 9, restricted colony 8 plasmid DNA; 10, restricted pOlePN3'.

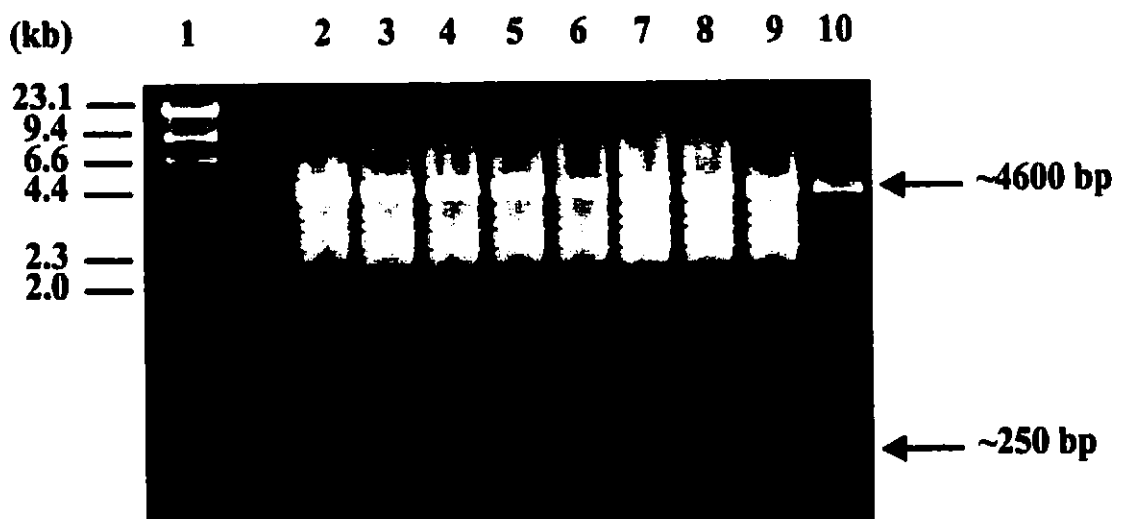
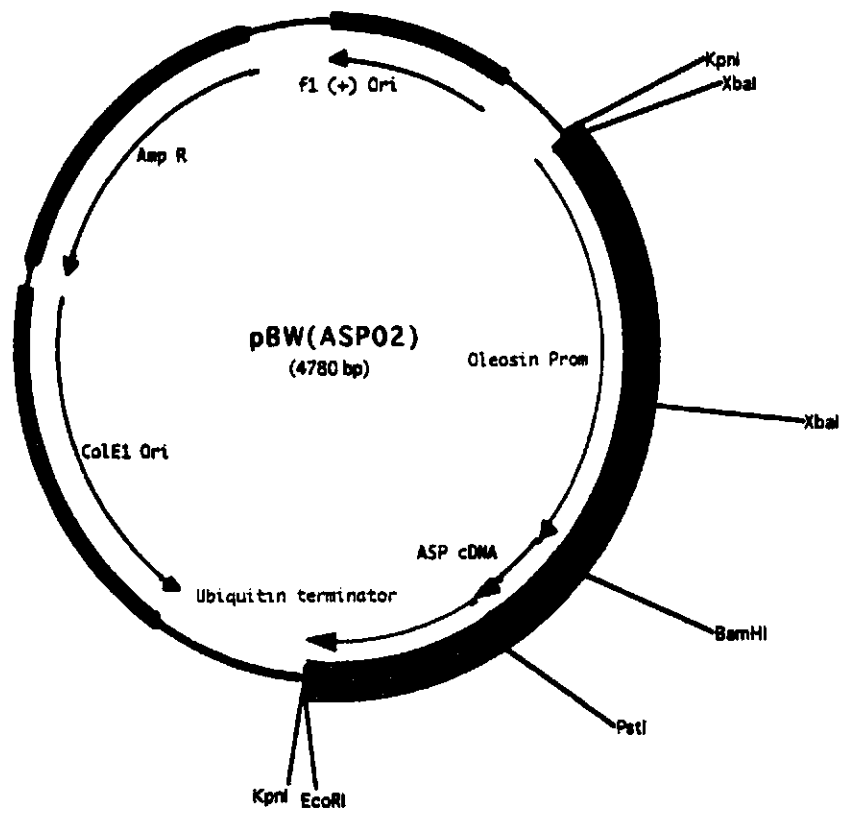


Figure 9. Plasmid map for pBW(ASP02). The vector contains an ampicillin resistance gene (solid black arrow) as well as the *ASP01* gene (purple arrow) that was inserted into the multiple cloning site of pOlePN3' via the *Bam*HI and *Pst*I restriction sites that were engineered on the 5' and 3' ends of the DNA sequence, respectively. An oleosin promoter (light blue arrow) drives the expression of the *ASP01* gene and a ubiquitin terminator (pink arrow) is also present. The detailed sequence of the insert as well as some flanking sequences is shown on the page following the plasmid map.



XbaI

GTACCCAAATACGATCTGATACTGATAACGCTAGATTTT TAGGGTTAAAGCAATCAATCACCTGACGATTCAAGGTGGTTGGATCATGAC
 GATTCAGAAAAATCAAGCAAGCTCTCAAAGCTACACTCTTTGGGATCATACTGAACTCTAACAACTCGTTATGTCCCGTAGTGCCAGT
 ACAGACATCCTCGTAACTCGGATTATGCACGATGCCATGGCTATACCCAACTCGGTCTTGGTCAACCAGGAACTCTCTGGTAAGCTAGC
 TCCACTCCCCAGAAACAAACGGCGCCAAATGCGCGAATTGCTGACCTGAAAGACGGAACTCATCGTGGGTCTTGGCGGATTGCGGCGG
 AAGATGGGTGAGCTTGGGCTTGGGACGAGACCCGAATCGAGTCTGTTGAAAGGTTGTTTATTGGGATTTGTATACGGAGATTGGTCTGCG
 AGAGGTTTGGGAAAGGACAAATGGGTTTGGCTCTGGAGAAAGAGAGTGGCGCTTAGAGAGAGAAATTGAGAGGTTTAGAGAGAGATGCG
 GCGCGATGACGGGAGGAGAGACGAGGACCTGCATTATCAAAGCACTGACGTTGGTAAATTTGGAACTTTTAAGAGGAGATAGATTT

XbaI

ATTATTTGTATCCATTTTCTTCTTCTAGAAATGTCGCGGAACAAATTTTAAACTAAATCTAAATTTTCTAATTTTGTGGCAATA
 GTGGATATGTGGCCGTATA GAAGGAATCTATTGAAAGGCCAAACCCATACTGACGAGCCCAAAGGTTCTGTTTTGCGTTTTATGTTTGGT
 TCGATGCCAACGCCACATTCTGAGCTAGGCAAAAA CAACGCTGCTTTGAAATAGACTCCTCTCGTTAACACATGCAGCGGCTGCATGGT
 ACGCCATTAACAGTGGCTACAATTGCATGATGTCTCCATTGACAGTGACTTCTCGTCTCTTTCTTAATATATCTAACAAACTCCT

BamHI

ACCTCTTCCAAAATATATACACATCTTTTGTATCAATCTCTCATTCAAATCTCATTCTCTCTAGTAAACAGGATCCATGTCGGTTCAGCT
 GACTGAAAAAGTATGGATAAAGTTGGTAAGTACCCGAAAGGAACTGCGTAAATGTTGCGAAGACGGGATGCGTGAAAAACCGATGCGTTTC
 TCTTGCCAACTGCTACCGCTTCTCTCCCTGGGCGAAGCTTGCAAAAAGGTTTTCTGGACTGCTGCAACTACATCACTGAACTGCGTC

PstI

GTCAGCACGCTCGTCTTCTCACCTGGGCTGGCTTAATGACTGCA GAATGAGTCCAGATGGTTGTGACGAAAGTTAGTTGGTTGTTTT
 TATGGAACTTTGTTAAGCTTGAATGTGAAAGAA CGTGTGGCTTTGTGGTTTTAAATGTTGGTGAATAAAGATGTTTCTTTGGATTA
 ACTAGTATTTTCTATTGGTTTCA TGGTTTTAGCACACAACTTTTAAATGCTGTTAGATGATGCTGCCTGCTTTATTATTTACTT
 ACCCTCACCTTCAGTTTCAAAGTTGTTGCAATGACTCTGTGAGTTAAGATCGAGTGAAAGTAGATTTTGTCTATTTATTAGGGGTA
 TTTGATATGCTAATGGTAAACATGGTTTATGACAGCGTACTTTTTGGTTATGGTGTGACGTTTCTTTTAAACATTATAGTAGCGTCCT

EcoRI KpnI

TGGTCTGTTCATTGGTTGAACAAAGGCACACTCACTGGAGATGCCGTCTCCACTGATATTTGAA CAAA GAATTCGGTACC

Figure 10. Plasmid map for the binary vector, pCGN1559. The vector contains a gentamycin resistance gene (solid black arrow), right and left borders (blue arrows), multiple cloning site (green) and a 35S promoter, NptII gene and terminator (shaded black segment with solid black arrow).

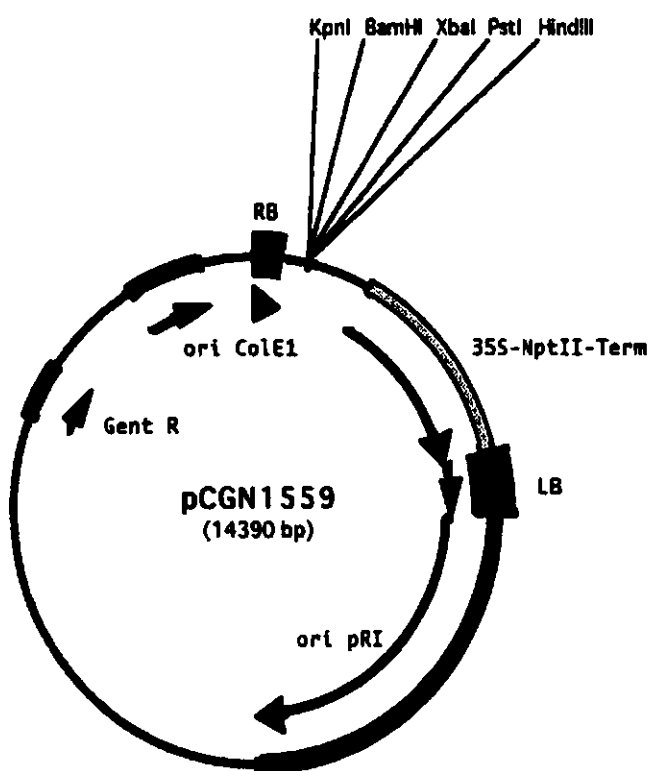
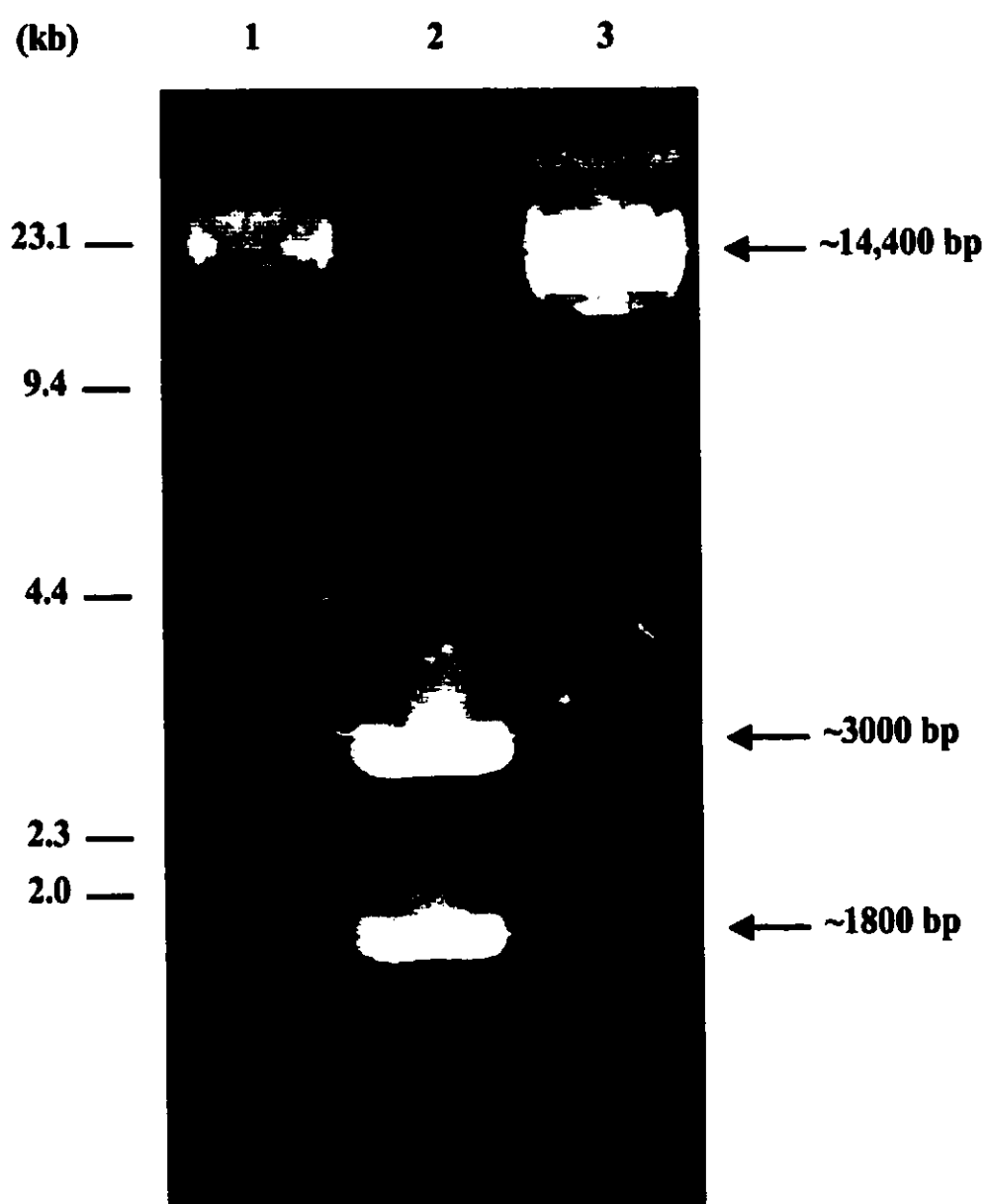


Figure 11. Restriction analysis using *KpnI* on pBW(ASP02) and pCGN1559. DNA (1.5 µg) was restricted for 3 hours at 37°C and run on a 0.8% (w/v) agarose gel prior to gene-cleaning. Lane 1 contains λ DNA/*HindIII* Ladder, and lanes 2 and 3 contain restricted pBW(ASP02) and pCGN1559, respectively.



used to transform *E. coli* the following day. Putative clones were first identified using α -complementation. A putative clone was screened for the correct insert and orientation by restricting the plasmid DNA with *Bam*HI and looking for a DNA fragment size of ~750 bp based on the restriction map and location of *Bam*HI sites within the plasmid (Figure 12). The plasmid DNA from this putative clone did produce a DNA fragment of ~750 bp when restricted with *Bam*HI, indicating that it contained the *ASP02* gene in the correct orientation. This plasmid DNA was sent for sequencing and the resultant sequence was compared to the known sequence for *Arabidopsis* oleosin promoter and DNA encoding ASP (Figure 13). The sequence of the putative plasmid DNA was correct for the *ASP02* gene. This final ASP binary vector was designated pBW(Bin-ASP03) (Figure 14) and was electroporated into *A. tumefaciens*.

4.1.3. Identification of Transgenic Plants: NptII Assay

Once cotyledons were dipped in an *A. tumefaciens* solution that was carrying pBW(Bin-ASP03), and tissue culture was carried out to regenerate plants, transgenic plantlets were identified using the NptII assay. This was possible because the binary vector had been engineered to carry a NptII gene driven by a 35S promoter and was inserted in tandem with the *ASP02* gene (Figure 14). A number of studies have used ubiquitously expressed NptII protein as a selectable marker (Chaudhary *et al.*, 1998; Parmenter *et al.*, 1995; Radke *et al.*, 1988). In each case approximately 75% of the plants that had successfully passed tissue culture screening were also NptII positive. For the putative ASP transgenic plants, a success rate of 73% was obtained for successful

Figure 12. Orientation analysis using *Bam*HI on plasmid DNA from a putative pBW(Bin-ASP03) clone. DNA (1.5 µg) was restricted for 3 hours at 37°C and run on a 0.8% (w/v) agarose gel. Lane 1 contains λ DNA/*Hind*III Ladder and lane 2 contains restricted plasmid DNA from the putative colony.

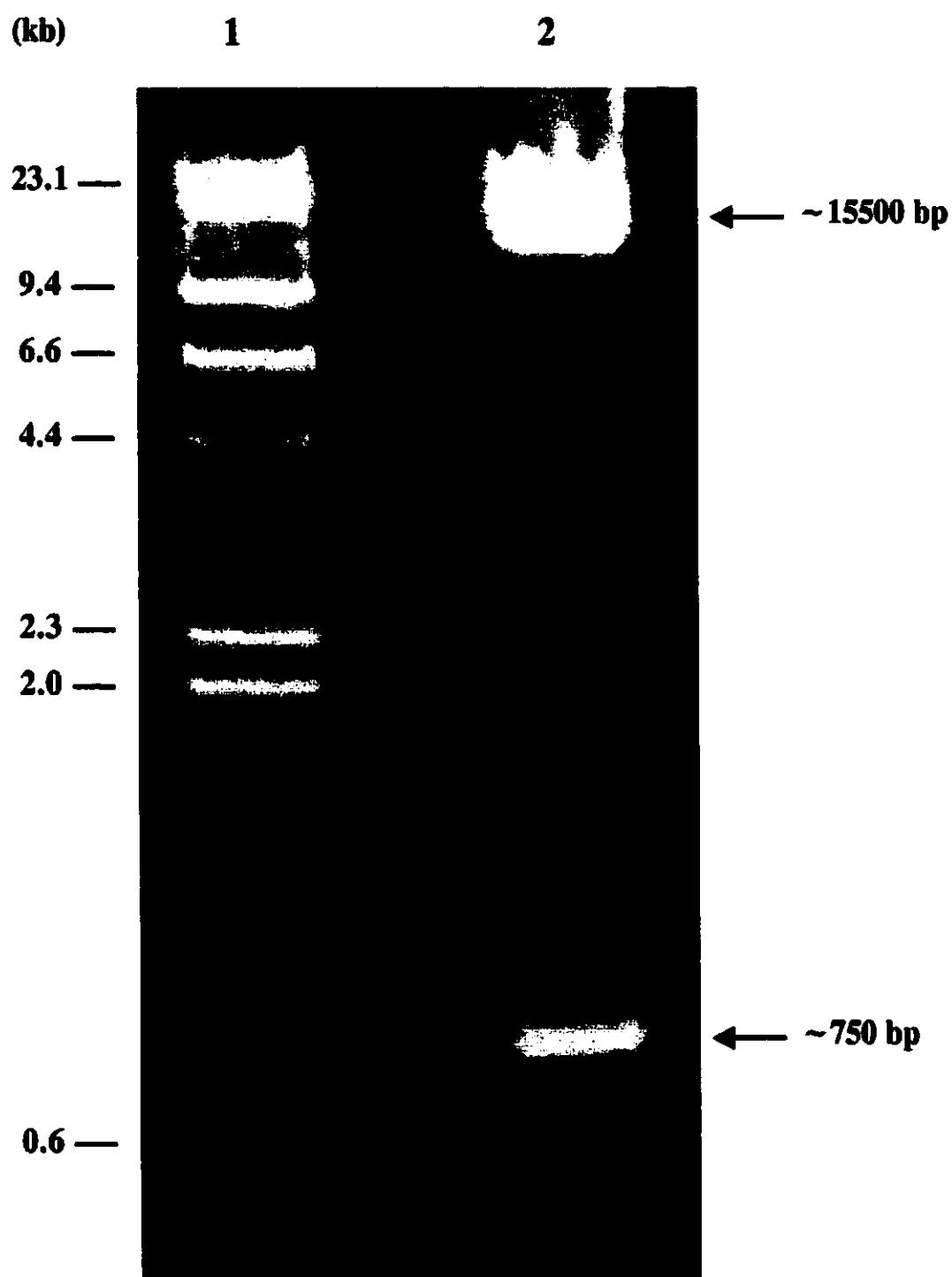
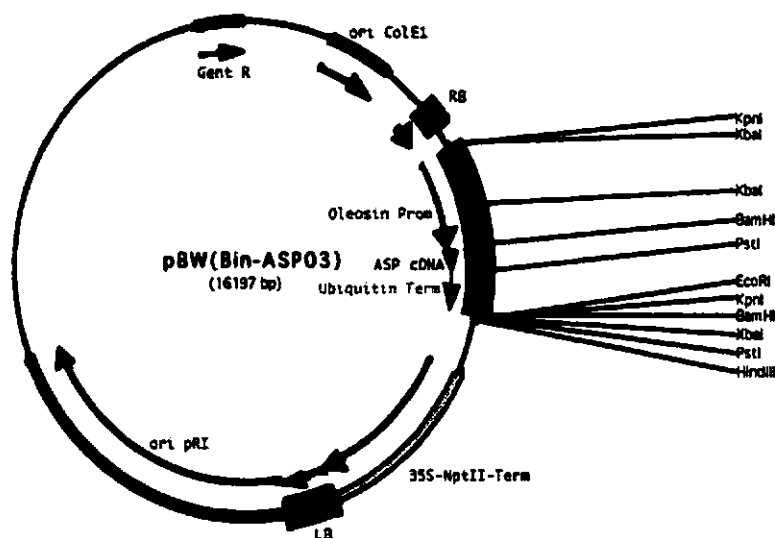


Figure 13. Sequence alignment for a putative *ASP03* gene in colony 1 against the known DNA sequence encoding oleosin promoter, ASP and ubiquitin terminator. Identical nucleotides are highlighted in yellow. The engineered *Bam*HI (5' end) and *Pst*I (3' end) restriction sites are shown in blue.

POTATIVE ASP03	TGGCTATACCCAACCTCGGTCTTGGTCACACCAGGAACCTCTCTGGTAAGCTAGCT	55
OLEOSIN PROMOTER	TGGCTATACCCAACCTCGGTCTTGGTCACACCAGGAACCTCTCTGGTAAGCTAGCT	55
POTATIVE ASP03	CCACTCCCAGAAACCAACCGGCGCAAATTCGCCGAATTGCTGACCTGAGACGG	110
OLEOSIN PROMOTER	CCACTCCCAGAAACCAACCGGCGCAAATTCGCCGAATTGCTGACCTGAGACGG	110
POTATIVE ASP03	AACATCATCGTCGGGTCCTTGGGCGATTGCGGCGAAGATGGGTCAGCTTGGGCT	165
OLEOSIN PROMOTER	AACATCATCGTCGGGTCCTTGGGCGATTGCGGCGAAGATGGGTCAGCTTGGGCT	165
POTATIVE ASP03	TGAGGACGAGACCCGAATCGAGTCTGTTGAAAGGTTGTTCAATGGGATTGTATA	220
OLEOSIN PROMOTER	TGAGGACGAGACCCGAATCGAGTCTGTTGAAAGGTTGTTCAATGGGATTGTATA	220
POTATIVE ASP03	CGGAGATTGGTCGTGAGAGGTTTGAGGGAAAGGACAAATGGGTTTGGCTCTGGA	275
OLEOSIN PROMOTER	CGGAGATTGGTCGTGAGAGGTTTGAGGGAAAGGACAAATGGGTTTGGCTCTGGA	275
POTATIVE ASP03	GAAAGAGAGTGGCGCTTTAGAGAGAGAATTGAGAGGTTTAGAGAGAGATGCGGCG	330
OLEOSIN PROMOTER	GAAAGAGAGTGGCGCTTTAGAGAGAGAATTGAGAGGTTTAGAGAGAGATGCGGCG	330
POTATIVE ASP03	GCGATGACGGGAGGAGAGACGACGAGGACCTGCATTATCAAAGCAGTGACGTGGT	385
OLEOSIN PROMOTER	GCGATGACGGGAGGAGAGACGACGAGGACCTGCATTATCAAAGCAGTGACGTGGT	385
POTATIVE ASP03	GAAATTTGGAACITTTAAGAGGCAGATAGATTTATTATTTGTATCCATTTCTTC	440
OLEOSIN PROMOTER	GAAATTTGGAACITTTAAGAGGCAGATAGATTTATTATTTGTATCCATTTCTTC	440
POTATIVE ASP03	ATTGTTCTAGAAATGTCGCGGAACAAATTTTAAACTAAATCCTAAATTTTCTAA	495
OLEOSIN PROMOTER	ATTGTTCTAGAAATGTCGCGGAACAAATTTTAAACTAAATCCTAAATTTTCTAA	495
POTATIVE ASP03	TTTTGTTGCCAATAGTGGATATGTGGCCGTATAGAAGGAATCTATTGAAGGCC	550
OLEOSIN PROMOTER	TTTTGTTGCCAATAGTGGATATGTGGCCGTATAGAAGGAATCTATTGAAGGCC	550
POTATIVE ASP03	AAACCCACTACTGACGAGCCCAAAGGTCGTTTGCCTTTTATGTTTCGGTTCGAT	605
OLEOSIN PROMOTER	AAACCCACTACTGACGAGCCCAAAGGTCGTTTGCCTTTTATGTTTCGGTTCGAT	605
POTATIVE ASP03	GCCAAACGCGACATCTGAGCTAGGCCAAAAACAAACGTGTCTTTGAATAGACTCC	660
OLEOSIN PROMOTER	GCCAAACGCGACATCTGAGCTAGGCCAAAAACAAACGTGTCTTTGAATAGACTCC	660
POTATIVE ASP03	TCTCGTTAACACATGCAGCGGCTGCATGGTGACCCATTAACACGTGGCTACAA	715
OLEOSIN PROMOTER	TCTCGTTAACACATGCAGCGGCTGCATGGTGACCCATTAACACGTGGCTACAA	715
POTATIVE ASP03	TTGCATGATGTCCTCATTGACACGTGACTTCTCGTCTCCTTCTTAATATATCTA	770
OLEOSIN PROMOTER	TTGCATGATGTCCTCATTGACACGTGACTTCTCGTCTCCTTCTTAATATATCTA	770
POTATIVE ASP03	ACAAACACTCCTACCTCTTCCAAATATATACACATCTTTTGATCAATCTCTCA	825
OLEOSIN PROMOTER	ACAAACACTCCTACCTCTTCCAAATATATACACATCTTTTGATCAATCTCTCA	825
POTATIVE ASP03	TTCAAAATCTCATTCTCTCTAGTAAACAGGATCCATGTCGGTTCAGCTGACTGAA	880
OLEOSIN PROMOTER/ASP	TTCAAAATCTCATTCTCTCTAGTAAACA ATGTCGGTTCAGCTGACTGAA	853/21
POTATIVE ASP03	AAACGTATGGATAAAGTTGGTAAGTACCCGAAGGAAGTGCCTAAATGTTGCCAAG	935
ASP	AAACGTATGGATAAAGTTGGTAAGTACCCGAAGGAAGTGCCTAAATGTTGCCAAG	76
POTATIVE ASP03	ACGGGATGCGTGAAAACCCGATGCGTTTCTTGGCCAACTGCTACCCGCTTCAT	990
ASP	ACGGGATGCGTGAAAACCCGATGCGTTTCTTGGCCAACTGCTACCCGCTTCAT	131
POTATIVE ASP03	CTCCCTGGGCGAAGCTTGCAAAAAGGTTTTCTGGACTGCTGCAACTACATCACT	1045
ASP	CTCCCTGGGCGAAGCTTGCAAAAAGGTTTTCTGGACTGCTGCAACTACATCACT	186
POTATIVE ASP03	GAACTGCGTCGTGAGCAGCCTCGTCTTCTCACCTGGGCTGGCTTAATGACTGC	1100
ASP	GAACTGCGTCGTGAGCAGCCTCGTCTTCTCACCTGGGCTGGCTTAATGACTGC	233
POTATIVE ASP03	AGAATGAGTCCAAAGATGGTTTGTGACGAAGTTAGTTGGTTGTTTTATGGAAC	1155
UBIQUITIN TERMINATOR	AATGAGTCCAAAGATGGTTTGTGACGAAGTTAGTTGGTTGTTTTATGGAAC	53
POTATIVE ASP03	TGTTTAAGCTTGTAAATGTGGAAAGAACGTGGCTTTGGTTTTTAA	1204
UBIQUITIN TERMINATOR	TGTTTAAGCTTGTAAATGTGGAAAGAACGTGGCTTTGGTTTTTAA	102

Figure 14. Plasmid map for pBW(Bin-ASP03). The binary vector contains a gentamycin resistance gene (solid black arrow). The *ASP02* gene, containing an oleosin promoter (light blue arrow), ASP cDNA (purple arrow) and ubiquitin terminator (pink arrow) was inserted into the multiple cloning site of pCGN1559 via the *KpnI* restriction sites on the 5' and 3' ends of the sequence. This gene was inserted in tandem with the kanamycin resistance gene driven by a separate 35S promoter (shaded black section with solid black arrow). The detailed sequence of the insert is shown along with some flanking sequences.



KpnI
 GGTACCCAAATACGATCTGATACTGATAACGTCTAGATTTTAGGGTTAAAGCAATCAATCACCTGACGATTCAAGGTGGTTGGATCATGACGATCCAGAAAAA
XbaI
 TCAAGCAAGCTCTCAAAGCTACACTCTTTGGGATCATACTGAACTCTAAACAACCTCGTTATGTCCCGTAGTGCCAGTACAGACATCCTCGTAACTCGGATTATGCA
 CGATGCCATGGCTATACCCAACTCGGTCTTGGTCACACCGAACTCTCTGGTAAGTAGCTCCACTCCCAGAAAACAACCGGCCAAATGCCGGAATTGCTG
 ACCTGAAGACGGAAATCATCGTCTGGTCTTGGGCGATTGCGGGCAAGATGGGTGAGCTTGGGCTTGAGGACGAGACCCGAATCGAGTCTGTTGAAAGGTTGTT
 CATTGGGATTTGTATACGGAGATTGTCGTCGAGAGTTTGGGGAAAGGACAAATGGGTTGGCTCTGGAGAAAAGAGAGTGGGCTTTAGAGAGAGAATTGAGAG
 GTTTAGAGAGAGATGCGCGGCGATGACGGGAGGAGACGACGAGGACCTGCATTATCAAAGCAATGACGTTGAAATTTGGAACTTTTAAGAGGCAGATAGAT
XbaI
 TTATTATTTGTATCCATTTCTTCATTGTTCTAGAAATGTCGGGAACAAATTTAAACTAAATCTAAATTTTTCTAATTTTGTGCCAATAGTGGATATGTTGG
 CCGTATAGAAGGAATCTATTGAAGGCCAAACCACTACTGACGAGCCCAAAGTTCTGTTTTGCGTTTTATGTTTCGGTTCGATGCCAACGCCACATTCTGAGCTAG
 GCAAAAAACAACGTTCTTTGAATAGACTCCTCTCGTTAACACATGACGGCTGCATGGTGACGCCATTAAACAGTGGCCTACAATTCATGATGTCTCCATTG
 ACACGTGACTTCTGCTCCTTTCTAATATATCTAAACAACACTCCTACCTTCCAAAAATATATACACATCTTTTTGATCAATCTCTCATTCAAATCTCATT
BamHI
 TCTCTAGTAAACAGGATCCATGTCCTCGTTCAGCTGACTGAAAAAGTATGGATAAAGTTGGTAAGTACCCGAAGGAACTGCGTAAATGTTGCGAAGACGGGATGCGT
 GAAAACCGATGCGTTTCTCTTGCCAACTGTCGTAACCGCTTCTCCTCGGCGAAGCTTGCAAAAAGTTTTCTGGACTGCTGCAACTACATCACTGAACTGC
PstI
 GTCGTCAGCAGCTCGTCTTCTCACTGGGCTGGCTTAATGACTGCAGAAATGAGTCCAAAGATGGTTGTGACGAAAGTGTGTTGTTTATGGAACCTTG
 FTTAAGCTTGTAAATGTGAAAGAAGCTGTGGCTTGTGGTTTTAAATGTTGGTGAATAAAGATGTTCCCTTGGATTAAGTATTTTCTATTGGTTTCATG
 GTTTAGCACACAATTTAAATATGCTGTTAGATGATATGCTGCTGCTTTATTTACTTACCCCTCACCTTCAGTTTCAAAGTGTGCAATGACTCTGTG
 TAGTTAAGATCGAGTGAAGTAGATTTTGTCTATATTTAGGGGATTTGATATGCTAATGGTAAACATGGTTATGACAGCGTACTTTTTGGTTATGGTGT
EcoRI
 TGACGTTTCTTTAAACATTATAGTAGGCTCCTTGGTCTGTGTTCAATGGTGAACAAAGGCACACTCACTGGAGATGCCGCTCCACTGATATTTGAACAAAG
KpnI BamHI XbaI PstI HindIII
 AATTCGGTACCGATCCTCTAGACTGCAGGATGCAAGCTT

integration of the NptII gene. This number is in close agreement with the studies listed above. This means that the selection/screening that was used during tissue culture was efficient. The frequency of genetic escapes with the putative ASP plants was in line with results obtained by other researchers (Chaudhary *et al.*, 1998; Parmenter *et al.*, 1995; Radke *et al.*, 1988). The data for the NptII assay for putative ASP plants is shown in Table 1. ASP 1, ASP 2, ASP 4, ASP 5, ASP 6, ASP 8, ASP 10, ASP 11, ASP 13, ASP 14 and ASP 15 plants had integrated the pBW(Bin-ASP03) construct. These NptII positive plants were transferred to a greenhouse whereas negative plants were autoclaved and discarded.

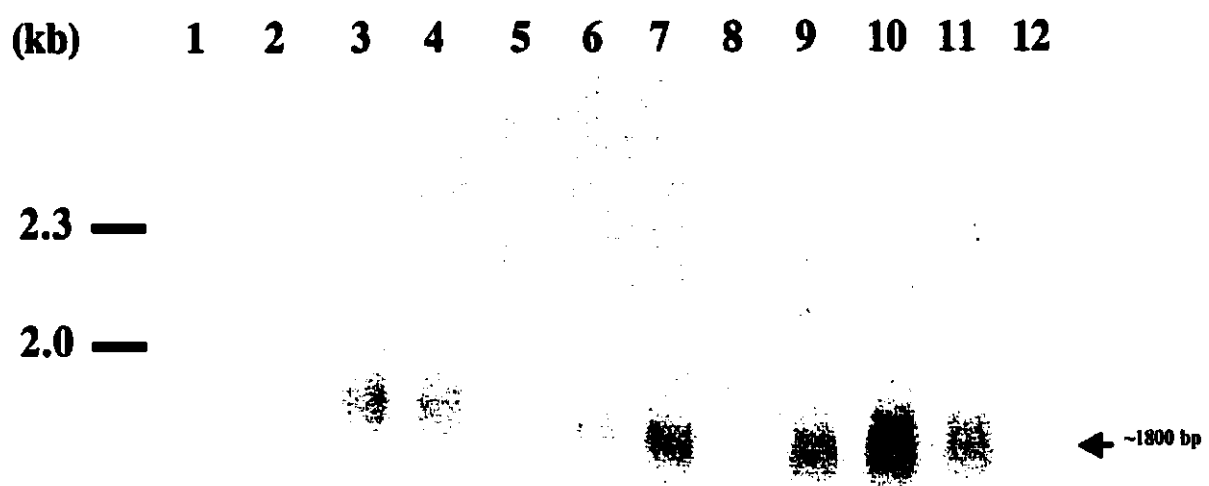
4.1.4. Integration of ASP cDNA into the Plant Genome

Southern blot analysis was performed to examine if the ASP insert had been integrated into the plant genome. The genomic DNA was isolated and subsequently restricted with *KpnI* overnight to excise the *ASP02* gene. The restricted genomic DNA was electrophoresed on a 1.0% (w/v) agarose gel and transferred to Hybond-N+ membrane (Amersham Canada Ltd., Oakville, ON). Following UV cross-linkage the blot was probed using the [³²P]-*ASP01* gene. The results of this Southern blot are shown in Figure 15. A DNA fragment of ~1800 bp was present for ASP 1, ASP 2, ASP 4, ASP 5, ASP 6, ASP 8, ASP 10, ASP 11, ASP 13, ASP 14 and ASP 15, corresponding to the size of the *ASP02* gene. Therefore, the *ASP02* gene was integrated into the genome of the aforementioned plants. According to the Southern blot, all of the plants that were NptII positive were also positive for the existence of the cDNA encoding ASP. This is in

Table 1. Results of NptII dot blot assay of extracts of leaves from putative transformed canola plants.

Plant	NptII Positive or Negative
ASP 1	Positive
ASP 2	Positive
ASP 3	Negative
ASP 4	Positive
ASP 5	Positive
ASP 6	Positive
ASP 7	Negative
ASP 8	Positive
ASP 9	Negative
ASP 10	Positive
ASP 11	Positive
ASP 12	Negative
ASP 13	Positive
ASP 14	Positive
ASP 15	Positive

Figure 15. Southern blot analysis of *Kpn*I digests of 30 µg of genomic DNA isolated from transformed *B. napus* plants. All transformants were obtained by integration of pBW(Bin-ASP03). Blots were probed with the [³²P]-*ASP01* gene. The contents of each lane are as follows: 1, restricted genomic DNA from ASP 1; 2, restricted genomic DNA from ASP 2; 3, restricted genomic DNA from ASP 4; 4, restricted genomic DNA from ASP 5; 5, restricted genomic DNA from ASP 6; 6, restricted genomic DNA from ASP 8; 7, restricted genomic DNA from ASP 10; 8, restricted genomic DNA from ASP 11; 9, restricted genomic DNA from ASP 13; 10, restricted genomic DNA from ASP 14; 11, restricted genomic DNA from ASP 15; 12, restricted genomic DNA from a negative control plant.

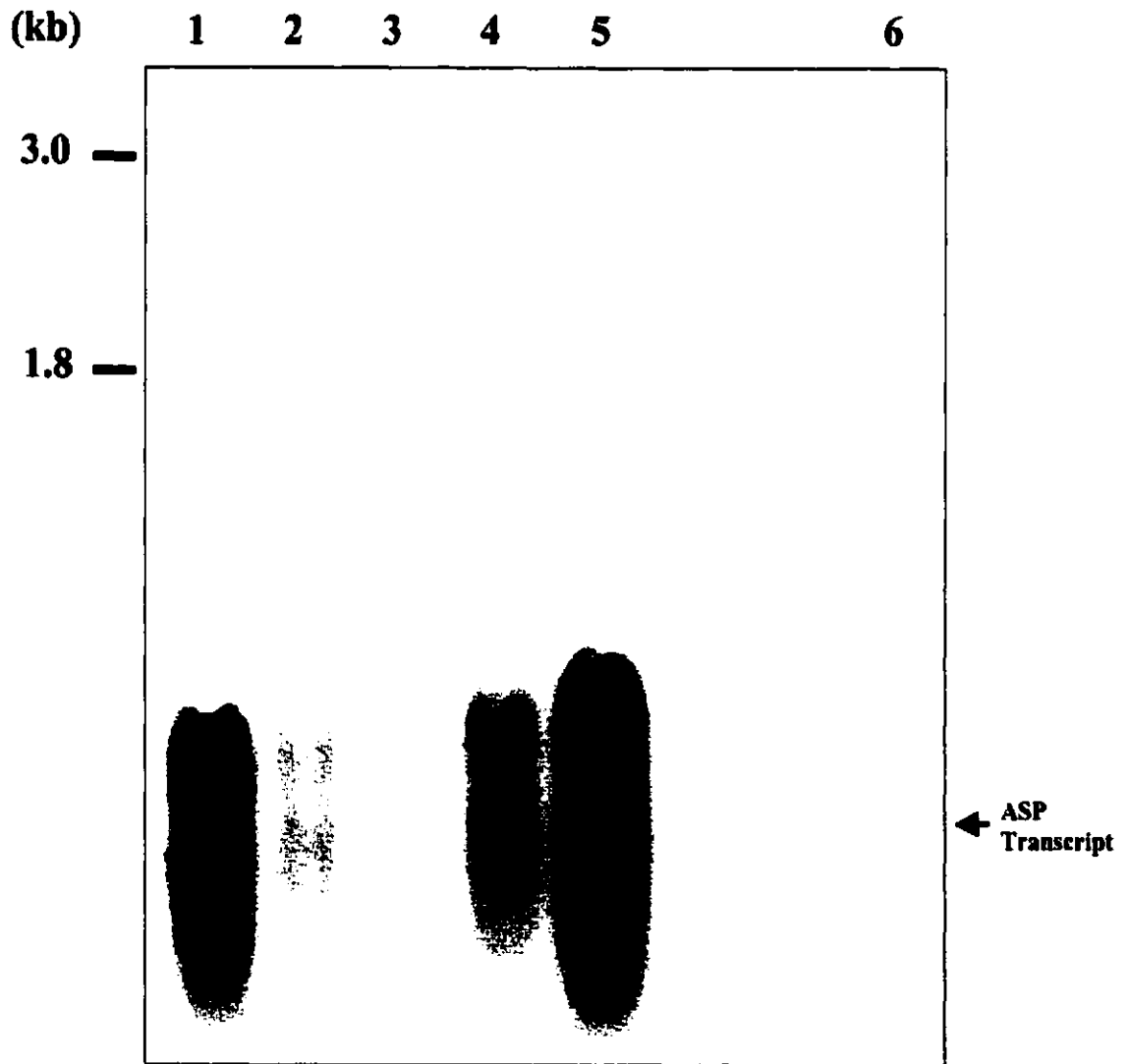


agreement with the results obtained by Parmenter *et al.* (1995) that found that all NptII positive plants were positive for the cDNA encoding hirudin. However, a mock restriction or restriction with a different enzyme to clearly distinguish the plasmid from the integrated gene sequence should have been performed (Cho *et al.*, 1998; Bommineni *et al.*, 1997).

4.1.5. Production of ASP Transcripts

Plants that had ASP cDNA integrated into the genome that also had sufficient numbers of developing seeds (25-30 days after flowering) (Weselake *et al.*, 1993), were further examined for their capacity to produce transcripts. To test this, RNA was extracted from developing seeds, electrophoresed on an agarose gel and transferred to Hybond-N+ membrane (Amersham Canada Ltd., Oakville, ON). Following UV cross-linkage, Northern blot analysis was performed using the [³²P]-*ASP01* gene as a probe (Figure 16). Because of limited availability of developing seeds, the RNA extraction and subsequent Northern blot could only be performed on ASP 1, ASP 2, ASP 6, ASP 13 and ASP 14. Of these five plants, ASP 6 was the only one that did not produce transcript. This may be due to transcriptional gene silencing. According to Vaucheret and Fagard (2001), increasing the copy number of a particular gene can lead to gene silencing. As a result of this, copy number should be investigated in subsequent generations. In addition, this may be due to insert rearrangement or reorganization that leads to loss of function (Cho *et al.*, 1998; Bommineni *et al.*, 1997). The remaining four plants, on the other hand, not only had the *ASP02* gene integrated into their genome, but the gene was also being

Figure 16. Northern blot analysis of 30 μ g of total RNA extracted from developing seeds of transformed *B. napus* plants. All transformants were obtained by integration of pBW(Bin-ASP03). Blots were probed with the [³²P]-ASP01 gene. The contents of each lane are as follows: 1, total RNA from ASP 1; 2, total RNA from ASP 2; 3, total RNA from ASP 6; 4, total RNA from ASP 13; 5, total RNA from ASP 14; 6, total RNA from a negative control plant.



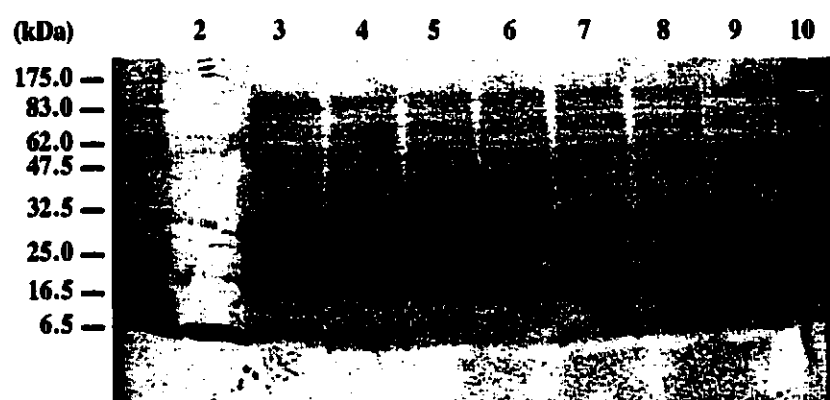
transcribed. ASP 1 and ASP 14 appeared to have higher transcript levels since the equivalent amounts of RNA were loaded in each well for all plants analyzed.

4.1.6. Production of ASP Protein

Protein extraction was attempted with four different extraction buffers (see Materials and Methods) and Western blotting was performed using monoclonal and polyclonal antibodies directed against the ASP protein, but no immunoreactive polypeptide was detected. This could be due to several factors. The antibody against ASP detected many different polypeptides in both control and transgenic seeds making it difficult to make an assessment regarding production of ASP polypeptide (Figure 17). It also could be that ASP was formed but is in such low levels that it could not be detected through conventional Western blotting techniques. As well, ASP may have been formed and degraded shortly after expression, or it simply may not have been translated. An additional possibility is that post-transcriptional gene silencing is occurring where mRNA is being degraded shortly after formation (Vaucheret and Fagard, 2001). Other research groups have had problems with low protein levels. Ishimaru *et al.* (1997) engineered transgenic *C3 Arabidopsis* plants to express maize *C4* pyruvate orthophosphate dikinase gene. The resultant plants had low protein levels that were not detectable with western blotting but were realized using ELISA. They attributed this problem either to post-transcriptional and/or translational regulation. Han *et al.* (1999) introduced potato virus Y coat protein into tobacco via *Agrobacterium*-mediated transformation and observed low levels of protein. They attributed this result to post-transcriptional gene silencing

Figure 17. Western blot analysis using monoclonal antibodies against ASP of negative control mature seed protein extract spiked with purified ASP polypeptide.

The contents of each lane are as follows: 1, Prestained Protein Marker, Broad Range Protein Ladder (New England Biolabs, Beverly, MA); 2, 50 ng ASP; 3, 200 μ g negative control protein extract spiked with 100 ng ASP; 4, 200 μ g negative control protein extract spiked with 75 ng ASP; 5, 200 μ g negative control protein extract spiked with 50 ng ASP; 6, 200 μ g negative control protein extract spiked with 25 ng ASP; 7, 200 μ g negative control protein extract spiked with 10 ng ASP; 8, 200 μ g negative control protein extract; 9, 200 μ g ASP 1 protein extract; 10, 200 μ g ASP 14 protein extract.



directed by homologous transgenes. Post-transcriptional gene silencing was also suggested when low levels of introduced P1 sequence of potato virus Y was found in transformed potato cultivar Pito (Valkama *et al.*, 2000). In addition, we may be facing a stability problem with the protein. Boothe *et al.* (1997) found that protein degradation and instability of proteins decreased when they were expressed as oleosin fusions rather than cytoplasmic expression. Oleosin fusions have been made with the ASP cDNA and these constructs are described in section 4.3.1.

4.1.7. Genetic Constructs for Cytosolic Expression of BSA

PCR was performed using *Pfu* polymerase on the original vector carrying the BSA cDNA. The primers BSA736 and BSA747 were selected so that the cDNA sequence encoding BSA would be amplified without the signal peptide (Figure 18). During biosynthesis of native BSA, a signal peptide on the N-terminus directs the protein to be excreted from the cytoplasm into the extracellular space. If the protein was excreted from the plant cells it could not stimulate the TAG forming enzymes that are located within the cells. *Bam*HI and *Pst*I restriction sites were engineered on the 5' and 3' ends of the BSA sequence, respectively, for compatibility with the multiple cloning site of pOlePN3' (Figure 3). A start codon was also engineered on the 5' end of the fragment following the *Bam*HI site to allow for translation. The amplified product was analyzed by agarose gel electrophoresis (Figure 19). A DNA fragment of ~1800 bp was produced, corresponding to the size of the mature BSA cDNA.

Figure 18. Primers developed to amplify the mature BSA cDNA sequence for incorporation into pOlePN3'. A *Bam*HI restriction site was incorporated into the BSA736 primer and a *Pst*I restriction site was incorporated into the BSA747 primer so that these sites would be present at the 5' and 3' ends of the sequence for compatibility with the multiple cloning site of pOlePN3'. A start codon was engineered following the *Bam*HI restriction site for translation purposes.

Forward Primer: BSA736**5' - GGATCCATGAGGGGTGTGTTTCGTCGA - 3'**

| | |
*Bam*HI Restriction Site

| |
Start Codon

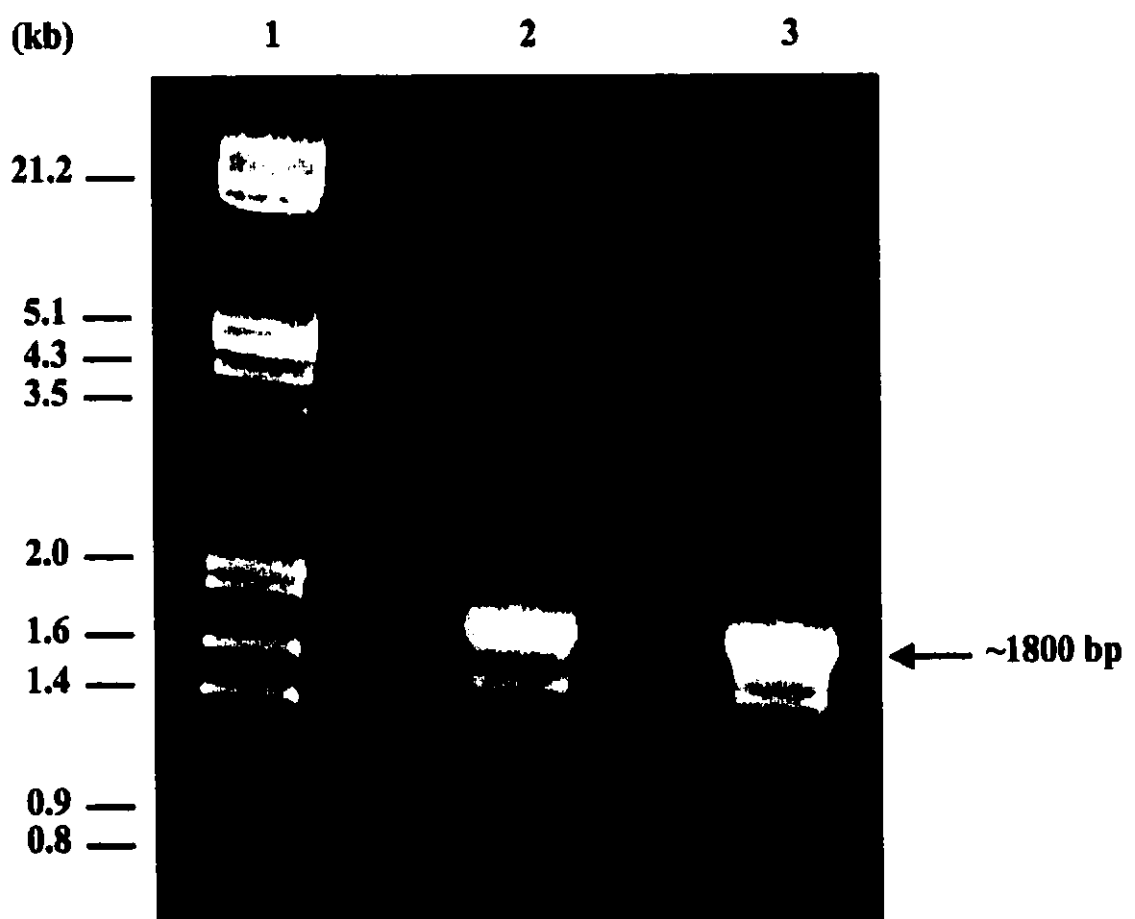
|
Bases Complementary to Lagging Strand of BSA cDNA

Reverse Primer: BSA747**5' - CTGCAGTTAGGCTAAGGCTGTTTG - 3'**

| |
*Pst*I Restriction Site

|
Bases Complementary to Leading Strand of BSA cDNA

Figure 19. Agarose gel electrophoresis analysis of PCR of BSA cDNA using *Pfu* polymerase and primers BSA736 and BSA747. The reaction conditions were 94°C for 1 minute (hot start) and 35 subsequent PCR cycles at 94°C for 45 seconds, 55°C for 1 minute and 72°C for 2 minutes. Following the 35 cycles, a final extension of 72°C for 5 minutes was performed. The amplified DNA was run on a 0.8% (w/v) agarose gel. Lane 1 contains λ DNA/ *EcoRI*+*HindIII* Ladder (MBI Fermentas, Flamborough, ON), and lanes 2 and 3 contain 5 μ L of the PCR reaction from two independent trials.



The blunt-ended PCR fragment was ligated into a BlueScript plasmid that was previously linearized with *EcoRV*. Using agar plates containing X-gal, putative clones were identified using α -complementation. The white colonies were inoculated into selective broth and restriction analysis was performed on the plasmid DNA (Figure 20). The restricted plasmid DNA from colony 2 and colony 4 produced DNA fragments of ~1800 bp and ~3000 bp. These DNA fragments corresponded to the size of the mature BSA cDNA and linearized pBlueScript, respectively. The plasmid DNA from colony 2 was sent for sequencing. The sequence for this plasmid was correct when compared to the known sequence for BSA cDNA and the engineered restriction sites and start codon were also intact (Figure 21). The BlueScript plasmid carrying this BSA cDNA with engineered fragments was designated pBW(BSA01) (Figure 22).

The cDNA fragment encoding BSA was extracted from pBW(BSA01) by restricting with *Bam*HI and *Pst*I restriction enzymes and the fragment was subsequently gel extracted and gene-cleaned. This fragment was ligated into gel purified pOlePN3' that had been previously restricted with *Bam*HI and *Pst*I. The ligation reaction was used to transform competent *E. coli* cells the following day. Several colonies were inoculated into broth and restriction analysis was performed (Figure 23). All putative clones contained plasmids that produced fragments of ~1800 bp and ~4600 bp when restricted with *Bam*HI and *Pst*I. These fragments corresponded to the *BSA01* gene and the linearized pOlePN3' parent plasmid. The new plasmid containing the *Arabidopsis* oleosin promoter, BSA cDNA and ubiquitin terminator was designated pBW(BSA02) (Figure 24).

Figure 20. Restriction analysis using *Bam*HI and *Pst*I for putative pBW(BSA01) clones. DNA (1.5 µg) was restricted for 3 hours at 37°C and run on a 0.8% (w/v) agarose gel. The contents of each lane are as follows: 1, λ DNA/ *Eco*RI+*Hind*III Ladder (MBI Fermentas, Flamborough, ON); 2, uncut colony 1 plasmid DNA; 3, restricted colony 1 plasmid DNA; 4, uncut colony 2 plasmid DNA; 5, restricted colony 2 plasmid DNA; 6, uncut colony 3 plasmid DNA; 7, restricted colony 3 plasmid DNA; 8, uncut colony 4 plasmid DNA; 9, restricted colony 4 plasmid DNA; 10, uncut pBlueScript; 11, restricted pBlueScript.

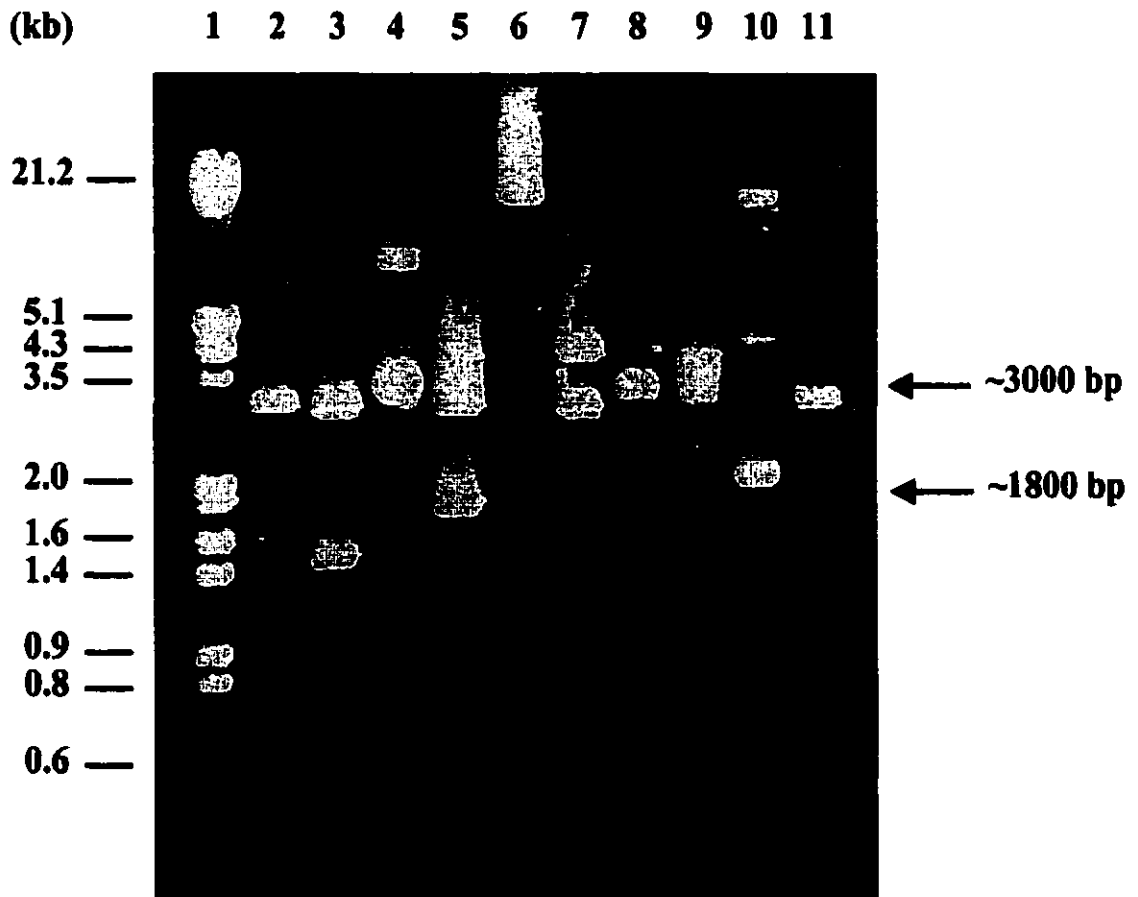
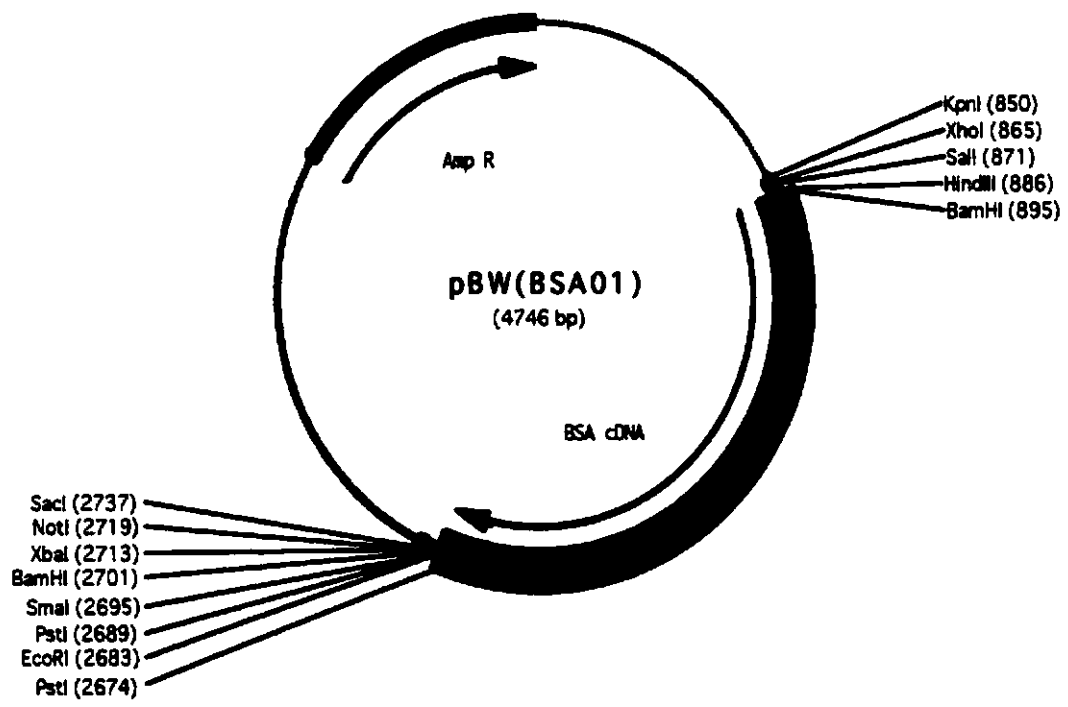


Figure 21. Sequence alignment for a putative *BSA01* gene in colony 2 against the known BSA cDNA sequence. Identical nucleotides are highlighted in yellow. The engineered *Bam*HI (5' end) and *Pst*I (3' end) restriction sites are shown in blue while the engineered start codon is shown in red.

PUTATIVE BSA01 BSA cDNA	GGATCCATGAGGGGTGTGTTTCGTCGAGATACACACAAGAGTGAGATTGCTCATC AGGGGTGTGTTTCGTCGAGATACACACAAGAGTGAGATTGCTCATC	55 46
PUTATIVE BSA01 BSA cDNA	GGTTTAAAGATTGGGAGAGAACAATTTTAAAGGCCTGGTACTGATTGCCTTTTC GGTTTAAAGATTGGGAGAGAACAATTTTAAAGGCCTGGTACTGATTGCCTTTTC	110 101
PUTATIVE BSA01 BSA cDNA	TCAGTATCTCCAGCAGTGTCCATTGATGAGCATGTAAAATTAGTGAACGAACTA TCAGTATCTCCAGCAGTGTCCATTGATGAGCATGTAAAATTAGTGAACGAACTA	165 156
PUTATIVE BSA01 BSA cDNA	ACTGAGTTGCAAAAACATGTGTGCTGATGAGTCCCATGCCGGCTGTGAAAAGT ACTGAGTTGCAAAAACATGTGTGCTGATGAGTCCCATGCCGGCTGTGAAAAGT	220 211
PUTATIVE BSA01 BSA cDNA	CACCTCACACTCTCTTTGGAGATGAATTGTGTAAAGTTGCATCCCTCGTGAAC CACCTCACACTCTCTTTGGAGATGAATTGTGTAAAGTTGCATCCCTCGTGAAC	275 266
PUTATIVE BSA01 BSA cDNA	CTATGGTGACATGGCTGACTGCTGTGAGAAACAGAGCCTGAAAGAAATGAATGC CTATGGTGACATGGCTGACTGCTGTGAGAAACAGAGCCTGAAAGAAATGAATGC	330 321
PUTATIVE BSA01 BSA cDNA	TTCTGAGCCCAAAGATGATAGCCAGACCTCCCTAAATTGAAACCAGACCCCA TTCTGAGCCCAAAGATGATAGCCAGACCTCCCTAAATTGAAACCAGACCCCA	385 376
PUTATIVE BSA01 BSA cDNA	ATACTTTGTGTGATGAGTTTAAAGCCAGATGAAAAGAAGTTTGGGGAAAATACCT ATACTTTGTGTGATGAGTTTAAAGCCAGATGAAAAGAAGTTTGGGGAAAATACCT	440 431
PUTATIVE BSA01 BSA cDNA	ATACGAAATTGCTAGAAGACATCCCTACTTTTATGCACCAGAACTCCTTTACTAT ATACGAAATTGCTAGAAGACATCCCTACTTTTATGCACCAGAACTCCTTTACTAT	495 486
PUTATIVE BSA01 BSA cDNA	GCTAATAAATAAATGGAGTTTTTCAAGAATGCTGCCAAGCTGAAGATAAAGGTG GCTAATAAATAAATGGAGTTTTTCAAGAATGCTGCCAAGCTGAAGATAAAGGTG	550 541
PUTATIVE BSA01 BSA cDNA	CCTGCCTGCTACCAAAGATTGAAACTATGAGAGAAAAGTACTGACTTCATCTGC CCTGCCTGCTACCAAAGATTGAAACTATGAGAGAAAAGTACTGACTTCATCTGC	605 596
PUTATIVE BSA01 BSA cDNA	CAGACAGAGACTCAGGTGTGCCAGTATTCAAAAATTTGGAGAAAGAGCTTTAAAA CAGACAGAGACTCAGGTGTGCCAGTATTCAAAAATTTGGAGAAAGAGCTTTAAAA	660 651
PUTATIVE BSA01 BSA cDNA	GCATGGTCAGTAGCTCGCCTGAGCCAGAAATTTCCCAAGGCTGAGTTTGTAGAAG GCATGGTCAGTAGCTCGCCTGAGCCAGAAATTTCCCAAGGCTGAGTTTGTAGAAG	715 706
PUTATIVE BSA01 BSA cDNA	TTACCAAGCTAGTGACAGATCTCACAAAAGTCCACAAGGAATGCTGCCATGGTGA TTACCAAGCTAGTGACAGATCTCACAAAAGTCCACAAGGAATGCTGCCATGGTGA	770 761
PUTATIVE BSA01 BSA cDNA	CCTACTTGAATGCGCAGATGACAGGGCAGATCTTGCCAAGTACATATGTGATAAT CCTACTTGAATGCGCAGATGACAGGGCAGATCTTGCCAAGTACATATGTGATAAT	825 816
PUTATIVE BSA01 BSA cDNA	CAAGATACAATCTCCAGTAACTGAAGGAATGCTGTGATAAGCCTTTGTTGGAAA CAAGATACAATCTCCAGTAACTGAAGGAATGCTGTGATAAGCCTTTGTTGGAAA	880 871
PUTATIVE BSA01 BSA cDNA	AATCCCACATGCAATGCTGAGGTAGAAAAAGATGCCATACCTGAAAACCTGCCCCC AATCCCACATGCAATGCTGAGGTAGAAAAAGATGCCATACCTGAAAACCTGCCCCC	935 926
PUTATIVE BSA01 BSA cDNA	ATTAACTGCTGACTTTGCTGAAGATAAGGATGTTTGCAAAAATATCAGGAAGCA ATTAACTGCTGACTTTGCTGAAGATAAGGATGTTTGCAAAAATATCAGGAAGCA	990 981
PUTATIVE BSA01 BSA cDNA	AAAGATGCCTTCCTGGGCTCGTTTTTGTATGAATATTCAGAAGGCATCCTGAAT AAAGATGCCTTCCTGGGCTCGTTTTTGTATGAATATTCAGAAGGCATCCTGAAT	1045 1036
PUTATIVE BSA01 BSA cDNA	ATGCTGTCTCAGTGCTATTGAGACTTGCCAAGGAATATGAAGCCCACTGGAGGA ATGCTGTCTCAGTGCTATTGAGACTTGCCAAGGAATATGAAGCCCACTGGAGGA	1100 1091
PUTATIVE BSA01 BSA cDNA	ATGCTGTGCCAAGATGATCCACATGCATGCTATTCCACAGTGTGACAACTT ATGCTGTGCCAAGATGATCCACATGCATGCTATTCCACAGTGTGACAACTT	1155 1146
PUTATIVE BSA01 BSA cDNA	AAGCATCTTGTGGATGAGCCTCAGAATTTAATCAAAACAAAATCTGACCAATTCG AAGCATCTTGTGGATGAGCCTCAGAATTTAATCAAAACAAAATCTGACCAATTCG	1210 1201

POTATIVE BSA01 BSA cDNA	AAAACCTGGAGAGTATGGATTCCAAAATGCGCTCATAGTTCGTTACACCAGGAA AAAACCTGGAGAGTATGGATTCCAAAATGCGCTCATAGTTCGTTACACCAGGAA	1265 1256
POTATIVE BSA01 BSA cDNA	AGTACCCCAAGTGTCAACTCCAACCTCTCGTGGAGGTTTCAAGAAGCCTAGGAAAA AGTACCCCAAGTGTCAACTCCAACCTCTCGTGGAGGTTTCAAGAAGCCTAGGAAAA	1320 1311
POTATIVE BSA01 BSA cDNA	GTGGTACTAGGTGTTGTACAAAGCCGGAATCAGAAGAATGCCCTGTAAGTAAAG GTGGTACTAGGTGTTGTACAAAGCCGGAATCAGAAGAATGCCCTGTAAGTAAAG	1375 1366
POTATIVE BSA01 BSA cDNA	ACTATCTGAGCTTGAATCCTGAACCGGTTGTGGTGCATGAGAAGACACCAGT ACTATCTGAGCTTGAATCCTGAACCGGTTGTGGTGCATGAGAAGACACCAGT	1430 1421
POTATIVE BSA01 BSA cDNA	GAGTGAAAAGTCACCAAGTGTGACAGAGTCATTGGTGAACAGACGGCCATGT GAGTGAAAAGTCACCAAGTGTGACAGAGTCATTGGTGAACAGACGGCCATGT	1485 1476
POTATIVE BSA01 BSA cDNA	TTCTCTGCTCTGACACCTGATGAACATATGTACCCAAAGCCTTTGATGAGAAT TTCTCTGCTCTGACACCTGATGAACATATGTACCCAAAGCCTTTGATGAGAAT	1540 1531
POTATIVE BSA01 BSA cDNA	TGTTACCTTCCATGCAGATATATGCACACTCCCGATACTGAGAAACAAATCAA TGTTACCTTCCATGCAGATATATGCACACTCCCGATACTGAGAAACAAATCAA	1595 1586
POTATIVE BSA01 BSA cDNA	GAAACAACTGCACTTGTGAGCTGTGAAACACAAGCCCAAGGCAACAGAGGAA GAAACAACTGCACTTGTGAGCTGTGAAACACAAGCCCAAGGCAACAGAGGAA	1650 1641
POTATIVE BSA01 BSA cDNA	CAACTGAAAACCGTCATGGAGAATTTGTGGCTTTGTAGACAAGTGTGTGCAG CAACTGAAAACCGTCATGGAGAATTTGTGGCTTTGTAGACAAGTGTGTGCAG	1705 1696
POTATIVE BSA01 BSA cDNA	CTGATGACAAAGAGGCCCTGCTTGTGTGGAGGGTCCAAAACCTTGTGTTTCAAC CTGATGACAAAGAGGCCCTGCTTGTGTGGAGGGTCCAAAACCTTGTGTTTCAAC	1760 1751
POTATIVE BSA01 BSA cDNA	TCAAACAGCCTTAGCCTAACTGCAG TCAAACAGCCTTAGCCTAA	1785 1770

Figure 22. Plasmid map for pBW(BSA01). The vector contains an ampicillin resistance gene (black arrow) as well as the mature BSA cDNA (purple arrow) that was inserted into the multiple cloning site (green) of pBlueScript via the blunt-ended *EcoRV* restriction site. The detailed sequence of the insert is shown along with some flanking sequences on the page following the plasmid map.



KpnI (850) Sall (871) BamHI (895)
 XhoI (865) HindIII (886)

GGTACCGGGCCCCCCTCGAGGTCGACGGTATCGATAAGCTTGATGGATCCATGAGGGGTGTGTTTCGTGAGATACACACA
 AGAGTGAGATTGCTCATCGGTTAAAGATTTGGGAGAAGAACATTTTAAAGGCCTGGTACTGATTGCCTTTCTCAGTATCT
 CCAGCAGTGCCATTTGATGAGCATGTAAATTAGTGAACGAACATACTGAGTTTGCAAAAACATGTGTTGCTGATGAGTCC
 CATGCCGGCTGTGAAAAGTCACTTCACTCTCTTTGGAGATGAATTGTGTAAGTTGCATCCCTTCGTGAAACCTATGGTG
 ACATGGCTGACTGCTGTGAGAAACAAGGCCTGAAAGAAATGAATGCTTCTGAGCCACAAAGATGATAGCCAGACCTCCC
 TAAATTGAAACCAGACCCCAATACTTTGTGTGATGAGTTAAGGCAGATGAAAAGAAAGTTTGGGAAAATACCTATACGAA
 ATTGCTAGAAGACATCCCTACTTTTATGCACCAGAACTCCTTACTATGCTAATAAATAATGGAGTTTTCAAGAATGCT
 GCCAAGCTGAAGATAAAGGTGCCTGCCTGCTACCAAGATTGAACTATGAGAGAAAAGTACTGACTTCATCTGCCAGACA
 GAGACTCAGGTGTGCCAGTATTCAAAAATTTGGAGAAAGAGCTTAAAGCATGGTCAGTAGCTCGCCTGAGCCAGAAATTT
 CCCAAGGCTGAGTTTGTAGAAGTTACCAAGCTAGTGACAGATCTCACAAAAGTCCACAAGGAATGCTGCCATGGTGACCTAC
 TTGAATGCGCAGATGACAGGGCAGATCTTGCCAAGTACATATGTGATAATCAAGATACAATCTCCAATAACTGAAGGAATG
 CTGTGATAAGCCTTTGTTGGAAAATCCCACTGCATTGCTGAGGTAGAAAAGATGCCATACCTGAAAACCTGCCCCATTA
 ACTGCTGACTTTGCTGAAGATAAGGATGTTTGCAAAAACATCAGGAAGCAAAGATGCCTTCTGGGCTCGTTTTGTATG
 AATATTCAAGAAGGCATCCTGAATATGCTGTCTCAGTGCTATTGAGACTTGCCAAGGAATATGAAGCCACACTGGAGGAATG
 CTGTGCCAAAGATGATCCACATGCATGCTATTCCACAGTGTGACAAACTTAAGCATCTTGTGGATGAGCCTCAGAATTA
 ATCAAACTAACTGTGACCAATTCGAAAACCTGGAGAGTATGGATTCCAAAATGCGCTCATAGTTGTTACACCGAAAG
 TACCCCAAGTGTCACTCAACTCTCGTGGAGGTTTCAAGAAGCCTAGGAAAAGTGGGTAAGTGGTGTGTACAAAGCCGGA
 ATCAGAAAGAAATGCCCTGACTGAAGACTATCTGAGCTTGATCCTGAACCGGTTGTGCGTGTGCATGAGAAGACCAAGTG
 AGTGAAAAGTACCAAGTGTGCACAGATCATTGGTGAACAGACGGCCATGTTTCTCTGCTGACACCTGATGAAACAT
 ATGTACCCAAAGCCTTTGATGAGAAATGTTACCTTCCATGCAGATATATGCACACTTCCCGATACTGAGAAACAAATCAA
 GAAACAACTGCACTTGTGAGCTGTTGAAACAAGCCCAAGGCAACAGAGGAACAACCTGAAAACCGTCATGGAGAATTTT
 GTGGCTTTGTAGACAAGTGCTGTGCAGCTGATGACAAAGAGGCTGCTTTGCTGTGGAGGGTCCAAAACCTGTTGTTCAA

PstI (2689) BamHI (2701) NotI (2719)
 PstI (2674) EcoRI (2683) SmaI (2695) XbaI (2713)

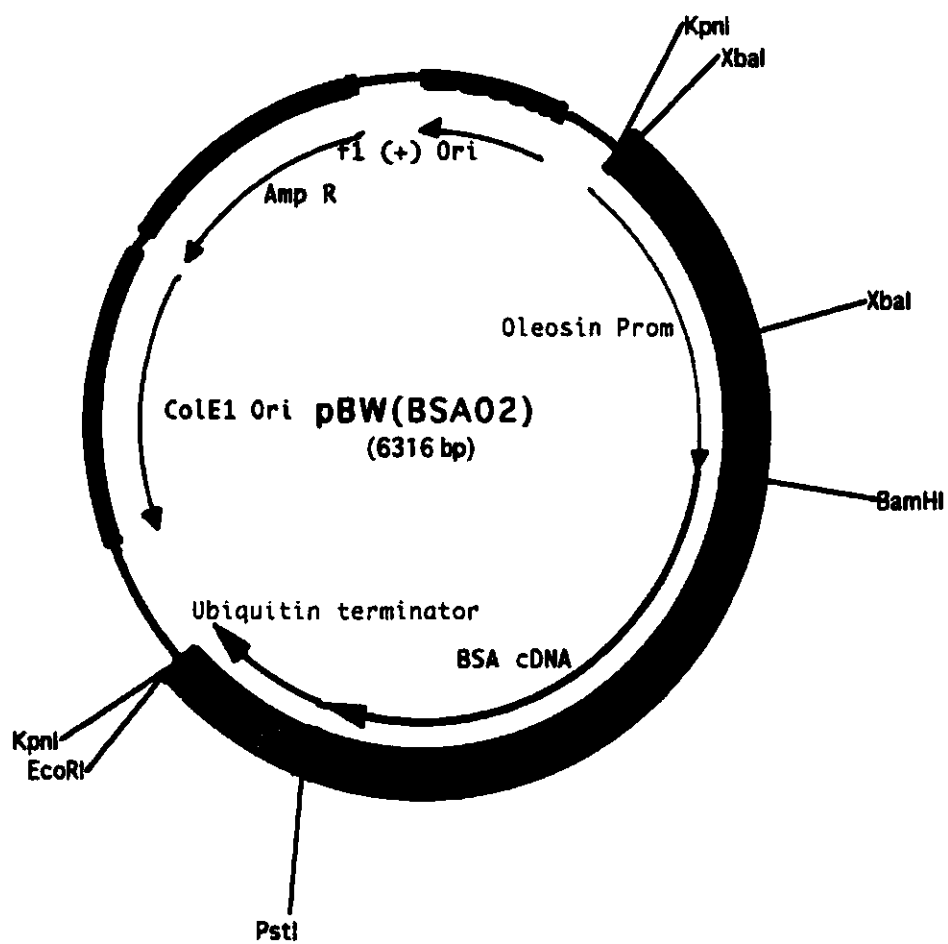
CTCAACAGCCTTAGCCTAACTGCAGATCGAATTCCTGCAGCCCGGGGATCCAAGTCTAGAGCGGCCGCCACCGCGGT

SacI (2737)
 GGAGCTC

Figure 23. Restriction analysis using *Bam*HI and *Pst*II on plasmid DNA from putative pBW(BSA02) clones. DNA (1.5 μ g) was restricted for 3 hours at 37°C and run on a 0.8% (w/v) agarose gel. The contents of each lane are as follows: 1, λ DNA/*Hind*III Ladder; 2, restricted pOlePN3'; 3, restricted colony 1 plasmid DNA; 4, restricted colony 2 plasmid DNA; 5, restricted colony 3 plasmid DNA; 6, restricted colony 4 plasmid DNA; 7, restricted colony 5 plasmid DNA; 8, restricted colony 6 plasmid DNA; 9, restricted colony 7 plasmid DNA; 10, restricted colony 8 plasmid DNA.



Figure 24. Plasmid map for pBW(BSA02). The vector contains an ampicillin resistance gene (solid black arrow) as well as the *BSA01* gene (purple) that was inserted into the multiple cloning site of pOlePN3' via the *Bam*HI and *Pst*I restriction sites that were engineered on the 5' and 3' ends of the DNA sequence, respectively. The *BSA01* gene is driven by a 1.1 Kb *Arabidopsis* oleosin promoter (light blue arrow) and is followed by a ubiquitin terminator (pink arrow). The detailed sequence of the insert is shown along with some flanking sequences on the page following the plasmid map.



KpnI XbaI
GGTACCCAAATACGATCTGATACTGATAACCTCTAGATTTTAAAGGTTAAAGCAATCAATCACCTGAGC
ATTCAGCTGGTGGATCATGACGATTCAGAAAACATCAAGCAAGCTCTCAAGCTACACTCTTTGGG
ATCATACTGAACTTAACAACCTGTTATGTCCGTAGTCCAGTACAGACATCTCGTAACCTGGATT
ATGCAGATGCCATGGCTATACCAACCTGGCTTGGTCAACCAAGCAACTCTCTGGTAAGCTAGCTC
CACTCCCCAAGAACCCGCGCCAAATTCGGAAATGCTGACCTGAAGACGGAAATCATCTGTCGGG
TCCCTGGGCAATTCGGCCGAAGATGGCTAGCTTGGCTTGGAGACGAGACCCGAATCGAGTCTGTTG
AAGGGTTGTTCAATGGGATTTGATACCGAGATGGCTGTCGAGAGCTTGAAGGAAAGGCAAAATGGG
TTTGGCTCTGGAGAAAGAGATGCGCTTACAGAGAGAAATTCAGAGCTTACAGAGAGATGCGCGCGC
GATGACGGGAGAGAGACGAGGACCTGCATTATCAAGCACTGACCTGGTGAATTTGGAACTTTT

XbaI
AAGAGGCAGATAGATTATTATTGTATCCATTTCTTCAATGTTCTAGAATGTCGGGAACTAATTTT
AAACTAACTCTAATTTTCTAATTTTCTTCCAAATGAGATATGTTGGCCGATAGAGAACT
ATTGAAGGCCAAACCCACTGACGAGCCCAAGGTTGCTTTGCGTTTATGTTGCGTTCGATGCGC
AAGCCCACTTCTGAGCTAGGCAAAAACAACCTGCTTAAATAGACTCTCTCGTAAACATGCA
CGGCTGATGGTACGGCATTACACCTGGCCCTCAATTCATGATGCTCTCATTGACAGTACTTC
TGGTCTCCTTCTAATATATCTAACAAACTCTACCTTCCAAAATATATACACATCTTTTGAAT

BamHI
CAATCTCTCATTCAAAATCTCATCTCTAGTAAACAGATCCATGAGGGGTGTGTTCTCAGAAATA
CACACAAGATGAGATGCTCATCGGTTAAGATTGGGAGAGAACATTTAAAGCCCTGGTACTGA
TTGCCCTTCTCAGTATCTCCAGCAGTCTCATTGATGAGCATGAAAATGAGCAACCACTAATG
AGTTGCAAAAACATGCTTGGCTGATGAGTCCCAAGCCGCTGTGAAAAGTCACTTCACACTCTTTG
GAGATCAATGCTAAAGTGCATCCCTCTGTGAACCTATGCTGACATGCTGACTGCTGTAAGAAC
AAGGCCCTGAAAGAAATGAATGCTTCTGAGCCACAAAGATGATAGCCAGACCTCCCTAAATGAAAC
CAGGCCCAATCTTTGATGATGATTTAAGCCAGATGAAAGAGTTTGGGAAATACCTATAGC
AAATGCTAGAAAGATCCCTACTTTTATGACCCAGAACTCTTTACTATGCTAATAAATATAATGAG
TTTTCAAGAACTGTCCAAGCTAAGATAAAGTGGCTGCTGCTACCAAGATTGAACATGAGAG
AAAAAGTACTGACTGACTGCTGCTGACAGAGACTGAGTGTGCGCATTTCAAAAATTTGGAGAAAG
CTTAAAGCATGCTCAGTACGCTGCTGAGCCAGAAATTTCCCAAGCTGAGTTTGTAGAAATACCA
AGCTAGTACAGATCTCACAAGATCCCAAGGAAATGCTGCTGCTGACTACTTGAATGCGCAATG
ACAGGCCAGATCTTGGCAAGTACATATGTAATACAGATACAACTCTCAAGTAACTGAGGAAATGCT
GTGATAAGCTTTGTTGCAAAAATCCACTGCTGCTGAGTACAAAAGATGCCATACCTGAAACCC
TGGCCCCATTAACTGCTGACTTGTGAGATAGGATGTTTCCAAAACATACAGGAGCAAAAGATG
CCTTCTGGCTGCTTTTGTATGAAATATTCAAGAAAGCATCTGAAATGCTGCTCAGTCTATTTGA
GACTTCCAAAGAAATGAAAGCCACACTGAGGAAATGCTGCTCAAGATGATCCACATGATGCTATT
CCACAGTGTGCAAACTTAAGCATCTTGTGATGAGCTCAGAAATTAATCAAAACAAATCTGAGCC
AATTCGAAAACCTGGAGATGGAATTCAAAATGCGCTCATAGTTGCTTACACAGGAAAGTACCC
AAGTGTCACTCAACTCTGCTGAGGTTTCAAGAAAGCTAGGAAAAGTGGTACTAGGTTGTGACAA
AGCCGAAATCAGAAAGATGCCCCTGACTGAAGACTATCTGAGCTTGAATCTTAAACCGTTGTGCTGC
TGCATGAGAGACACCACTGAGTGAAGAACTCCAAATGCTGCAAGATGATTTGTTGACACAGAGC
CATGTTCTCTGCTGACACTGATGAACATATGATCCCAAGCCCTTGTGATGAGAAATGTTCACT
TCCATGAGATATATCCACTTCCGATACTGAGAAACAAATCAAGAAACAACTGCACTTGTGAGC
TGTGAAACCAAGCCCAAGCAAGAGAACTGAAAACCGCTCATGGAGAAATTTTGGCTTTG
TAGACAAGTCTGTGAGCTGATGCAAGAGGCTGCTTTGCTGTGAGGGTCCAAAATCTGTGTTT

PstI
CAACTCABAACGCTTAGCTAAGTGCAGAAATGATTCACAGATGTTTGTGACCAAGTTAGTGGTTG
TTTTATGGAACTTTGTTAAGCTTGAATGTGGAAAGAACGTGGCTTTGTTGTTTTAAATGTTGG
TGAATAAGATGTTTCTTTGGATTAACTAGTATTTTCTATAATGTTTCAATGTTTGTGACACACA
TTTTAAATATGCTGTAGATGATAGCTGCTGCTTTAATTAATTAACCTCACCTCAGTTTCAA
AGTTGTTGCAATGACTCTGTGATGTTAAGATCGAGTAAAGTAAATTTGCTATAATTAATGAGCT
ATTTGATAGCTAATGGAACAATGTTTATGACAGCTACTTTTGGTATGCTGTTGACGTTTCTCT
TTTTAACTATAGTGGCTTCTGCTGTTTCAATGTTGAAACAAGGCACTCACTTGGAGATG

EcoRI KpnI
CCGCTCCACTGATTTTGAACAAGAAATTCGGTACC

4.1.8. Insertion of the *BSA02* Gene into the Binary Vector, pCGN1559

The *BSA02* gene, containing the *Arabidopsis* oleosin promoter, BSA cDNA and ubiquitin terminator, was extracted from pBW(*BSA02*) by restricting the plasmid with *KpnI* for compatibility with the multiple cloning site of pCGN1559 (Figure 10). This restriction as well as pCGN1559 linearized with *KpnI* were electrophoresed on an agarose gel (Figure 25). The restricted pBW(*BSA02*) revealed DNA fragments of ~3400 bp and ~3000 bp, corresponding to the *BSA02* gene and the remainder of the pOlePN3' parent plasmid. In addition, the restricted pCGN1559 was ~14,400 bp. The *BSA02* gene and linearized pCGN1559 were excised from the agarose gel and subsequently gel extracted and gene-cleaned. The two pieces were ligated and used to transform *E. coli* the following day. Putative clones were first identified using α -complementation. The white clones were screened for the correct insert by restricting the plasmid DNA with *KpnI* (Figure 26). The restricted plasmid DNA revealed DNA fragments of ~3400 bp and ~14,400 bp, corresponding to the *BSA02* gene and pCGN1559 parent plasmid DNA. Orientation analysis was also performed by restricting the plasmid DNA from three putative clones with *BamHI* (Figure 27). In addition to a DNA fragment of ~15,500 bp, the restricted plasmid DNA from colony 3 also produced a DNA fragment of ~2300 bp. This indicated that colony 3 contained a plasmid with the *BSA02* gene in the correct orientation. This plasmid was sent for sequencing and the resultant sequence was compared to the known sequence for *Arabidopsis* oleosin promoter and BSA cDNA (Figure 28). This final BSA binary vector was designated pBW(Bin-*BSA03*) (Figure 29) and was electroporated into *A. tumefaciens*.

Figure 25. Restriction analysis using *KpnI* on pCGN1559 and pBW(BSA02). DNA (1.5 µg) was restricted for 3 hours at 37°C and run on a 0.8% (w/v) agarose gel prior to gene-cleaning. Lane 1 contains λ DNA/*HindIII* Ladder, and lanes 2 and 3 contain restricted pCGN1559 and pBW(ASP02), respectively.

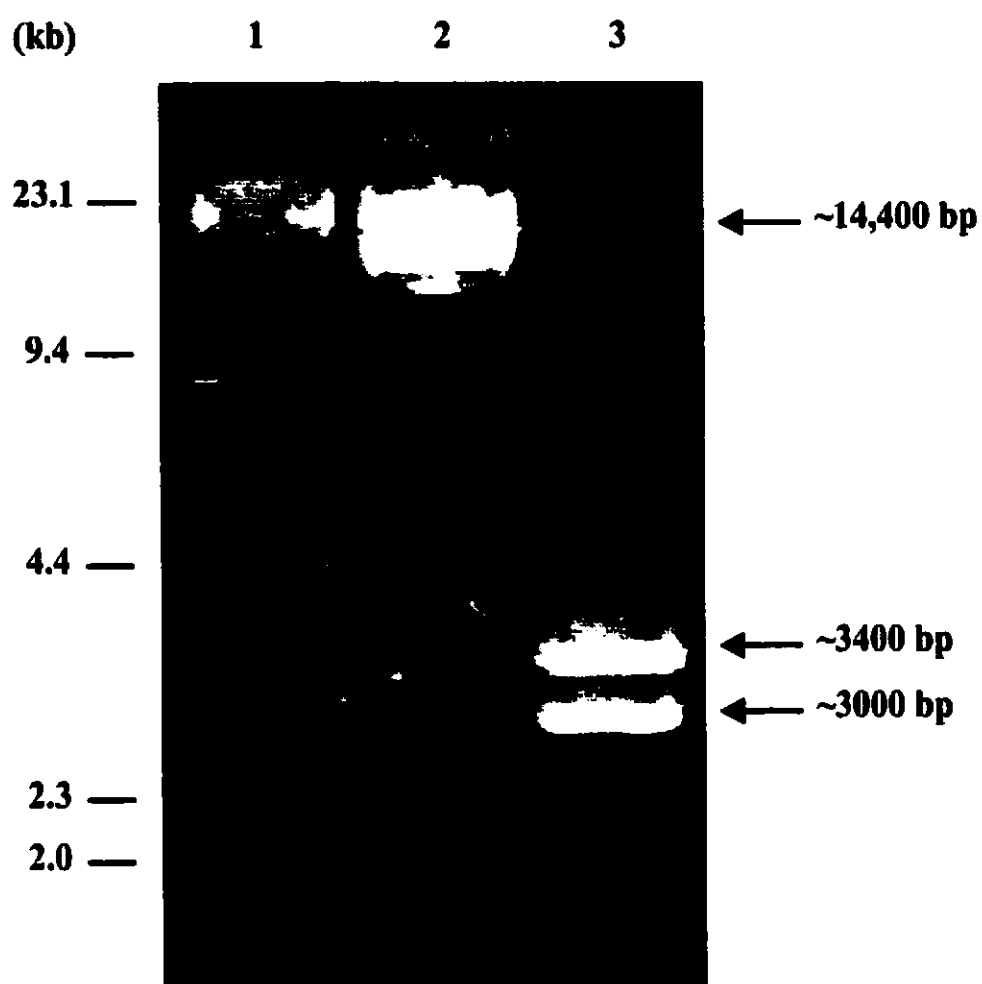


Figure 26. Restriction analysis using *KpnI* on plasmid DNA from a putative pBW(Bin-BSA03) clone. DNA (1.5 µg) was restricted for 3 hours at 37°C and run on a 0.8% (w/v) agarose gel. Lane 1 contains λ DNA/*HindIII* Ladder, and lane 2 contains restricted plasmid DNA from the putative colony.

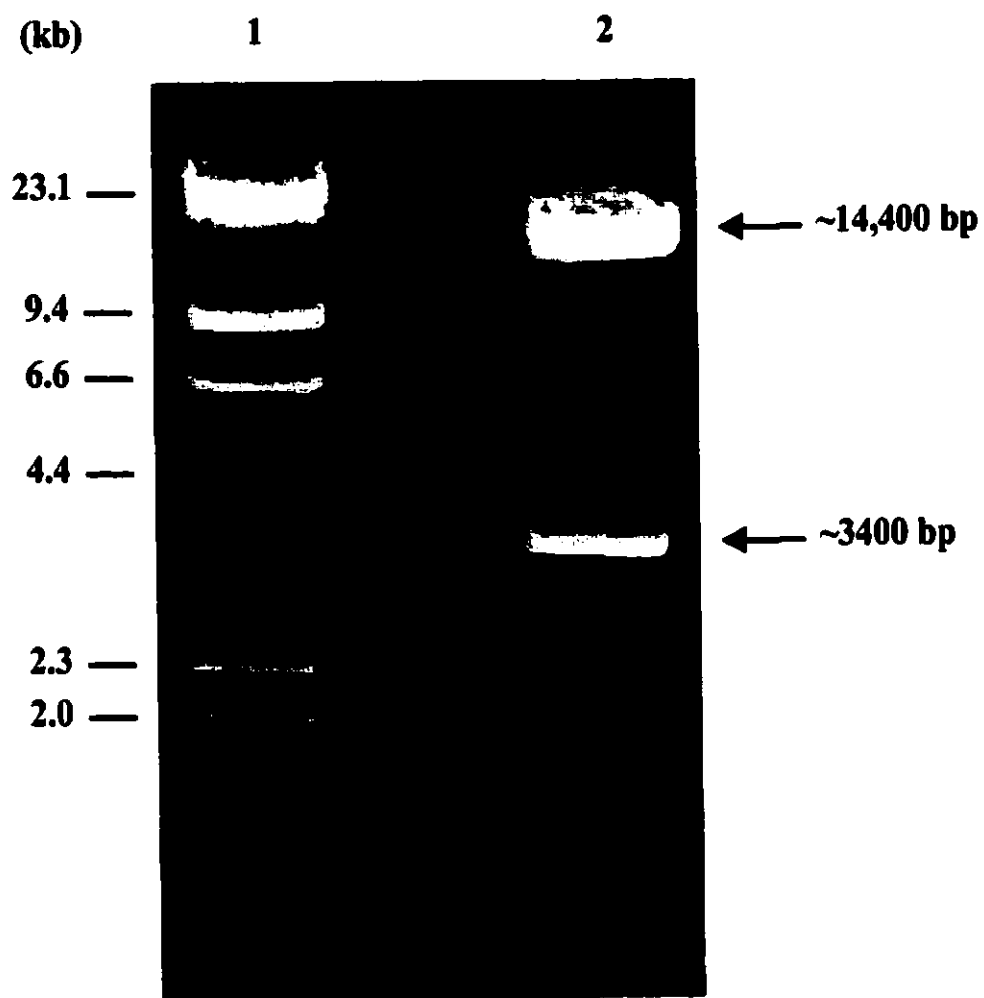


Figure 27. Orientation analysis using *Bam*HI on plasmid DNA from putative pBW(Bin-BSA03) clones. DNA (1.5 μ g) was restricted for 3 hours at 37°C and run on a 0.8% (w/v) agarose gel. Lane 1 contains λ DNA/*Hind*III Ladder, and lanes 2 through 4 contain restricted plasmid DNA from colonies 1 through 3, respectively.

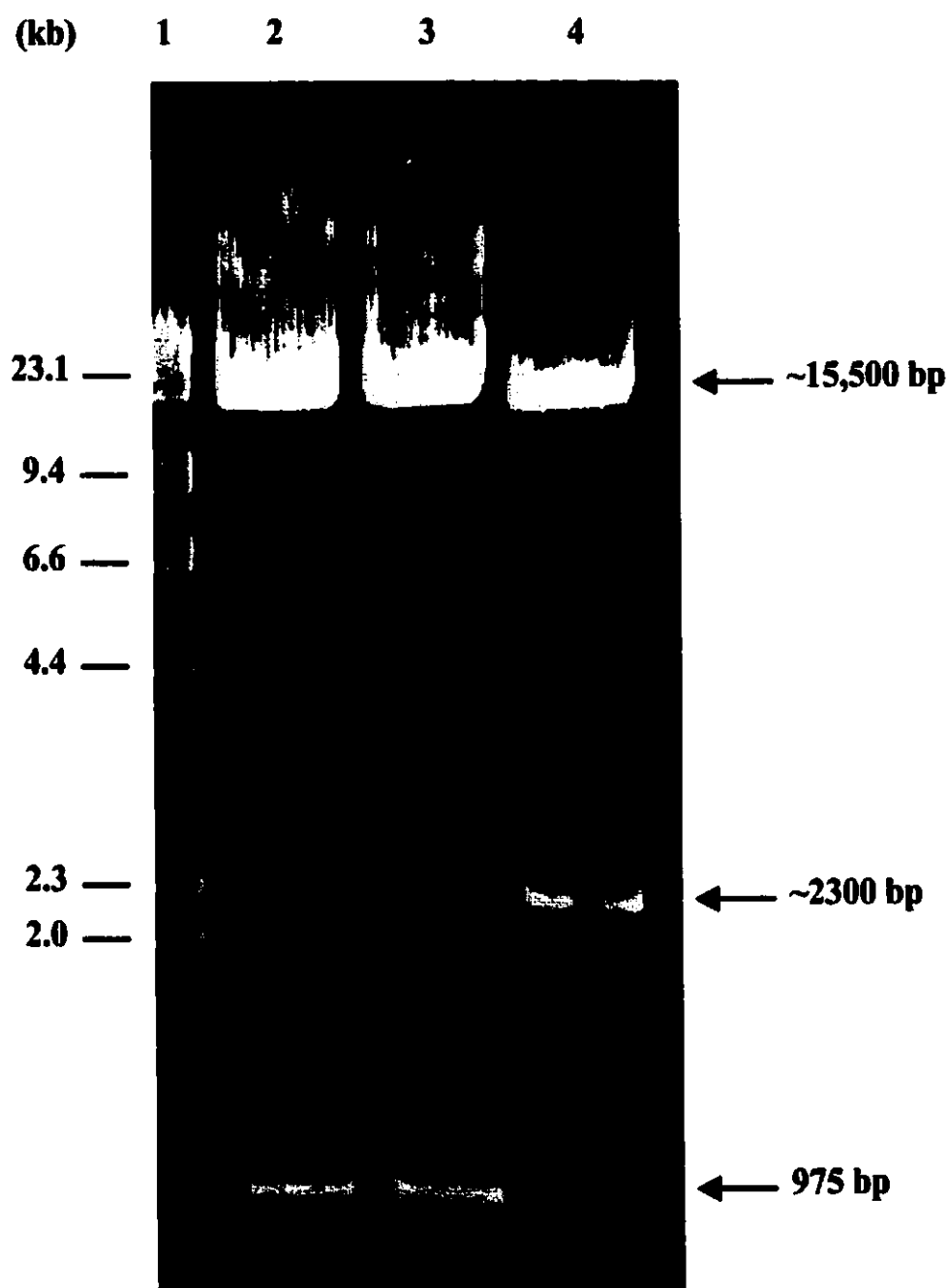


Figure 28. Sequence alignment for a putative *BSA03* gene in colony 3 against the known DNA sequence encoding oleosin promoter and BSA cDNA. Identical nucleotides are highlighted in yellow. The engineered *Bam*HI (5' end) and *Pst*I (3' end) restriction sites are shown in blue. Also, the engineered start codon is shown in red.

PUTATIVE BSA03 OLEOSIN PROMOTER	TGGCTATACCCACCTCGGTCTTGGTCACACCAGGAACCTCTCTGGTAAGCTAGCT TGGCTATACCCACCTCGGTCTTGGTCACACCAGGAACCTCTCTGGTAAGCTAGCT	55 55
PUTATIVE BSA03 OLEOSIN PROMOTER	CCACTCCCAGAAACACCGGCGCAAATGCGCGAATTGCTGACCTGAAGACGG CCACTCCCAGAAACACCGGCGCAAATGCGCGAATTGCTGACCTGAAGACGG	110 110
PUTATIVE BSA03 OLEOSIN PROMOTER	AACATCATCGTCCGGTCTTGGGCGATTGCGGCGGAAGATGGGTGAGCTTGGGCT AACATCATCGTCCGGTCTTGGGCGATTGCGGCGGAAGATGGGTGAGCTTGGGCT	165 165
PUTATIVE BSA03 OLEOSIN PROMOTER	TGAGGACGAGACCCGAATCGAGTCTGTTGAAAGGTTGTTCAATGGGATTTGTATA TGAGGACGAGACCCGAATCGAGTCTGTTGAAAGGTTGTTCAATGGGATTTGTATA	220 220
PUTATIVE BSA03 OLEOSIN PROMOTER	CGGAGATTGGTCGTGAGAGGTTTGGAGGAAAGGACAAATGGGTTTGGCTCTGGA CGGAGATTGGTCGTGAGAGGTTTGGAGGAAAGGACAAATGGGTTTGGCTCTGGA	275 275
PUTATIVE BSA03 OLEOSIN PROMOTER	GAAAGAGAGTGCGGCTTTAGAGAGAGAATTGAGAGGTTTAGAGAGAGATGCGGCG GAAAGAGAGTGCGGCTTTAGAGAGAGAATTGAGAGGTTTAGAGAGAGATGCGGCG	330 330
PUTATIVE BSA03 OLEOSIN PROMOTER	GCGATGACGGGAGGAGAGACGACGAGGACCTGCATTATCAAAGCAGTGACGTGGT GCGATGACGGGAGGAGAGACGACGAGGACCTGCATTATCAAAGCAGTGACGTGGT	385 385
PUTATIVE BSA03 OLEOSIN PROMOTER	GAAATTTGGAACTTTTAAGAGGCAGATAGATTATTATTGTATCCATTTCTTC GAAATTTGGAACTTTTAAGAGGCAGATAGATTATTATTGTATCCATTTCTTC	440 440
PUTATIVE BSA03 OLEOSIN PROMOTER	ATTGTTCTAGAAATGTCGCGGAACAAATTTAAAACCTAAATTTTCTAA ATTGTTCTAGAAATGTCGCGGAACAAATTTAAAACCTAAATTTTCTAA	495 495
PUTATIVE BSA03 OLEOSIN PROMOTER	TTTTGTTGCCAATAGTGGATATGTGGCCGTATAGAAGGAATCTATTGAAGGCC TTTTGTTGCCAATAGTGGATATGTGGCCGTATAGAAGGAATCTATTGAAGGCC	550 550
PUTATIVE BSA03 OLEOSIN PROMOTER	AAACCCACTGACGAGCCCAAGGTTTCGTTTTGCGTTTTATGTTTCGGTTCGAT AAACCCACTGACGAGCCCAAGGTTTCGTTTTGCGTTTTATGTTTCGGTTCGAT	605 605
PUTATIVE BSA03 OLEOSIN PROMOTER	GCCAACGCCACATCTGAGCTAGGCCAAAAACAACGTGTCTTTGAATAGACTCC GCCAACGCCACATCTGAGCTAGGCCAAAAACAACGTGTCTTTGAATAGACTCC	660 660
PUTATIVE BSA03 OLEOSIN PROMOTER	TCTCGTTAACACATGCAGCGGCTGCATGGTGACGCCATTAACACGTGGCTACAA TCTCGTTAACACATGCAGCGGCTGCATGGTGACGCCATTAACACGTGGCTACAA	715 715
PUTATIVE BSA03 OLEOSIN PROMOTER	TTGCATGATGTCTCCATTGACACGTGACTTCTCGTCTCCTTTCTTAATATATCTA TTGCATGATGTCTCCATTGACACGTGACTTCTCGTCTCCTTTCTTAATATATCTA	770 770
PUTATIVE BSA03 OLEOSIN PROMOTER	ACAAACACTCCTACCTCTCCAAAATATATACACATCTTTTGTATCAATCTCTCA ACAAACACTCCTACCTCTCCAAAATATATACACATCTTTTGTATCAATCTCTCA	825 825
PUTATIVE BSA03 OLEOSIN PROMOTER/BSA	TTCAAATCTCATTCTCTCTAGTAAACAGGATCCATGAGGGGTGTGTTTCGTGGA TTCAAATCTCATTCTCTCTAGTAAACA AGGGGTGTGTTTCGTGGA	880 847/18
PUTATIVE BSA03 BSA	GATACACACAAGAGTGAGATTGCTCATCGGTTTAAAGATTTGGGAGAAGAACATT GATACACACAAGAGTGAGATTGCTCATCGGTTTAAAGATTTGGGAGAAGAACATT	935 73
PUTATIVE BSA03 BSA	TTAAAGGCCCTGGTACTGATTGCCTTTTCTCAGTATCTCCAGCAGTGTCCATTGA TTAAAGGCCCTGGTACTGATTGCCTTTTCTCAGTATCTCCAGCAGTGTCCATTGA	990 128
PUTATIVE BSA03 BSA	TGAGCATGTAAAATTAGTGAACGAACTAAGTGTGCAAAAACATGTGTGCT TGAGCATGTAAAATTAGTGAACGAACTAAGTGTGCAAAAACATGTGTGCT	1045 183
PUTATIVE BSA03 BSA	GATGAGTCCCATGCCGCTGTGAAAAGTC GATGAGTCCCATGCCGCTGTGAAAAGTC	1074 212

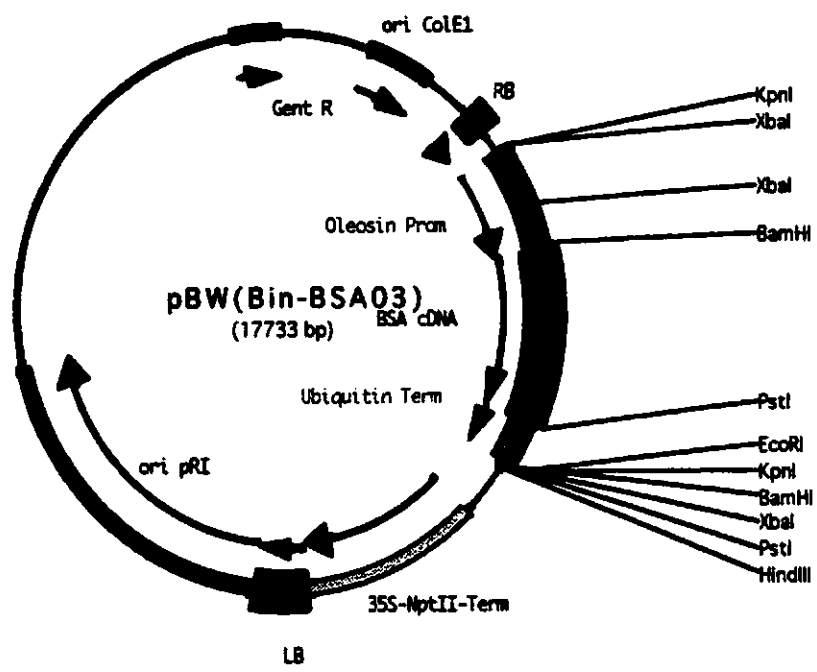
4.1.9. Identification of Transgenic Plants: NptII Assay

Once cotyledons were dipped in an *A. tumefaciens* solution that was carrying pBW(Bin-BSA03), and tissue culture was carried out to regenerate plants, transgenic plantlets were identified using the NptII assay. This was possible because the binary vector had been engineered to carry a NptII gene driven by a 35S promoter and was inserted in tandem with the *BSA02* gene (Figure 29). The data for this is shown in Table 2. BSA 2, BSA 3, BSA 5, BSA 7, BSA 11, BSA 12, BSA 14 and BSA 17 had integrated the pBW(Bin-BSA03) construct. Previous studies have been performed using the NptII gene as a selectable marker inserted in tandem with a gene of interest. For example, Parmenter *et al.* (1995) made transgenic plants expressing hirudin in the seeds of *B. napus* and found a 75% frequency of plants that successfully passed tissue culture screening were also NptII positive. This frequency was also in agreement with the results obtained from Chaudhary *et al.* (1998) and Radke *et al.* (1988) when using the same selectable marker in independent studies. For the putative BSA plants, a frequency of 47% was obtained for plants that were NptII positive that had successfully passed tissue culture screening. This represents a lower efficiency of screening for these plants as compared to other studies and the putative ASP plants. The NptII positive plants were transferred to a greenhouse whereas negative plants were autoclaved and discarded.

4.1.10. Integration of BSA cDNA into the Plant Genome

Southern blot analysis was performed to examine if the BSA cDNA had been integrated into the plant genome. The genomic DNA was isolated and subsequently

Figure 29. Plasmid map for pBW(Bin-BSA03). The binary vector contains a gentamycin resistance gene (solid black arrow). The *BSA02* gene, containing an oleosin promoter (light blue arrow), BSA cDNA (purple arrow) and ubiquitin terminator (pink arrow) was inserted into the multiple cloning site of pCGN1559 via the *KpnI* restriction sites on the 5' and 3' ends of the sequence. This gene was inserted in tandem with the kanamycin resistance gene driven by a separate 35S promoter (shaded black section with solid black arrow). The detailed sequence of the insert is shown along with some flanking sequences.



KpnI XbaI
GGTACCCAAATACGATCTGATACTGATAACGCTAGATTTTGGGTTAAAGCAATCAATCACTGACGATTCAAGGTGGTGGG
TCATGACGATTCAGAAACATCAAGCAAGCTCTCAAGCTACACTCTTTGGGATCATCTGAACCTAACAACCTCGTATGTC
CCGTAGTGCCAGTACAGACACTCTGTAACCTGGATTATGCAGATGCCATGGCTATACCCAACTCGGTCTTGGTCAACCCAGG
AACTCTCTGGTAAGCTAGCTCCACTCCCAAGAAACACCGGCGCAAAATGCCGGAATTGCTGACCTGAAGACGGAAACATCATCG
TCGGGCTCTGGGGGATTGCGGGGAAGATGGGTGAGCTTGGGCTTGGGACGAGACCCGAATCGAGTCTGTTGAAGGTTGTYC
ATTGGGATTTGATACGGGATTGGTCTGAGAGGTTTGGGGAAAGGACAAATGGGTTTGGCTCTGGAGAAAGAGAGTGGGG
TTTAGAGAGAGAAATGAGAGGTTTGAAGAGAGATGCGGGCGGATGACGGGAGGAGAGACGACGAGGACCTGCATTATCAAGCA
GTGACGTGGTAAATTTGAACTTTAAGAGGGCAGATGATTTATTGATCCATTTCTTCATTGTTCTAGAAATGTCGGGG
AAACAAATTTTAAACTAAATCTAAATTTTCTAATTTTGTGCCAATAGTGGATATGGGGCCGTATAGAAGGAATCTATTGAA
GGCCAAACCCACTGACGAGCCAAAGGTTGTTTTGCTTTTATGTTTCGGTTCGATGCCAACGCCACATTCGAGCTAGGC
AAAAACAACGTGCTTTGAATAGACTCTCTCGTTAACACATGCAGCGGCTGCATGGTGACGCCATTAAACAGTGGCCATACA
TTGCATGATGCTCCATTGACACGTGACTTCTGCTCTCTTCTTAATATATCTAACAAACACTCTACCTCTTCCAAATATAT
ACACATCTTTTGTCAATCTCTCATTCAAATCTCATTCTCTCTAGTAAACAGGATCCATGAGGGGTGTGTTTCGTCCAGATAC
ACACAAGGTGAGATTGCTCATCGTTTAAAGATTGGGAGAAACATTTTAAAGCCCTGGTACTGATGCTCTTTCTCAATAT
CTCCAGCAGTGCATTTGATGAGCATGTAAATTAAGTAAACGAACTAACTGAGTTTCAAAAACATGCTGTTGATGATGCC
ATGCCGGCTGTAAAAGTCACTTCAACTCTCTTTGGAGATGAATGTGTAAGTTGCACTCCCTTCTGAAACCTATGGTGACAT
GGCTGACTGCTGAGAAACAAGCCCTGAAAGAAATGAATGCTTCTGAGCCACAAAGATGATAGCCACAGCCTCCCTAAATG
AAACAGACCCCAATCTTTGTGTGATGATTTAAGGACAGTGAAGAAAGATTTTGGGAAATACTATACGAATTTGCTAGAA
GACATCCCTACTTTTATGACCAAGACTCTTTACTATGCTAATAAATATAATGGAGTTTTCAAGATGCTGCCAAGCTGAGA
TAAAGTGGCTGCTGCTACCAAGATTGAACTATGAGAGAAAGTACTGACTTCACTGCCAGACAGACTCAAGTGTGCC
AGTATTCAAATTTGAGAAAGAGCTTTAAAGCATGCTCAGTAGCTGCCCTGAGCCAGAAATTTCCCAAGGCTGAGTTGTAG
AAGTACCAGCTAGTACAGATCTCAAAAAGTCCCAAGGAATGCTGCCATGGTACTCTTGAATGCCAGATGACAGGGC
AGATCTTCCCAAGTACATATGTGATAATCAAGATCAATCTCCAATAACTGAAGGAATGCTGTATAGCCCTTTGTTGAAAAA
TCCACTGCTTGTGAGTGAAGAAAGATGCCATACCTGAAACCTGCCCAATTAAGTGTGACTTGTGAGATAGGATG
TTTGCAAAACATACAGGAAGCAAGATGCTTCTGGCTGTTTTGTATGAATTTCAAGAAAGCATCTGAATATGCTGT
CTCAGTCTATTGACACTTCCCAAGGAATGAAAGCACTGAGGAATGCTGTCCCAAGATGATCCACATGATGCTATCCC
ACAGTGTGACAACTTAAGCATCTTGTGATGAGCTCAGATTTAATCAACAAAACCTGACCAATTCGAAAACCTTGGAG
AGTATGGATCCAAATCCGCTCATGTTCTGTTACCCAGGAAGTACCCCAAGTGTCAACTCCAATCTCTGAGGAGTTTCAG
AAGCCTAGGAAAGTGGTACTAGGTTGTAAGAAAGCGGAATCAGAAAGATGCCCTGACTGAAAGTATCTGAGCTTGTG
CTGACCGGTTGCTGCTGATGAGAGACCACTGAGTGAAGAAAGTCAAGTGTGACAGAGTATTGTTGAAACAGC
GGCATGTTCTCTGCTGACACCTGATGAAACATATGATCCAAAGCCTTTGATGAGAAATTTGTTCACTTCCATGCAATAT
ATGACACTTCCCGATCTGAGAAACAAATCAAGAAACAACTGCACTTTGAGCTTTGAAACAGAGCCCAAGGCAACAGAG
GAAACACTGAAACCGTCAAGAAATTTTGGCTTTGTAGCAAGTGTGTCAGCTGATGACAAAGAGGCTGCTTGTCTG
TGGAGGTCAAAACCTTGTGTTCAACTCAACAGCCTTAGCCTAAGTCAAGATGAGTTCCAAGATGGTTTGTGACGAAGTTA
GTTGGTGTGTTTATGGAACCTTGTAAAGCTTGAATGTGGAAGAACTGTGGCTTTGTTTAAATGTTGGTGAATAAA
GATGTTCCCTTGGATTAAGTATGATTTTCTAATGGTTTCAAGTTTGGCACACAACTTTAAATATGCTGTTAGATGATA
TCTGCTGCTTTATTTAATTAECTTCACTTCAAGTTGTTGCAATGACTCTGTGATTTAAGATGAGTGA
AGTAGATTTGCTATATTTAAGGGTATTTGATGCTAATGGTAAACATGGTTTATGACAGGCTACTTTTGGTTATGGT
GTTGACGTTTCTTTAAACATTAATAGGCTCTTGTCTGTTGTTGTTGTTGAAACAAAGGCACTCACCTTGGATGCCG
TCTCCACTGATATTTGAACAAAGAAATCCGTACCGATCCTAGACTGCAGGATGCAAGCTT

BamHI
ACACATCTTTTGTCAATCTCTCATTCAAATCTCATTCTCTCTAGTAAACAGGATCCATGAGGGGTGTGTTTCGTCCAGATAC

PstI
TGGAGGTCAAAACCTTGTGTTCAACTCAACAGCCTTAGCCTAAGTCAAGATGAGTTCCAAGATGGTTTGTGACGAAGTTA

EcoRI KpnI BamHI XbaI PstI HindIII
TCTCCACTGATATTTGAACAAAGAAATCCGTACCGATCCTAGACTGCAGGATGCAAGCTT

Table 2. Results of NptII dot blot assay of extracts of leaves from putative transformed canola plants.

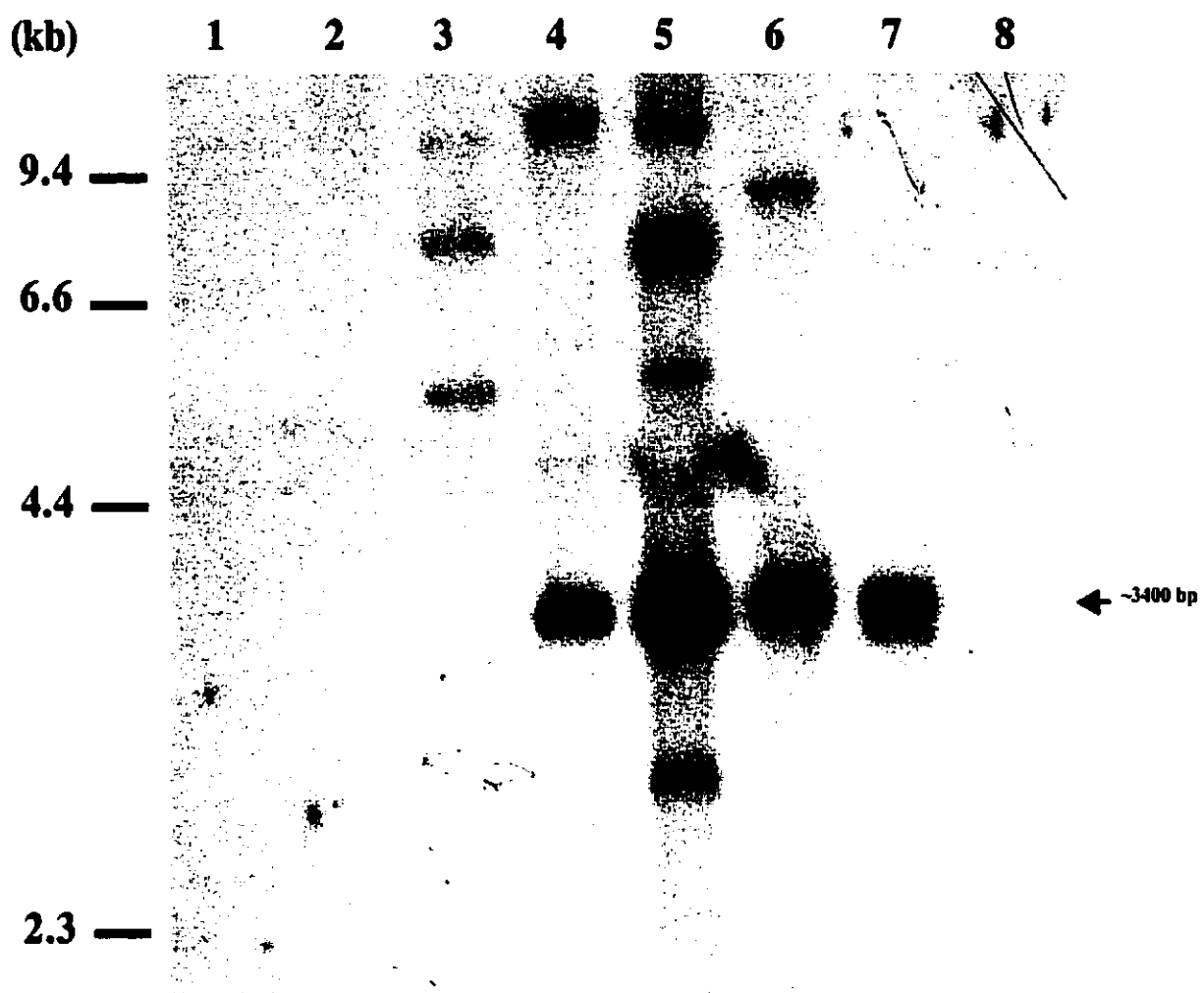
Plant	NptII Positive or Negative
BSA 1	Negative
BSA 2	Positive
BSA 3	Positive
BSA 4	Negative
BSA 5	Positive
BSA 6	Negative
BSA 7	Positive
BSA 8	Negative
BSA 9	Negative
BSA 10	Negative
BSA 11	Positive
BSA 12	Positive
BSA 13	Negative
BSA 14	Positive
BSA 15	Negative
BSA 16	Negative
BSA 17	Positive

restricted with KpnI overnight to excise the *BSA02* gene. The restricted genomic DNA was electrophoresed on a 1.0% (w/v) agarose gel and transferred to Hybond-N+ membrane (Amersham Canada Ltd., Oakville, ON). Following UV cross-linkage the blot was probed using the [³²P]-*BSA01* gene. The results of this Southern blot are shown in Figure 30. A DNA fragment of ~3400 bp was present in plants BSA 11, BSA 12, BSA 14 and BSA 17. The DNA fragment corresponded to the size of the *BSA02* gene. Therefore, the BSA cDNA was integrated into the genome of those plants. Previous studies with hirudin expression in *B. napus* have revealed that all NptII positive plants were also positive for the integration of hirudin cDNA into the genome (Parmenter *et al.*, 1995). Aside from BSA 2, because there were insufficient numbers of leaves to perform a genomic DNA extraction, 4 of the remaining 7 BSA plants that were NptII positive, also showed the BSA insert presence. However, a mock restriction or restriction with a different enzyme to clearly distinguish the plasmid from the integrated gene sequence should have been performed (Cho *et al.*, 1998; Bommineni *et al.*, 1997).

4.1.11. Production of BSA Transcripts

Putative BSA plants that also had sufficient numbers of developing seeds (25-30 days after flowering) (Weselake *et al.*, 1993), were further examined for their capacity to produce transcripts. Northern blotting failed to reveal the presence of BSA transcripts. RT-PCR was used in an attempt to amplify potentially less abundant transcripts. To ensure that the RT-PCR reaction would work on the RNA extractions from the plants, control reactions were performed by attempting to amplify a fragment of cDNA encoding

Figure 30. Southern blot analysis of *Kpn*I digests of 30 µg of genomic DNA isolated from transformed *B. napus* plants. All transformants were obtained by integration of pBW(Bin-BSA03). Blots were probed with the [³²P]-*BSA01* gene. The contents of each lane are as follows: 1, restricted genomic DNA from BSA 3; 2, restricted genomic DNA from BSA 5; 3, restricted genomic DNA from BSA 7; 4, restricted genomic DNA from BSA 11; 5, restricted genomic DNA from BSA 12; 6, restricted genomic DNA from BSA 14; 7, restricted genomic DNA from BSA 17; 8, restricted genomic DNA from a negative control plant.



DGAT (Nykiforuk *et al.*, 1999). The amplified products were electrophoresed on an agarose gel (Figure 31). A cDNA fragment of ~300 bp was present for BSA 5, BSA 7, BSA 11, BSA 17, 1559G and pDGAT3-3 (positive control). This DNA fragment corresponded to the internal DGAT fragment that was expected to amplify. Therefore, the RT-PCR reaction worked for the aforementioned samples. It is theorized that the RT-PCR reaction failed for BSA 12, BSA 14 and 1559H because the RNA had degraded before it was reverse transcribed. Regardless of results, the putative BSA plants were then tested for the presence of BSA transcript by performing RT-PCR on total RNA extractions using primers BSA736 and BSA747. The amplified products were electrophoresed on an agarose gel (Figure 32). A cDNA fragment of ~1800 bp was present for the BSA 11 plant and pBSA (positive control). This DNA fragment corresponded to the mature BSA cDNA. Therefore, the DNA sequence encoding BSA was transcribed in the BSA 11 plant to form RNA.

4.1.12. Production of BSA Protein

Protein extraction was attempted with four different extraction buffers (see Materials and Methods) and Western blotting was performed using antibodies directed against the BSA polypeptide, but no immunoreactive polypeptide was detected. This could be due to several factors. The antibody against BSA detected many different polypeptides in both control and transgenic seeds making it difficult to make an assessment regarding production of BSA polypeptide. This was similar to the effect seen for ASP protein extracts (Figure 17). It also could be that BSA was formed but is in such

Figure 31. Agarose gel electrophoresis of an internal DGAT cDNA fragment that was amplified from total RNA extractions of developing seeds of transformed *B. napus* using RT-PCR. Total RNA was reverse transcribed to obtain a single stranded cDNA. The cDNA was amplified using two primers designed to amplify an internal DGAT fragment. The PCR reaction conditions were 94°C for 1 minute (hot start) and 35 subsequent PCR cycles at 94°C for 1 minute, 52°C for 1 minute and 72°C for 1 minute. Following the 35 cycles, a final extension of 72°C for 10 minutes was performed. The amplified DNA was electrophoresed on a 0.8% (w/v) agarose gel. The contents of each lane are as follows: 1, DNA ladder (gene ruler); 2, 5µL of RT-PCR reaction from BSA 5; 3, 5 µL of RT-PCR reaction from BSA 7; 4, 5 µL of RT-PCR reaction from BSA 11; 5, 5 µL of RT-PCR reaction from BSA 12; 6, 5 µL of RT-PCR reaction from BSA 14; 7, 5 µL of RT-PCR reaction from BSA 17; 8, 5 µL of RT-PCR reaction from 1559 G (negative control plant); 9, 5 µL of RT-PCR reaction from 1559 H (negative control plant); 10, PCR mixture without template (negative control); 11-13, 5 µL of RT-PCR reaction from pDGAT3-3 (positive control); 14, DNA ladder (gene ruler).

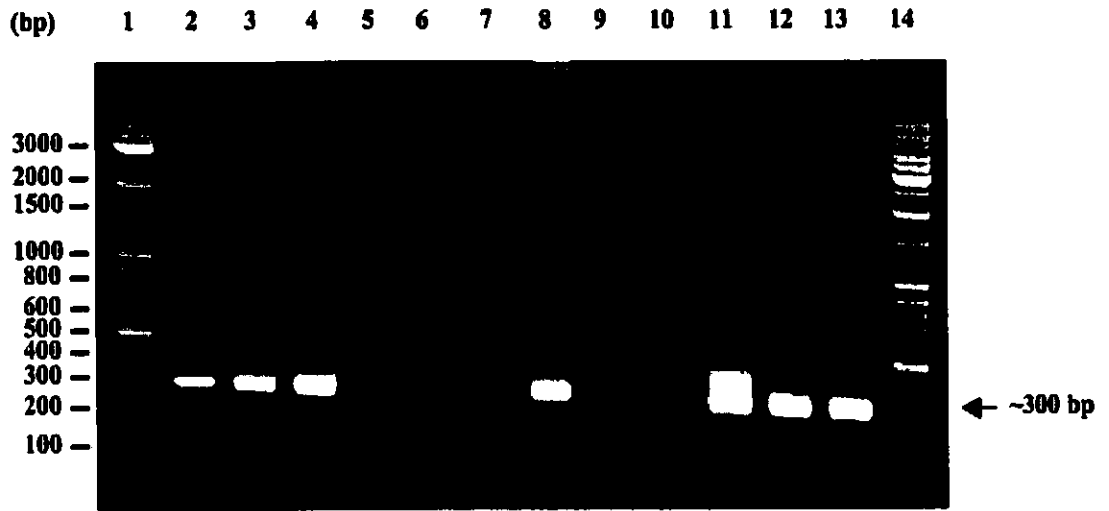
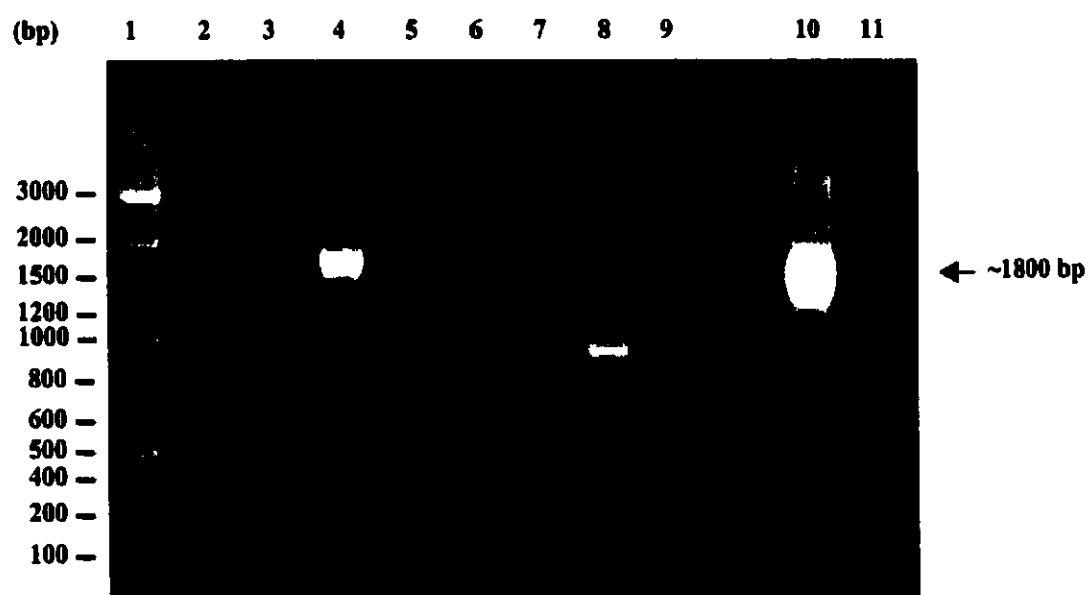


Figure 32. Agarose gel electrophoresis of BSA cDNA that was amplified from total RNA extractions of developing seeds of transformed *B. napus* using RT-PCR. Total RNA was reverse transcribed to obtain a single stranded cDNA. The cDNA was amplified using the BSA736 and BSA747 primers (Figure 17). The PCR reaction conditions were 94°C for 1 minute (hot start) and 35 subsequent PCR cycles at 94°C for 1 minute, 55°C for 1 minute and 72°C for 1 minute. Following the 35 cycles, a final extension of 72°C for 10 minutes was performed. The amplified DNA was electrophoresed on a 0.8% (w/v) agarose gel. The contents of each lane are as follows: 1, DNA ladder (gene ruler); 2, 5 µL of RT-PCR reaction from BSA 5; 3, 5 µL of RT-PCR reaction from BSA 7; 4, 5 µL of RT-PCR reaction from BSA 11; 5, 5 µL of RT-PCR reaction from BSA 12; 6, 5 µL of RT-PCR reaction from BSA 14; 7, 5 µL of RT-PCR reaction from BSA 17; 8, 5 µL of RT-PCR reaction from 1559 G (negative control plant); 9, 5 µL of RT-PCR reaction from 1559 H (negative control plant); 10, 5 µL of RT-PCR reaction from pBSA (positive control); 11, PCR mixture without template (negative control).



low levels that it could not be detected through conventional Western blotting techniques. As well, BSA may have been formed and degraded shortly after expression, or it simply may not have been translated. Another possibility is that post-transcriptional gene silencing is occurring where mRNA is being degraded shortly after formation (Vaucheret and Fagard, 2001). Other research groups have had problems with low protein levels that they attributed to post-transcriptional and/or translational regulation (Ishimaru *et al.*, 1997), post-transcriptional gene silencing directed by homologous transgenes (Han *et al.*, 1999; Valkama *et al.*, 2000). In addition, we may be facing a stability problem with the protein. Boothe *et al.* (1997) found that protein degradation and instability of proteins decreased when they were expressed as oleosin fusions rather than cytoplasmic expression. Oleosin fusions have been made with the BSA cDNA and these constructs are described in section 4.3.2.

4.2. Characteristics of Mature Seeds From Transgenic Plants

Mature seeds from all transgenic plants were harvested. This included negative control plants transformed with the binary vector pCGN1559, ASP plants that were transformed with pBW(Bin-ASP03) and BSA plants transformed with pBW(Bin-BSA03). These mature seeds were analyzed for total lipid (gravimetrically and low-resolution (LR-NMR), seed weight, percent lipid on a per seed basis and FA composition of total acyl lipids. LR-NMR uses magnetic field and radiofrequency pulses to determine the amount of oil in a sample of seeds. This technique has several advantages over conventional methods. For example, samples remain unchanged so the seeds can be used

for other analysis. This is especially important for transgenic seeds because typically in the first generation there are minimal numbers of seeds produced and sacrificing any of these seeds is undesirable. Also the analysis time is under one minute whereas conventional techniques take up to 5 hours per sample. In addition, there are no solvents or hazardous waste using LR-NMR.

4.2.1. Total Lipid for Negative Control Plants

A number of studies have been performed in an attempt to increase seed oil content in plants. Rousler *et al.* (1997) and Zou *et al.* (1997) performed two of the more notable studies. In the first case, Rousler *et al.* (1997) engineered transgenic *B. napus* using a napin promoter to drive the expression of cytosolic ACCase and attached a chloroplast transit peptide to direct the enzyme to the plastid. In doing this, they achieved a 5% increase in seed oil content. In the second case, Zou *et al.* (1997) engineered transgenic *A. thaliana* using a 35S promoter to drive the expression of a LPAAT from yeast that was capable of accepting a range of acyl-CoAs. In doing this, they achieved increases of 8 to 48% in seed oil content and increases in both overall proportions and amounts of very-long-chain fatty acids in seed TAGs (Zou *et al.*, 1997). Although our approach for affecting total seed lipid was different than the introduction of enzymes discussed above, there was a noticeable decrease in total lipid from the mature seeds of BSA 11 as described below. Total lipid was measured for negative control plants gravimetrically and using LR-NMR (Table 3). This data was used to determine a baseline lipid content of mature seeds taken from *B. napus* that were subjected to

Table 3. Total lipid expressed as percent in mature seeds of transgenic negative control plants analyzed gravimetrically and with low-resolution NMR.

Gravimetric % Lipid
36.3
37.1
35.0
34.8
28.6
33.5
34.2
3.0

transformation using *A. tumefaciens*. This set acted as a negative control because the other transgenic plants were transformed with the same binary vector with the only difference being that the DNA sequence for either BSA or ASP was also inserted with the 1.1 kb *Arabidopsis* oleosin promoter and ubiquitin terminator. The average \pm one standard deviation was $34.2 \pm 3.0\%$ for gravimetric analysis and $37.0 \pm 3.4\%$ for NMR analysis for the negative control seeds.

4.2.2. Total Lipid for ASP Plants

Total lipid for ASP plants was measured the same way as the negative control plants discussed above. This data is shown in Table 4. Knowing the mean and standard deviation of the negative control plants allowed for the calculation of a probability (*P*)-value for each ASP plant with respect to whether or not it was significantly different in the total lipid of mature seeds for either gravimetric or NMR analysis (Sokal and Rohlf, 1995). None of the transgenic plants were statistically different in total lipid component compared to negative control plants.

4.2.3. Total Lipid for BSA Plants

Total lipid for BSA plants was also measured the same way as the negative control plants discussed above. This data is shown in Table 5. Knowing the mean and standard deviation of the negative control plants allowed for the calculation of a *P*-value for each BSA plant with respect to whether or not it was significantly different in the total lipid of mature seeds for either gravimetric or NMR analysis (Sokal and Rohlf, 1995).

Table 4. Total lipid expressed as percent in mature seeds of transgenic ASP plants analyzed gravimetrically and with low-resolution NMR. The *P*-value was determined as compared to the negative control plants.

Grav. % Lipid	NMR % Lipid
33.7	35.9
35.0	41.0
37.7	39.2
35.3	37.0
35.3	35.6
35.2	34.5
36.6	37.8
34.5	37.5
35.0	36.8
34.7	38.0
35.7	37.0
32.3	34.5

Table 5. Total lipid expressed as percent in mature seeds of transgenic BSA plants analyzed gravimetrically and with low-resolution NMR. The *P*-value was determined as compared to the negative control plants.

	Grav. % Lipid		NMR % Lipid	
	32.9		34.0	
	32.9		35.7	
	36.3		40.9	
	32.3		35.8	
	28.1		30.3	
	33.4		37.0	
	31.1		30.5	

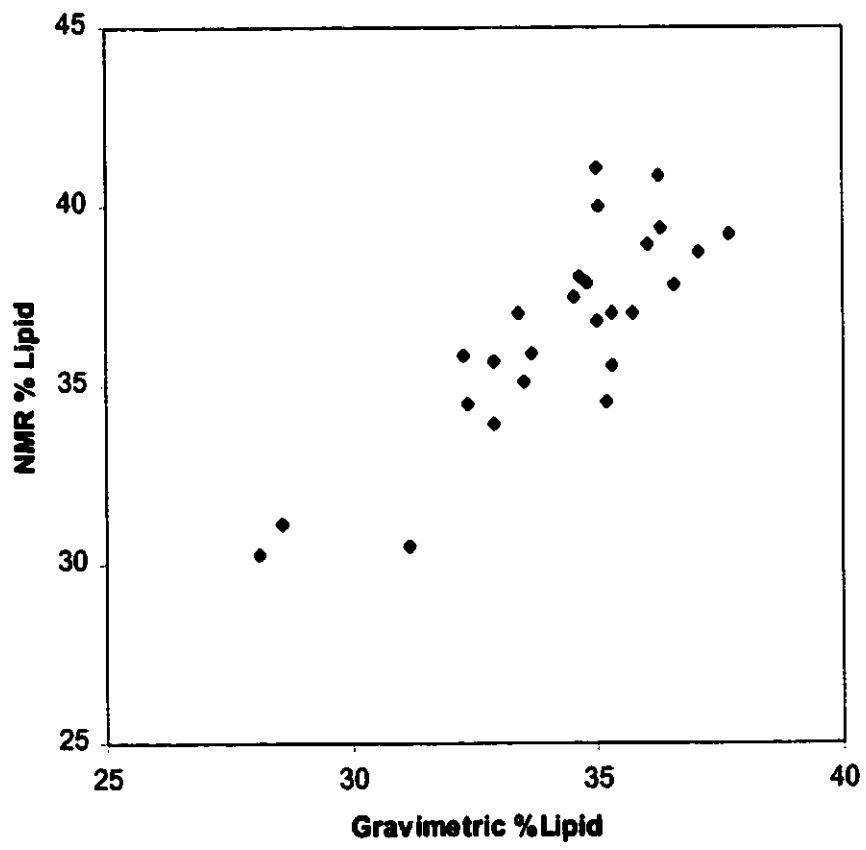
None of the transgenic plants were statistically different in total lipid component compared to negative control plants. Having said that, BSA 11 did have noticeably lower total lipid than the negative control plants, even though the numbers were not statistically significant. When measured gravimetrically, the mature seeds had 28.1% lipid and using LR-NMR the total lipid was 30.3%. These numbers are noticeably lower than the negative control seeds that had total lipid of $34.2 \pm 3.0\%$ gravimetrically and $37.0 \pm 3.4\%$ using LR-NMR. This may mean that BSA is inhibiting some aspect of lipid biosynthesis.

4.2.4. Correlation Between Gravimetric and NMR Analysis for Total Lipid From Mature Seeds

NMR was used before gravimetric lipid analysis because NMR uses high magnetic fields to give a non-destructive measure of the total lipid within the seeds (Hutton *et al.*, 1999). More than one method for measuring seed oil levels was desirable so that potential agreement could be obtained between independent methods. In addition, studies using NMR on plant seeds have found that this technique agrees with results obtained gravimetrically and with gas chromatography (Gunstone and Wolff, 1996; Hutton *et al.*, 1999). There were differences in the total lipid for mature seeds when comparing the results obtained using gravimetric methods versus NMR analysis. To test if there was a correlation between the methods, however, a graph was plotted comparing the results of the two methods for all plants analyzed (Figure 33). Although the results differ between the two methods of total lipid analysis, there was a correlation between them that was highly significant ($r^2 = 0.70$, $P < 0.0001$).

Figure 33. Relationship between lipid percentage obtained gravimetrically and lipid percentage obtained using LR-NMR from mature seeds of all transgenic plants.

There was a highly significant correlation between these two methods of analysis ($r^2 = 0.70, P < 0.0001$).



4.2.5. Seed Weight and Amount of Lipid Per Seed for Negative Control Plants

Many research groups report lipid data on a percent weight basis (Zou *et al.*, 1997; Löhden and Frentzen, 1992) or percent molar basis (Griffiths *et al.*, 1988b; Frentzen *et al.*, 1983). We felt that it was important to explore the data on a per seed basis as well.

Seeds were weighed for all negative control plants prior to performing a hexane-isopropanol lipid extraction. The gravimetric amount of lipid in these seeds was also used to determine the amount of lipid per seed (Table 6). The experiments were used to determine a baseline measure for this type of seed data with respect to mature seeds analyzed from *B. napus* that was subjected to transformation using *A. tumefaciens*. This set acted as a negative control because the other transgenic plants were transformed with the same binary vector with the only difference being that the DNA sequence for either BSA or ASP was also inserted with the 1.1 kb *Arabidopsis* oleosin promoter and ubiquitin terminator. The seed weight was 4.1 ± 0.5 mg/seed and the amount of lipid per seed was 1.4 ± 0.2 mg/seed for mature seeds from negative control plants. Due to limited availability of seeds, water content was not measured for seeds from any plants. In the next generation, however, this would be a useful variable to analyze for an indication of the proportion of the total seed weight that is due to water content.

4.2.6. Seed Weight and Amount of Lipid Per Seed for ASP Plants

Seed weight and amount of lipid per seed for mature seeds from ASP plants were measured the same way as the seeds from negative control plants discussed above. This

Table 6. Seed weight and amount of oil per seed measured gravimetrically for mature seeds of negative control plants.

Seed Weight (mg/seed)
3.3
3.9
4.3
4.6
4.3
4.1
4.1
0.5

data is shown in Table 7. Knowing the mean and standard deviation of the negative control plants allowed for the calculation of a *P*-value for each ASP plant with respect to whether or not it was significantly different in the seed weight or amount of oil per seed (Sokal and Rohlf, 1995). Although the seed weight from all mature seeds of ASP plants was not significantly different from the negative control plants, the amount of oil per seed was significantly higher for ASP 8 ($P = 0.04$) and ASP 10 ($P = 0.03$). The mature seeds from ASP 8 had 1.8 mg oil/seed and from ASP 10 had 1.8 mg oil/seed. This means that although the seeds were statistically the same weight as negative control plants, the mature seeds from ASP 8 and ASP 10 were producing more oil in each seed. This may be a measure of the efficiency of the lipid-forming enzymes within these seeds where fewer seeds are required to make the same amount of oil as negative control plants. Unfortunately, it was not possible to test these plants for transcript presence or absence because of the shortage of developing seeds. Therefore, these results may be due to the altered effects on the genome since insertion of the T-DNA using *A. tumefaciens* with a Ti plasmid is random. Even though seed weight was not statistically different as compared to the mature seeds from negative control plants, the seeds from ASP 8 and ASP 10 were noticeably higher (4.9 mg/seed and 5.3 mg/seed, respectively). Therefore, the significant differences in amount of oil per seed may be a function of the seeds being larger. Next generation plants will enable further analysis of these preliminary results.

Table 7. Seed weight and amount of oil per seed measured gravimetrically for the mature seeds of transgenic ASP plants. The *P*-value was determined as compared to the negative control plants (4.1 ± 0.5 mg/seed for seed weight and 1.4 ± 0.2 mg/seed for amount of oil per seed). The seed weight from all mature seeds of ASP plants was not significantly different from the negative control plants.

Seed Weight (mg/seed)	Oil Per Seed (mg/seed)
4.4	1.5
4.2	1.5
3.8	1.5
3.8	1.3
4.1	1.5
4.0	1.4
4.9	1.8
5.3	1.8
4.5	1.6
4.5	1.5
3.7	1.3
4.4	1.3

4.2.7. Seed Weight and Amount of Lipid Per Seed for BSA Plants

Seed weight and amount of lipid per seed for mature seeds from BSA plants were measured the same way as the mature seeds from negative control plants discussed above. This data is shown in Table 8. Knowing the mean and standard deviation of the negative control plants allowed for the calculation of a *P*-value for each BSA plant with respect to whether or not it was significantly different in the seed weight or amount of oil per seed (Sokal and Rohlf, 1995). The seed weights as well as the amount of oil per seed from the mature seeds of all transgenic BSA plants were not significantly different from the negative control plants. Although not statistically different, BSA 3 and BSA 17 did show noticeably different seed characteristics. BSA 3 had an average seed weight of 3.1 mg and had 1.0 mg of oil/seed. These measurements are lower than those obtained from negative control seeds. In addition, mature seeds from BSA 17 showed the opposite effect where the average seed weight was 5.3 mg and had 1.7 mg of oil per seed (both higher than seeds from negative control plants). These results may be an effect of random gene insertion, or could be the result of BSA acting on developmental enzymes within the plant. A second generation of plants is required to see if the same effect is evident or if it was a random event in the first generation.

4.2.8. Fatty Acid Composition of the Total Acyl Lipids From Mature Seeds From Negative Control Plants

Much experimentation has been performed to change the FA profile of lipids within the seeds of plants. For example, the *Umbellularia californica* form of the

Table 8. Seed weight and amount of oil per seed measured gravimetrically for the mature seeds of transgenic BSA plants. The *P*-value was determined as compared to the negative control plants (4.1 ± 0.5 mg/seed for seed weight and 1.4 ± 0.2 mg/seed for amount of oil per seed).

Seed Weight (mg/seed)	Oil Per Seed (mg/seed)
4.7	1.6
3.1	1.0
3.8	1.4
4.8	1.5
4.3	1.2
4.6	1.5
5.3	1.7

enzyme acyl-ACP hydrolase is specific for medium chain ACP-thioesters (Pollard *et al.*, 1991). The cDNA encoding this enzyme was expressed in the plastids of *A. thaliana* and resulted in the accumulation of medium chain FAs in the transgenic seeds (Voelker *et al.*, 1992). A canola line was subsequently developed that expressed this acyl-ACP hydrolase, and the seeds of this line contained up to 50% of the seed FA as C12:0 (Voelker *et al.*, 1996). Native canola LPAAT is incapable of utilizing erucoyl-CoA as a substrate (Bernerth and Frentzen, 1990). To overcome this obstacle, the cDNA encoding LPAAT from developing meadowfoam seeds was incorporated into oilseed rape under the expression of a napin promoter (Brough *et al.*, 1996). The reason for this was LPAAT from meadowfoam seeds has been shown to incorporate C22:1 on the *sn*-2 position of LPA (Laurent and Huang, 1992). This resulted in increased levels of TAGs having C22:1 at the *sn*-2 position (Brough *et al.*, 1996).

Desaturation experiments have also been performed. A rat liver Δ -9 desaturase was successfully transformed into soybeans (Liu *et al.*, 1996). This resulted in the levels of C16:0 in transformed embryos dropping from 25% to approximately 5% of total FAs. When a yeast Δ -9 desaturase, under the control of a CaMV 35S promoter, was expressed in *Petunia hybrida* there were significantly increased levels of palmitoleic acid (Choudhary *et al.*, 1994). A similar effect was observed when the yeast Δ -9 desaturase was expressed in tobacco with a CaMV 35S promoter. A 10-, 11- and 6-fold increase was found for C16:1 in the leaf, stem and root tissues, respectively. The seeds showed a 2-3 fold increase in the levels of C16:1. Yadav *et al.* (1993) expressed an *Arabidopsis* microsomal Δ -12 desaturase gene in transgenic plant tissues such as carrot hairy roots

and the level of C18:3 was significantly increased. They found that when this desaturase gene was expressed with a seed specific promoter, the level of C18:3 was increased 12-fold in second generation transgenic *Arabidopsis* plants. When the rapeseed version of this gene was expressed in *Arabidopsis*, the roots showed a 1.6-fold increase in C18:3 (Arondel *et al.*, 1992). Although we used a different approach to affect lipid metabolism, a difference in FA profiles was also evident for ASP and BSA plant lines as described below.

Following hexane-isopropanol extraction of lipids from mature seeds, methyl esters were generated and the samples were analyzed using a GLC to obtain the FA composition of the total acyl lipids within those seeds. These values were converted to a percentage of the total acyl lipids (Table 9). The experiments were used to determine a baseline measure for this lipid component data with respect to mature seeds analyzed from *B. napus* that was subjected to transformation using *A. tumefaciens*. This set acted as a negative control because the other transgenic plants were transformed with the same binary vector with the only difference being that the DNA sequence for either BSA or ASP was also inserted with the 1.1 kb *Arabidopsis* oleosin promoter and ubiquitin terminator. The average and standard deviation for each FA in the mature seeds of these negative control plants are shown in Table 9. The total of all FA percentages do not add up to 100% because there were a number of other FAs in the samples, in very low proportions, that collectively added up to ~1-2%.

Table 9. Fatty acid composition of total acyl lipids expressed as percent in mature seeds of transgenic negative control plants. Hexane-isopropanol extraction of lipids were performed on mature seeds, methyl esters were generated and the samples were run on a GLC to obtain a profile of all the components contributing to the total lipid within those seeds. These values were converted to a percentage of the total. The average and standard deviations of all fatty acids are shown below. TTL = Total.

SAMPLE	16:0	16:1	18:0	18:1	18:2	18:3	20:0	20:1	22:0	22:1	22:2	24:0	24:1	TTL
1559 B	4.2	0.2	3.0	67.7	15.5	4.5	0.9	1.2	0.2	0.5	0.3	0.3	0.3	98.8
1559 C	4.2	0.2	3.2	66.4	15.9	5.3	0.9	1.2	0.2	0.5	0.3	0.3	0.3	98.8
1559 D	4.1	0.2	2.9	67.9	15.3	5.0	0.9	1.3	0.1	0.5	0.1	0.1	0.3	98.7
1559 E	4.3	0.2	2.7	64.3	17.5	6.2	0.8	1.3	0.2	0.5	0.3	0.3	0.3	98.7
1559 H	5.2	0.5	5.3	61.7	15.8	5.7	1.4	1.0	0.2	0.7	0.2	0.2	0.5	98.4
1559 J	4.5	0.3	3.3	66.0	15.0	5.3	1.1	1.3	0.2	0.7	0.2	0.2	0.5	98.7

4.2.9. Fatty Acid Composition of the Total Acyl Lipids From Mature Seeds From ASP Plants

The FA composition of the total acyl lipids within the mature seeds of transgenic ASP plants were analyzed the same way as the negative control plants discussed above. This data is shown in Table 10. Knowing the mean and standard deviation of the negative control plants allowed for the calculation of a *P*-value for each ASP plant with respect to whether or not it was significantly different in that particular FA (Sokal and Rohlf, 1995). The level of C18:2 was 13.3% in mature seeds of ASP 3, which was significantly lower than the levels obtained with the seeds of negative control plants ($P = 0.05$). Similarly, the level of C18:3 was 3.6% in mature seeds of ASP 14, which was significantly lower than the levels obtained with the seeds of negative control plants ($P = 0.04$). Although Northern blotting could not be performed on ASP 3 due to the lack of developing seeds, Northern blotting was performed on developing seeds of ASP 14 and was positive for expression of transcript (Figure 16). These preliminary results suggested that ASP had the effect of lowering levels of one unsaturated FA of 18-carbon chains from mature seeds on a percent basis. On the other hand, these results may be due to altered effects on the genome because a gene encoding a lipid biosynthetic enzyme could have been disrupted. For example, the DNA sequence encoding Δ -9 and Δ -12 desaturase enzymes could have been disrupted resulting in lower levels of unsaturated FAs. Regardless, the decrease in C18:2 in mature seeds from ASP 3 appeared to come at the expense of increasing C18:1. Even though the percentage of C18:1 (70.5%) is not statistically significant, it is noticeably larger than the level for negative control plants

Table 10. Fatty acid composition of total acyl lipids expressed as percent in mature seeds of transgenic ASP plants. Hexane-isopropanol extraction of lipids were performed on mature seeds, methyl esters were generated and the samples were run on a GLC to obtain a profile of all the components contributing to the total lipid within those seeds. These values were converted to a percentage of the total. TTL = Total.

SAMPLE	16:0	16:1	18:0	18:1	18:2	18:3	20:0	20:1	22:0	22:1	22:2	24:0	24:1	TTL
ASP 1	4.2	0.2	3.9	66.6	15.3	5.0	1.1	1.1	0.1	0.5	0.1	0.1	0.4	98.6
ASP 2	4.2	0.2	3.2	67.2	15.8	4.9	1.0	1.3	0.1	0.5	0.1	0.1	0.3	98.7
ASP 3	3.8	0.2	3.7	70.5	13.3 ^A	4.1	1.0	1.2	0.1	0.5	0.1	0.1	0.3	98.8
ASP 4	4.3	0.3	4.0	67.0	15.5	4.1	1.1	1.2	0.1	0.5	0.1	0.1	0.4	98.7
ASP 5	4.2	0.2	3.4	68.2	14.4	4.9	1.0	1.2	0.1	0.5	0.1	0.1	0.4	98.7
ASP 6	4.1	0.2	3.0	66.5	16.6	4.9	1.0	1.3	0.1	0.5	0.1	0.1	0.3	98.7
ASP 8	4.0	0.2	2.7	66.4	15.6	6.6	0.9	1.4	0.1	0.5	0.1	0.1	0.3	98.7
ASP 10	3.9	0.2	3.1	66.6	15.3	6.1	0.9	1.3	0.1	0.5	0.1	0.1	0.3	98.6
ASP 11	4.1	0.2	3.1	65.5	16.2	6.0	0.9	1.3	0.1	0.5	0.1	0.1	0.3	98.3
ASP 13	4.1	0.2	3.5	68.7	14.7	4.2	1.0	1.2	0.1	0.5	0.1	0.1	0.3	98.6
ASP 14	4.2	0.3	4.0	68.6	14.3	3.6 ^B	1.1	1.2	0.1	0.6	0.1	0.1	0.3	98.5
ASP 15	4.3	0.2	3.7	67.3	15.0	4.5	1.1	1.2	0.1	0.6	0.1	0.1	0.4	98.4

^AP = 0.05

^BP = 0.04

(65.7 ± 2.3%). A second generation of plants is required to see if these results are carried through or if they are random effects and to further assess the biological significance of these results. In addition, leaf tissue must be analyzed in the next generation to determine if the ASP cDNA had integrated into the genome and developing seeds must be obtained in the next generation to determine whether transcript is being expressed. These tests were not performed on the first generation due to limited availability of tissue.

4.2.10. Fatty Acid Composition of the Total Acyl Lipids From Mature Seeds from BSA Plants

The FA composition of the total acyl lipids within the mature seeds of transgenic BSA plants were analyzed the same way as the negative control plants discussed above. This data is shown in Table 11. Knowing the mean and standard deviation of the negative control plants allowed for the calculation of a *P*-value for each BSA plant with respect to whether or not it was significantly different in that particular FA (Sokal and Rohlf, 1995). The level of C18:2 was 18.6% in mature seeds of BSA 11, which was significantly higher than the levels obtained with the seeds of negative control plants (*P* = 0.04). Similarly, the level of C18:3 was 7.6% in the mature seeds of BSA 11, which was significantly higher than the levels obtained with the seeds of negative control plants (*P* = 0.02). BSA 11 was the only plant in which transcript was detected in developing seeds (Figure 32). Since it is also the only plant that showed a significant difference in FA composition compared to negative control plants it appears that BSA had the effect of increasing levels of unsaturated FAs of 18-carbon strains from mature seeds on a percent

Table 11. Fatty acid composition of total acyl lipids expressed as percent in mature seeds of transgenic BSA plants. Hexane-isopropanol extraction of lipids were performed on mature seeds, methyl esters were generated and the samples were run on a GLC to obtain a profile of all the components contributing to the total lipid within those seeds. These values were converted to a percentage of the total. TTL = Total.

SAMPLE	16:0	16:1	18:0	18:1	18:2	18:3	20:0	20:1	22:0	22:1	22:2	24:0	24:1	TTL
BSA 2	4.0	0.2	3.1	65.5	16.4	5.9	1.0	1.3	0.1	0.6	0.1	0.1	0.3	98.5
BSA 3	3.9	0.2	2.9	64.0	17.9	6.5	0.9	1.4	0.0	0.5	0.1	0.1	0.3	98.6
BSA 5	3.9	0.2	2.8	68.6	14.2	5.7	0.9	1.3	0.1	0.5	0.1	0.1	0.3	98.6
BSA 7	4.1	0.2	3.2	67.4	14.4	5.5	1.0	1.4	0.1	0.6	0.1	0.1	0.4	98.3
BSA 11	4.7	0.3	4.0	59.4	16.6 ^A	7.6 ^B	1.1	1.1	0.1	0.6	0.1	0.1	0.5	98.1
BSA 14	4.0	0.2	2.9	66.3	16.2	5.8	0.9	1.3	0.1	0.5	0.1	0.1	0.3	98.5
BSA 17	4.1	0.2	3.7	64.3	17.3	5.5	1.1	1.2	0.0	0.6	0.1	0.1	0.4	98.5

^AP = 0.04

^BP = 0.02

basis. These effects may be due to altered effects on the genome resulting in the upregulation of Δ -9 and Δ -12 desaturases. This could account for the increase in the levels of C18:2 and C18:3 FAs. The alteration of FA composition in this BSA plant may also have involved modulation of acyltransferase specificity. Bafor *et al.* (1990) noticed a shift in the *in vitro* selectivity of LPAAT using microsomal preparations of developing safflower when BSA was in the reaction mixture. Concentrations of BSA greater than 0.25 mg/mL caused LPAAT to have a preference for C18:2-CoAs over more saturated FAs (Ichihara, 1984). This effect was presumably because BSA was binding the acyl acceptor, LPA. In the current study, mature seeds of BSA 11 has C18:2 proportions that were significantly increased over control plants. Although it is doubtful that the concentration of BSA was greater than 0.25 mg/mL during seed development, the *in vivo* concentrations to notice this effect have not yet been studied. Therefore, the *in vivo* concentrations to alter selectivity of *B. napus* LPAAT may be different than the *in vitro* concentrations noted by Ichihara (1984) in the *in vitro* concentrations using preparations of developing safflower. Regardless, the apparent increase in C18:2 and C18:3 levels in mature seeds of BSA 11 seemed to come at the expense of decreasing levels of C18:1. Although the level of C18:1 (59.4%) is not statistically different than the level in mature seeds from negative control plants ($65.7 \pm 2.3\%$), it is noticeably lower. Another generation of plants is required to further analyze the results.

4.3. Towards Expression of ASP and BSA in the Endoplasmic Reticulum (ER)

In developing seed tissues, FAs are formed in the plastids and are exported to the ER for assembly into TAGs (Murphy *et al.*, 1993). These TAGs are stored in oil bodies that bud from the ER. Oleosins are embedded within the oil bodies and are thought to stabilize them and prevent coalescence with other oil bodies (Murphy, 1990; Murphy and Cummins, 1989). In addition, expression of oleosins is precisely coordinated with oil accumulation in developing seeds of oilseed rape (Tzen *et al.*, 1993; van Rooijen *et al.*, 1992). To test the stimulatory effect of ASP and BSA at the site of TAG formation, targeting these proteins was desirable. To achieve this localization, the obvious candidate for a fusion was an oleosin since this protein is present at the site of oil body formation and is expressed at the time that oil is accumulating within developing seeds. The progress made creating the genetic constructs to facilitate the fusion of these mammalian proteins with oleosins is described below.

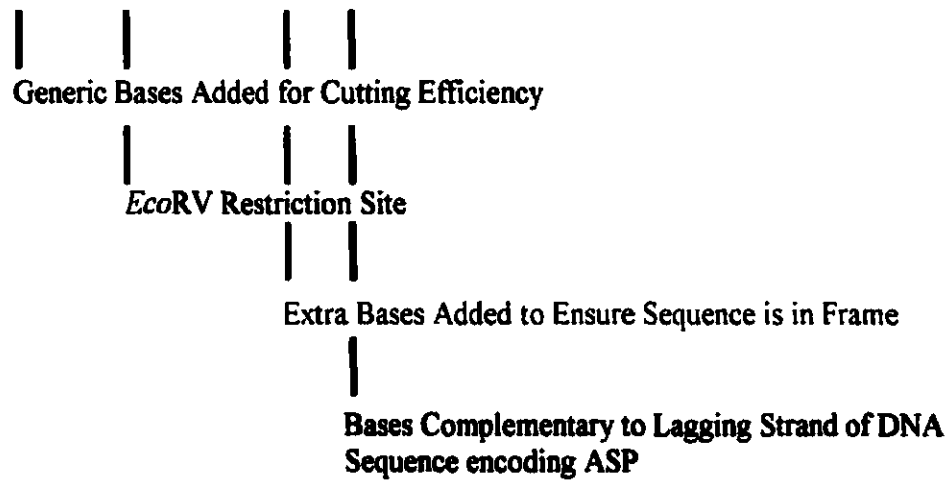
4.3.1. Genetic Constructs to Facilitate Expression of ASP in the ER

PCR was performed using *Pfu* polymerase on the original vector carrying the ASP cDNA. The primers ASP871 and ASP872 (Figure 34) were selected so that the DNA sequence encoding ASP would be amplified. *EcoRV* restriction sites were engineered on the 5' and 3' ends of the ASP DNA sequence, respectively, for compatibility with the multiple cloning site of pSBS2050. Two extra Gs were added following the *EcoRV* restriction site on the 5' end to ensure that the ASP DNA sequence would be in frame with the DNA encoding the oleosin protein. The restriction enzyme *SwaI* was selected to

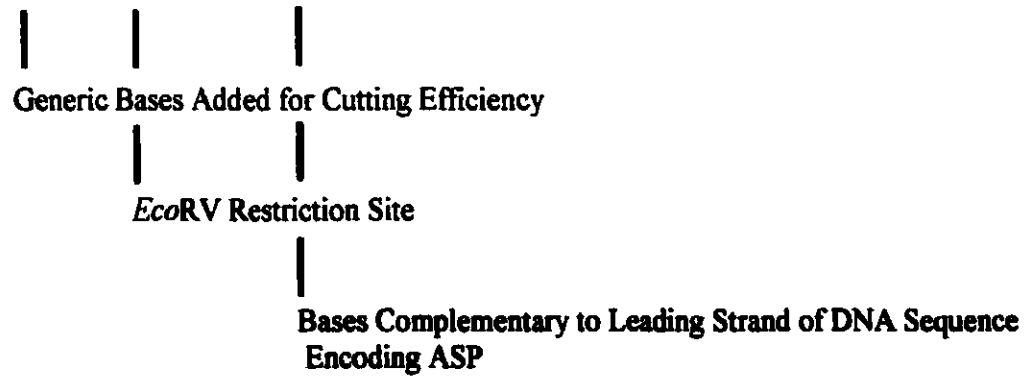
Figure 34. Primers designed to amplify the DNA sequence encoding ASP for insertion into pSBS2050. An *EcoRV* restriction site was incorporated into the ASP871 and ASP872 primers so that these sites would be present at the 5' and 3' ends of the sequence for compatibility with the blunt ended *SwaI* site of pSBS2050. Two extra Gs were added after the restriction site on the 5' end of the fragment to ensure that the DNA sequence encoding ASP would be in frame with the DNA sequence encoding oleosin protein in pSBS2050.

Forward Primer: ASP871

5' - GCGCGATATCGGATGTCCGTTTCAGCTGACTGAA - 3'

**Reverse Primer: ASP872**

5' - GCGCGATATCTCATTAAGCCAGACCCAGGTG - 3'



disrupt the stop codon of the DNA encoding the oleosin protein so that the DNA encoding ASP could also be translated as a fusion product (Figure 35). The amplified product was analyzed by agarose gel electrophoresis (Figure 36). A DNA fragment of ~250 bp was amplified, corresponding to the size of the DNA sequence encoding ASP.

The blunt ended PCR fragment was ligated into a pUC19 plasmid that was previously linearized with *Sma*I. Using agar plates containing 5-bromo-4-chloro-3-indolyl- β -D-galactoside (X-gal), putative clones were identified using α -complementation. A patch plate was constructed from the white colonies and colony PCR was performed. The amplified products were analyzed by agarose gel electrophoresis (Figure 37). A DNA fragment of ~250 bp was expected, corresponding to the size of the DNA sequence encoding ASP. The colony PCR revealed that all three colonies were carrying plasmid DNA that produced a DNA fragment size of ~250 bp following PCR with primers ASP871 and ASP872. To ensure that the engineered *Eco*RV sites were intact, restriction analysis with *Eco*RV was performed on the plasmid DNA from all three putative clones (Figure 38). The fragment of ~250 bp was produced when restricted with *Eco*RV confirming that the *Eco*RV sites were intact. The plasmid DNA from colony 1 was sent for sequencing. The sequence for this plasmid was identical when compared to the known sequence of DNA encoding ASP. The engineered restriction sites and two additional Gs on the 5' end of the DNA sequence were also included in the sequence (Figure 39). The pUC19 plasmid carrying the DNA sequence encoding ASP with engineered fragments was designated pBW(ASP04) (Figure 40). The

Figure 35. Integration of DNA sequence encoding ASP into pSBS2050 by disrupting the stop codon in the DNA sequence encoding oleosin protein. Originally there was a stop codon at the end of the DNA sequence encoding oleosin protein, but after restricting with *Swa*I, the stop codon was disrupted and the DNA sequence encoding ASP was integrated into the plasmid. The remaining base pairs of the engineered *Eco*RV site following restriction are shown in blue and the additional two Gs that were engineered to keep the DNA sequences in frame are shown in green.

DNA Sequence Encoding Oleosin Protein

5' ...CAC ACT ACT ATT TAA AT 3'
 3' ...GTG TGA TGA TAA ATT TA 5'

↑
 Stop Codon

DNA Sequence Encoding Oleosin Protein After Restriction with *Swa*I

5' ...CAC ACT ACT ATT T AA 3'
 3' ...GTG TGA TGA TAA A TT 5'

↑
 Stop Codon Disrupted

Insertion of Blunt Ended DNA Sequence Encoding ASP with Two Extra "G's" into the Blunt Ended DNA Sequence Encoding Oleosin Protein

5' ...CAC ACT ACT ATT TAT CGG ATG TCC GTT...3'
 3' ...GTG TGA TGA TAA ATA GCC TAC AGG CAA...5'

DNA Sequence Encoding
 Oleosin Protein without a
 Stop Codon

DNA Sequence
 Encoding ASP

Figure 36. Agarose gel electrophoresis analysis of PCR amplification of pASP using *Pfu* polymerase and primers ASP871 and ASP872. The reaction conditions were 94°C for 1 minute (hot start) and 35 subsequent PCR cycles at 94°C for 45 seconds, 55°C for 1 minute and 72°C for 1 minute. Following the 35 cycles, a final extension of 72°C for 5 minutes was performed. The amplified DNA was run on a 0.8% (w/v) agarose gel. Lane 1 contains a λ DNA/*EcoRI*+*HindIII* Ladder (MBI Fermentas, Flamborough, ON) and lane 2 contains 5 μ L of the PCR reaction.

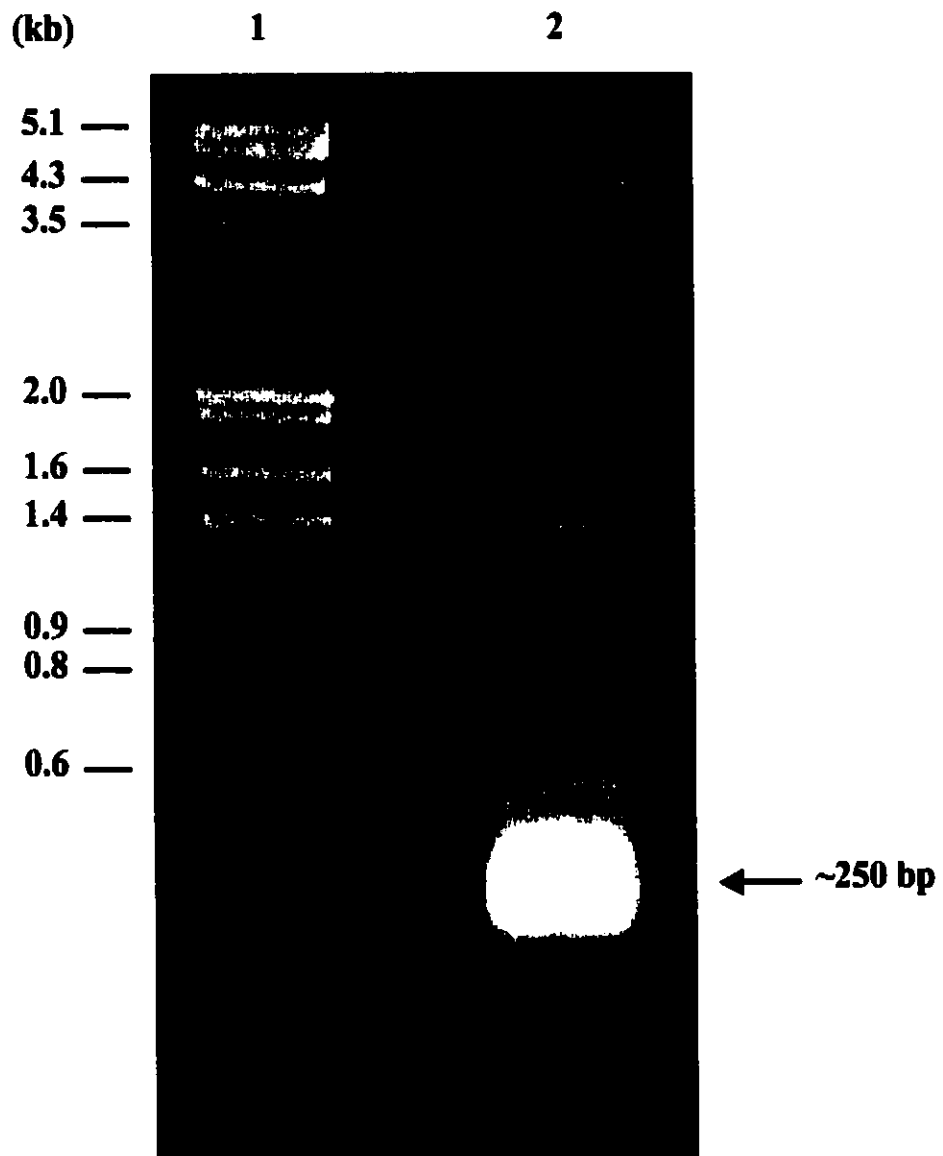


Figure 37. Electrophoretic analysis of DNA following colony PCR using *Taq* polymerase and primers ASP871 and ASP872. The reaction conditions were 94°C for 1 minute (hot start) and 35 subsequent PCR cycles at 94°C for 45 seconds, 55°C for 1 minute and 72°C for 1 minute. Following the 35 cycles, a final extension of 72°C for 5 minutes was performed. The amplified DNA was run on a 0.8% (w/v) agarose gel. The contents of each lane are as follows: 1, λ DNA/*EcoRI*+*HindIII* Ladder (MBI Fermentas, Flamborough, ON); 2, 5 μ L of the PCR reaction from colony 1; 3, 5 μ L of the PCR reaction from colony 2; 4, 5 μ L of the PCR reaction from colony 3.

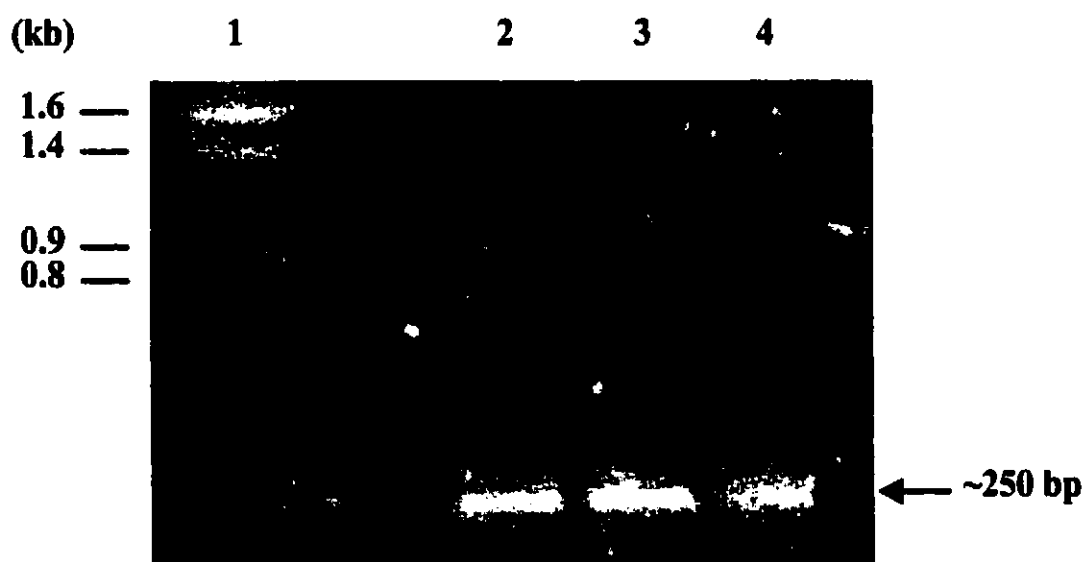


Figure 38. Restriction analysis using *EcoRV* on putative pBW(ASP04) clones. DNA (1.5 µg) was restricted for 3 hours at 37°C and run on a 0.8% (w/v) agarose gel. The contents of each lane are as follows: 1, λ DNA/*EcoRI*+*HindIII* Ladder (MBI Fermentas, Flamborough, ON); 2, uncut colony 1 plasmid DNA; 3, restricted colony 1 plasmid DNA; 4, uncut colony 2 plasmid DNA; 5, restricted colony 2 plasmid DNA; 6, uncut colony 3 plasmid DNA; 7, restricted colony 3 plasmid DNA.

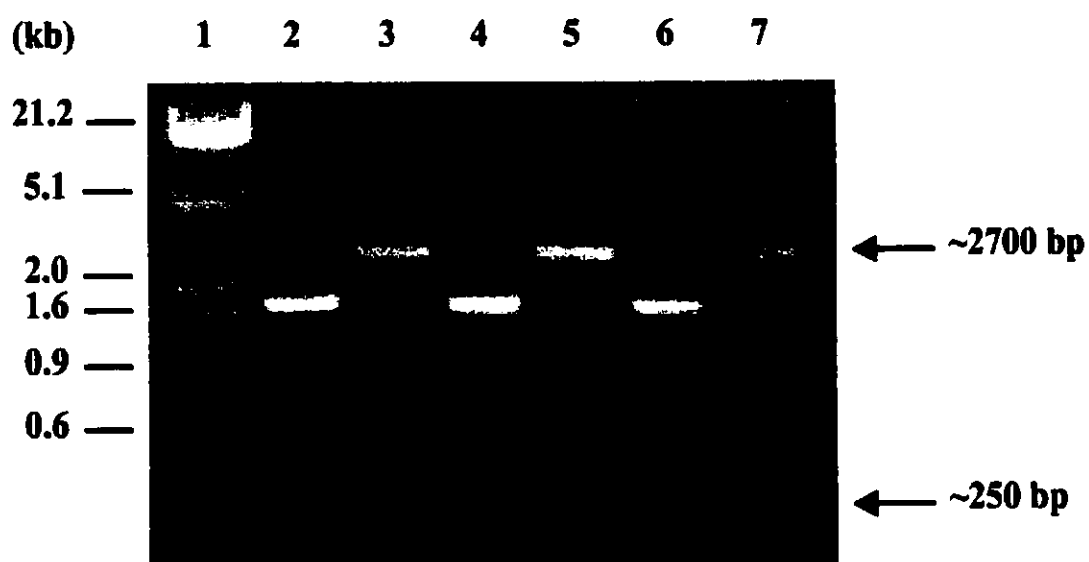
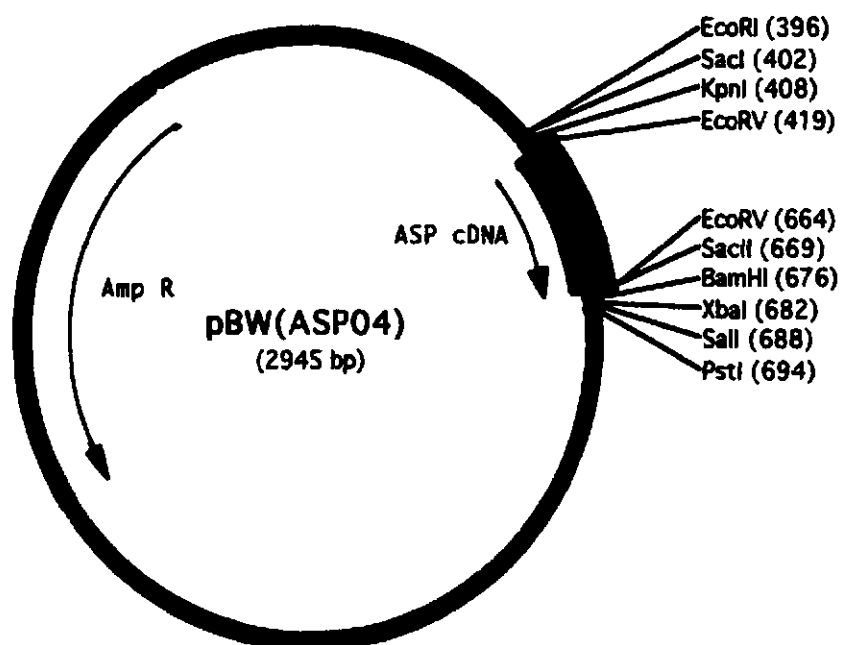


Figure 39. Sequence alignment for a putative *ASP04* gene in colony 1 against the known DNA sequence encoding ASP. Identical nucleotides are highlighted in yellow. The engineered generic bases designed for cutting efficiency are in pink, *EcoRV* sites are in blue, and additional Gs are in green.

PUTATIVE ASP04	GC CGATATCGGATGTC CG TT CA GCTGACTGAAAA CG TATGGATA AA AGTTGGTA	55
ASP	AT GTCCGTT CA GCTGACTGAAAA CG TATGGATA AA AGTTGGTA	43
PUTATIVE ASP04	AG TACCCGAAGGA ACT GC GT AAATGTTGCGAAGACGGGATGC GT GAAAACCCGAT	110
ASP	AG TACCCGAAGGA ACT GC GT AAATGTTGCGAAGACGGGATGC GT GAAAACCCGAT	98
PUTATIVE ASP04	GC GT TT CTCTTGCCA AG TC GT ACCC GT CA TC CTC CT GGCGAAG CT GC AAA	165
ASP	GC GT TT CTCTTGCCA AG TC GT ACCC GT CA TC CTC CT GGCGAAG CT GC AAA	153
PUTATIVE ASP04	AAG GT TT CTC CT GG ACT GC TG CA ACT AC AT CA CT GA ACT GC GT CG TC AG CAC GC TC	220
ASP	AAG GT TT CTC CT GG ACT GC TG CA ACT AC AT CA CT GA ACT GC GT CG TC AG CAC GC TC	208
PUTATIVE ASP04	GT GC TT CTC AC CTGG GT CTGG CT TA AT GAG AT AT CG CG C	259
ASP	GT GC TT CTC AC CTGG GT CTGG CT TA AT GA	237

Figure 40. Plasmid map for pBW(ASP04). The vector contains an ampicillin resistance gene (black arrow). The ASP cDNA (purple arrow) was inserted into the multiple cloning site of pUC19 (green) via the blunt ended *Sma*I restriction site. The detailed sequence of the insert is shown along with some flanking sequences.



SacI (402)
EcoRI (396) KpnI (408) EcoRV (419)
GAATTCGAGCTCGGTACCCGCGGATATCGGATGTCCGTTGAGCTGACTGAAAAACGTATGGATAAAGTT
GGTAAGTACCCGAAGGAACTGCGTAAATGTTGCGAAGACGGGATGCGTGAAAACCCGATGCGTTTCTCTT
GCCAACGTCGTACCCGCTTCATCTCCCTGGGCGAAGCTTGCAAAAAGGTTTTCTGGACTGCTGCAACTA
CATCACTGAACTGCGTCGTCAGCACGCTCGTGCTTCTCACCTGGGTCTGGCTTAATGAGATATCCGCGGG
XbaI (682) PstI (694)
BamHI (676) SalI (688)
GGATCCTCTAGAGTCGACCTGCAG

SacII (669)
EcoRV (664)

ASP04 gene should be in frame with the DNA encoding oleosin protein after the pSBS2050 plasmid is restricted with *SwaI*.

4.3.2. Genetic Constructs to Facilitate Expression of BSA in the ER

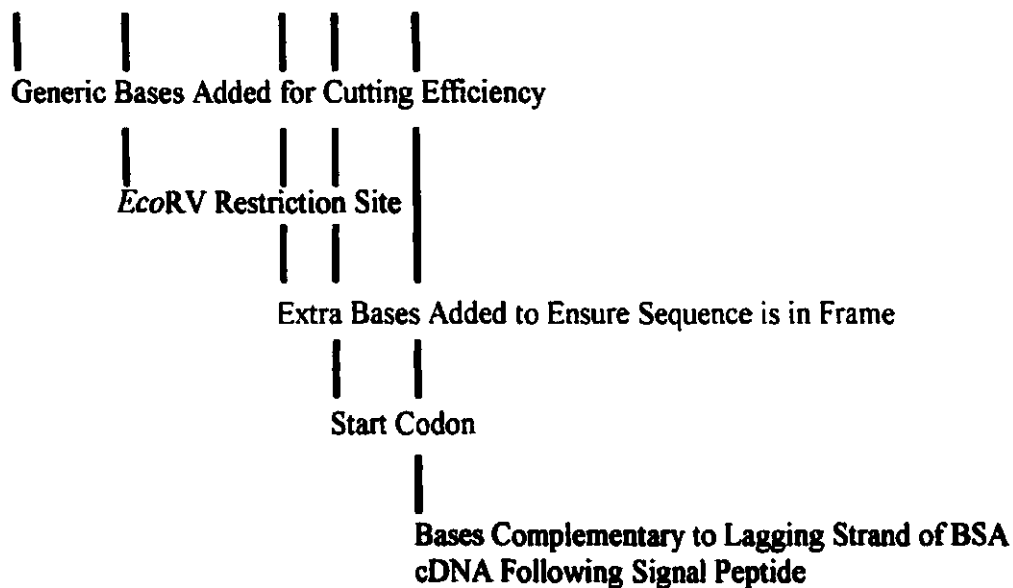
PCR was performed using *Pfu* polymerase on the original vector carrying the BSA cDNA. The primers BSA861 and BSA862 (Figure 41) were selected so that the cDNA sequence encoding BSA would be amplified without the signal peptide. *EcoRV* restriction sites were engineered on the 5' and 3' ends of the BSA sequence, respectively, for compatibility with the multiple cloning site of pSBS2050. Two extra Gs were added following the *EcoRV* restriction site on the 5' end followed by a start codon. This step was taken to ensure that the BSA cDNA would be in frame with the DNA encoding the oleosin protein from pSBS2050 (Figure 42). The restriction enzyme *SwaI* was selected to disrupt the stop codon of the DNA encoding the oleosin protein so that the DNA encoding BSA could also be translated (Figure 43). The amplified PCR products were analyzed using agarose gel electrophoresis (Figure 44). A DNA fragment of ~1800 bp was produced, corresponding to the size of the mature BSA cDNA.

The blunt ended PCR fragment was ligated into a pUC19 plasmid that was previously linearized with *SmaI*. Using agar plates containing X-gal, putative clones were identified using α -complementation. A patch plate was constructed from the white colonies and colony PCR was performed. Results of electrophoresis of the amplified products are shown in Figure 45. A DNA fragment of ~1800 bp was expected, corresponding to the size of the *BSA05* gene. The PCR revealed that colony 1 was

Figure 41. Primers designed to amplify the mature BSA cDNA for insertion into pSBS2050. An *EcoRV* restriction site was incorporated into the BSA861 and BSA862 primers so that these sites would be present at the 5' and 3' ends of the sequence for compatibility with the multiple cloning site of pSBS2050. Two extra Gs were added after the restriction site on the 5' end of the fragment to ensure that the BSA cDNA would be in frame with the DNA sequence encoding oleosin protein in pSBS2050. A start codon was engineered after the generic bases for translational purposes.

Forward Primer: BSA861

5' - GCGCGATATCGGATGAGGGGTGTGTTTCGTCGAGAT - 3'

**Reverse Primer: BSA862**

5' - GCGCGATATCTTAGGCTAAGGCTGTTTGAGT - 3'

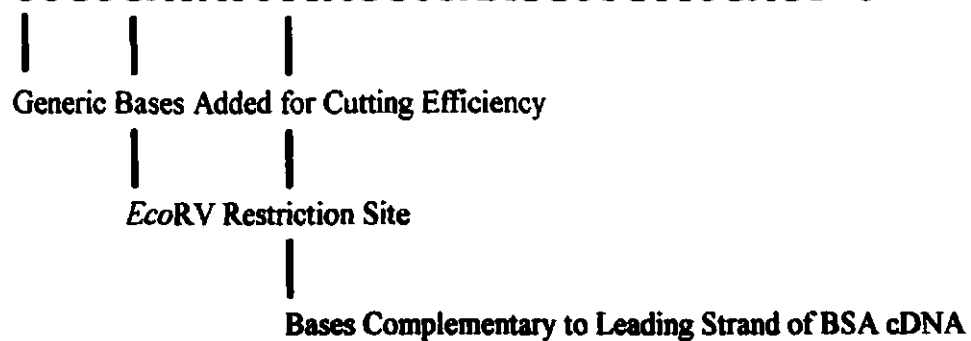
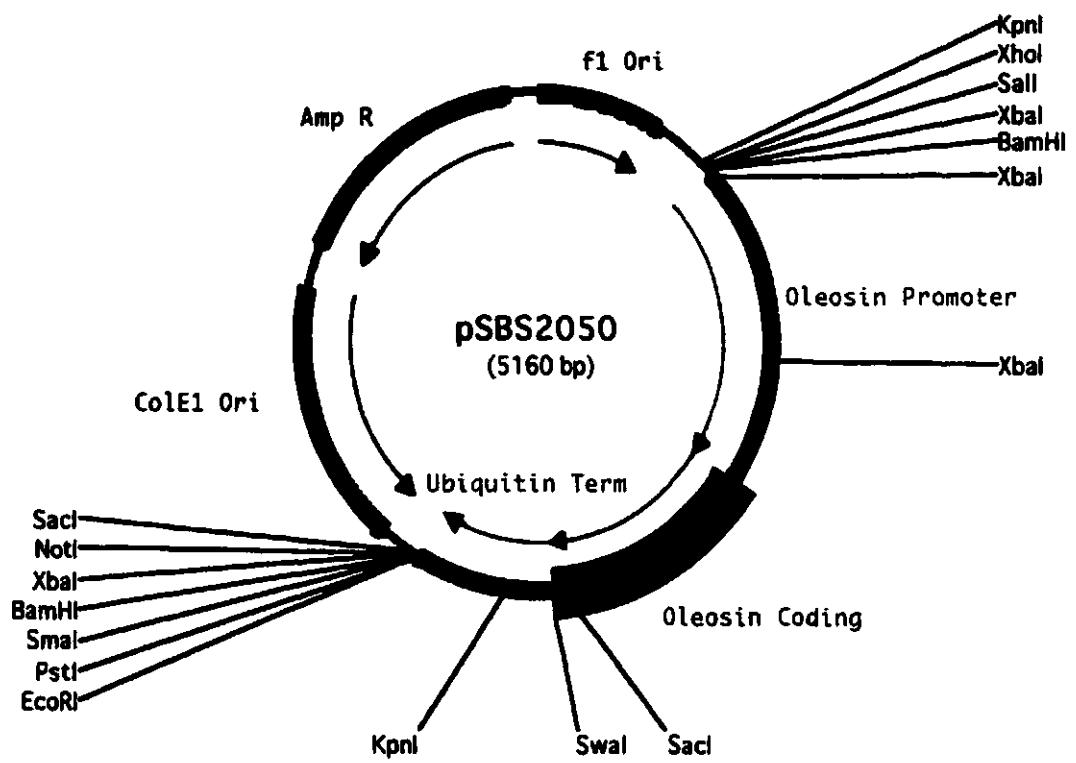


Figure 42. Plasmid map for pSBS2050. The vector contains an ampicillin resistance gene (solid black section and solid black arrow) as well as a 1.1 Kb *Arabidopsis* oleosin promoter (light blue arrow), an oleosin cDNA (solid green arrow) and a ubiquitin terminator (second light blue arrow). The detailed sequences of the oleosin promoter, coding region and ubiquitin terminator are shown on the page following the plasmid map.



KpnI XhoI Sall XbaI BamHI
 GGTACC GGCCCCCTCGAGGTCGACTCTAGAGGATCCCCCTATTGATTCAAATTACGATCTGATACTGA

XbaI
 TAACGTCTAGATTTTAGGGTTAAAGCAATCAATCACCTGACGATTCAAGTGGTGGATCATGACGATTCCA
 GAAACATCAAGCAAGCTCTCAAAGCTACACTCTTTGGGATCATACTGAACTCTAACAACTCGTTATGTCCC
 GTAGTGCCAGTACAGACATCCTCGTAACTCGGATTGTGACGATGCCATGGCTATACCCAACCTCGGCTTGG
 TCACACCAGGAACCTCTCTGGTAAGCTAGCTCCACTCCCCAGAAACAACCGGCCAAATTGCCGAATTGCTG
 ACCTGAAGACGGAAACATCATCGTCGGGTCCTTGGCCGATTGCGCCGGAAGATGGGTCAGCTTGGGCTTGAGGA
 CGAGACCCGAATCCGAGTCTGTTGAAAAGTTGTTCAATGGGATTTGTATACGGAGATTGGTCGTCGAGAGGT
 TTGAGGAAAGGACAAATGGGTTTGGCTCTGGAGAAAGAGAGTCCGGCTTAGAGAGAGAATTGAGAGGTTTA
 GAGAGAGATCGCCGGCGGATGACGGGAGGAGAGACGACGAGGACCTGCATTATCAAAGCAGTGACGTGGTGAA

XbaI
 ATTTGGAACTTTAAAGAGGCAGATAGATTTATTATTTGTATCCATTTTCTTCATTGTTCTAGAATGTCGGCGA
 ACAAATTTAAACTAAATCTAAATTTTCTAATTTGTTGCCAATAGTGGATATGTGGCCGTATAGAAGG
 AATCTATTGAAGGCCCAAACCTACTGACGAGCCCAAAGGTTGTTTTGCGTTTTATGTTTGGTTCGATGC
 CAACGCCACATTCTGAGTAGGCAAAAAACAACGTCCTTTGAATAGACTCCTCTCGTTAACACATGCAGCG
 GCTGCATGGTGACGCCATTAACACGTCGCTCAAAATGTCATGATGTCCTCATTGACACGTCGACTTCTCGTCTC
 CTTTCTTAATATATCTAACAAACCTCCTACCTCTTCCAAAATATATACACATCTTTTGTCAATCTCTCAT
 TCAAAATCTCATTCTCTAGTAAACAAGAACAAAAAATGGCCGATACAGCTAGAGGAACCCATCAGGATAT
 CATCGGCAGAGACCAGTACCCGATGATGGCCGAGACCGAGACCAGTACCAGATGTCGGACGAGGATCTGAC
 TACTCCAAGTCTAGGCAGATTGCTAAAGCTGCAACTGCTGTCAAGCTGGTGGTTCCCTCCTTGTCTCTCCA
 GCCTTACCCTTGTGGAACTGTCATAGCTTTGACTGTTGCAACACCTCTGCTCGTTATCTTCAGCCCAATCCT
 TGTCCCGGCTCTCATCACAGTTGCACTCCTCATCACCGGTTTTCTTCTCTGGAGGGTTTGGCAATTGCCGCT
 ATAACCGTTTTCTCTGGATTTCAAGTAAGCACACATTTATCATCTACTTCATAATTTGTGCAATATGTG
 CATGCATGTGTTGAGCCAGTAGCTTTGGATCAATTTTTTGGTGAATAACAAATGTAACAATAAGAAATTGC
 AAATCTAGGGAACTTTGGTAACTAAATACGAAATTTGACCTAGCTAGCTTGAATGTGCTGTGTATATCA
 TCTATATAGGTAATGCTTGGTATGATACCTATTGATTGTGAATAGGTACGCAACGGGAGAGCACCACAGG

SacI
 GATCAGACAAGTTGGACAGTGAAGGATGAAGTTGGGAAGCAAAGCTCAGGATCTGAAAGACAGAGCTCAGTA

Swal
 CTACGGACAGCAACATACTGGTGGGAAACATGACCGTGACCGTACTCGTGGTGGCCAGCACACTACTATTTAA
 ATGCAAGCTTGTACCCCACTGATGTCATCGTCATAGTCCAATAACTCCAATGTCGGGAGTTAGTTTATGAG
 GAATAAAGTGTTFAGAATTTGATCAGGGGGAGATAATAAAGCCGAGTTTGAATCTTTTGTATAAGTAATG

KpnI
 TTTATGTGTGTTTCTATATGTTGTCAAATGGTACCATGTTTTTTTTCCCTCTTTTGTAACTTGAAGTGTG
 TGTTGACTTTTATTGGCTTCITTTGTAAGTTGGTAACGGTGGTCTATATATGGAAAAGGCTTGTTTTGTAA
 ACTTATGTTAGTTAACTGGATTGCTCTTAAACCACAAAAAGTTTTCAATAAGCTACAAATTTAACACGCAAGC
 CGATCGAGTCATTAGTACATATATTTATTGCAAGTGATTACATGGCAACCCAAACTTCAAAAACAGTAGGTTG

EcoRI PstI SmaI BamHI
 CTCCATTTAGTAACTGAATTGCCTCCTGATTCTAGTTGATCCCATCGAATTCCTGCAGCCCGGGGATCCAC

XbaI NotI SacI
 TAGTCTAGAGCGGCCCCACCCGGTGGAGCTC

Figure 43. Integration of BSA cDNA into pSBS2050 by disrupting the stop codon in the DNA sequence encoding oleosin protein. Originally there was a stop codon at the end of the DNA sequence encoding oleosin protein, but after restricting with *SwaI*, the stop codon was disrupted and the BSA cDNA was integrated into the plasmid. The remaining base pairs of the engineered *EcoRV* site following restriction are shown in blue text, the additional two Gs that were engineered to keep the DNA sequences in frame are shown in green text and the engineered start codon is shown in red text.

DNA Sequence Encoding Oleosin Protein

5' ...CAC ACT ACT ATT TAA AT 3'
 3' ...GTG TGA TGA TAA ATT TA 5'

↑
 Stop Codon

DNA Sequence Encoding Oleosin Protein After Restriction with *Swa*I

5' ...CAC ACT ACT ATT T AA 3'
 3' ...GTG TGA TGA TAA A TT 5'

↑
 Stop Codon Disrupted

Insertion of Blunt Ended BSA cDNA with Engineered Start Codon and Two Extra "G's" into the Blunt Ended DNA Sequence Encoding Oleosin Protein

5' ...CAC ACT ACT ATT TAT CGG ATG AGG GGT...3'
 3' ...GTG TGA TGA TAA ATA GCC TAC TCC CCA...5'

⏟
 DNA Sequence Encoding
 Oleosin Protein without a
 Stop Codon

⏟
 BSA cDNA

Figure 44. Agarose gel electrophoresis analysis of PCR amplification of pBSA using *Pfu* polymerase and primers BSA861 and BSA862. The reaction conditions were 94°C for 1 minute (hot start) and 35 subsequent PCR cycles at 94°C for 45 seconds, 55°C for 1 minute and 72°C for 2 minutes. Following the 35 cycles, a final extension of 72°C for 5 minutes was performed. The amplified DNA was run on a 0.8% (w/v) agarose gel. Lane 1 contains a λ DNA/*EcoRI*+*HindIII* Ladder (MBI Fermentas, Flamborough, ON) and lane 2 contains 5 μ L of the PCR reaction.

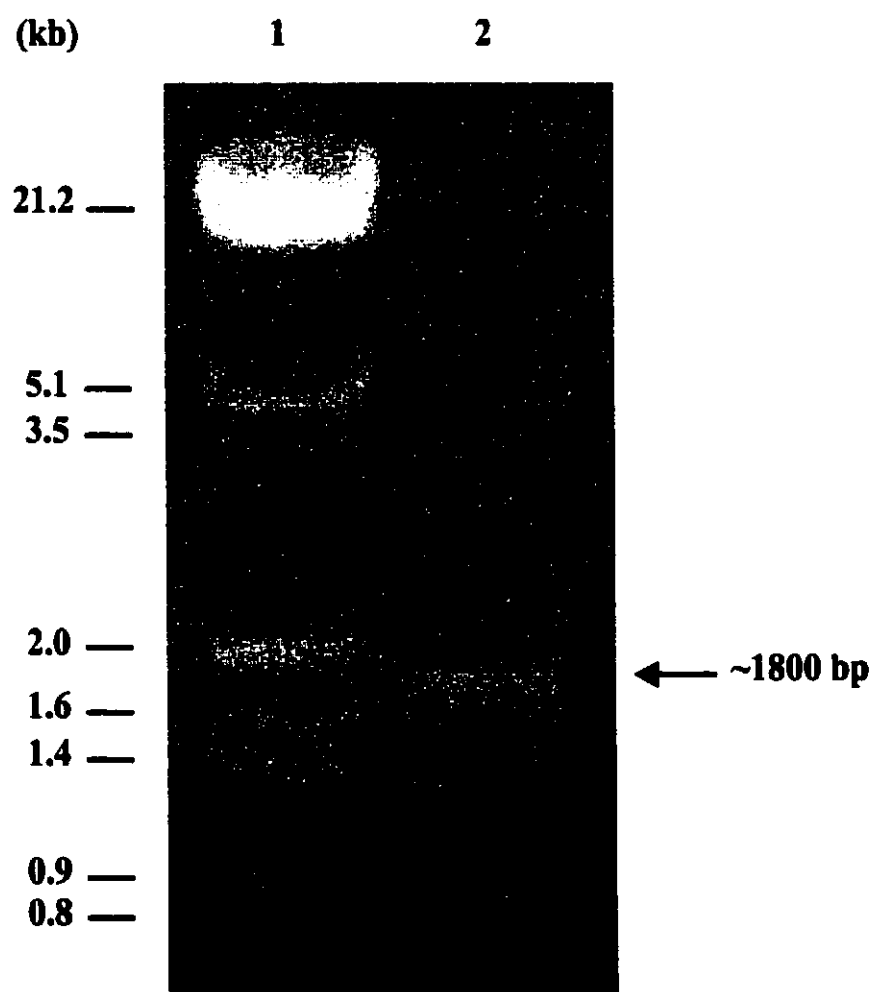
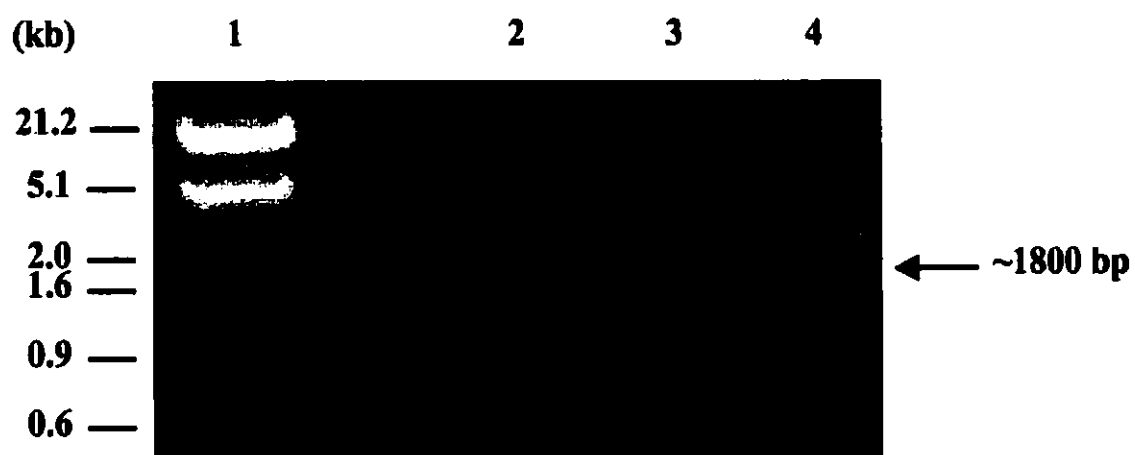


Figure 45. Electrophoretic analysis of DNA following colony PCR using *Taq* polymerase and primers BSA861 and BSA862. The reaction conditions were 94°C for 1 minute (hot start) and 35 subsequent PCR cycles at 94°C for 45 seconds, 55°C for 1 minute and 72°C for 2 minutes. Following the 35 cycles, a final extension of 72°C for 5 minutes was performed. The amplified DNA was run on a 0.8% (w/v) agarose gel. The contents of each lane are as follows: 1, λ DNA/*EcoRI*+*HindIII* Ladder (MBI Fermentas, Flamborough, ON); 2, 5 μ L of the PCR reaction from colony 1; 3, 5 μ L of the PCR reaction from colony 2; 4, 5 μ L of the PCR reaction from colony 3.



carrying plasmid DNA that produced a DNA fragment of ~1800 bp when amplified using Primers BSA861 and BSA862. Restriction analysis with *EcoRV* was performed on the plasmid DNA from colony 1 as compared to linearized pUC19 to ensure that the engineered *EcoRV* restriction sites were intact (Figure 46). The fragment of ~1775 bp was observed following agarose gel electrophoresis of the restriction reaction. This indicated that the analyzed vector putatively contained the *BSA05* gene with intact *EcoRV* restriction sites. The plasmid DNA from this colony 1 was sent for sequencing. The sequence for this plasmid was correct when compared to the known sequence for BSA cDNA and the engineered restriction sites, two extra Gs and start codon were also intact (Figure 47). The pUC19 plasmid carrying this BSA cDNA with engineered fragments was designated pBW(BSA05) (Figure 48).

The *BSA05* gene was extracted from pBW(BSA05) by restricting with *EcoRV* and the fragment was subsequently gel extracted and gene-cleaned. This fragment was ligated into gel purified pSBS2050 that had been previously restricted with *SmaI*. The ligation reaction was used to transform *E. coli* the following day. Several colonies were inoculated into broth and restriction analysis was performed (Figure 49). The restricted plasmid DNA from colony 1 and colony 5 produced DNA fragments of ~4100 bp and ~2800 bp, corresponding to the size of the *BSA06* gene and the remainder of the parent pSBS2050 plasmid. Since blunt-ended cloning was being performed, in addition to simple restriction analysis, orientation analysis was also performed. Plasmid DNA from these two putative clones was restricted with *PstI* and *ScaI* and electrophoresed on an agarose gel (Figure 50). The distinguishing DNA fragment that was expected for a

Figure 46. Restriction analysis using *EcoRV* on plasmid DNA from colony 1, which was presumed to contain the *BSA05* gene. DNA (1.5 μ g) was restricted for 3 hours at 37°C and run on a 0.8% (w/v) agarose gel. The contents of each lane are as follows: 1, λ DNA/*EcoRI*+*HindIII* Ladder (MBI Fermentas, Flamborough, ON); 2, pUC19 linearized *SmaI*; 3, restricted colony 1 plasmid DNA.

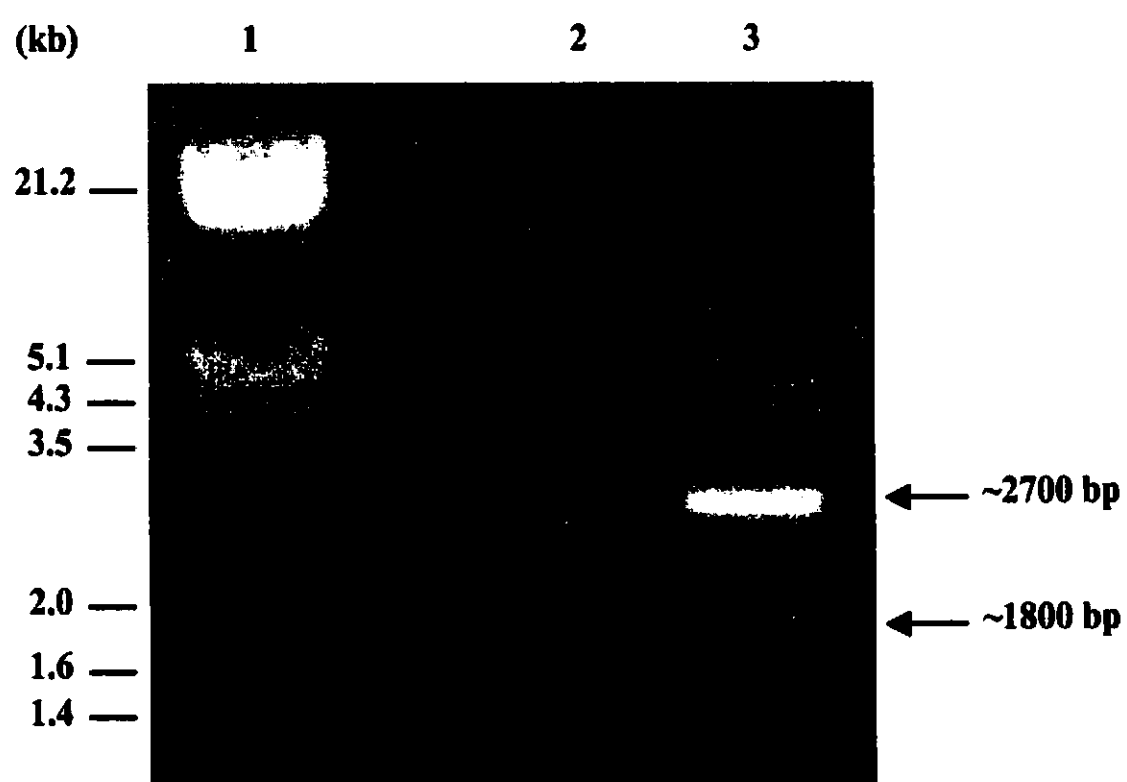
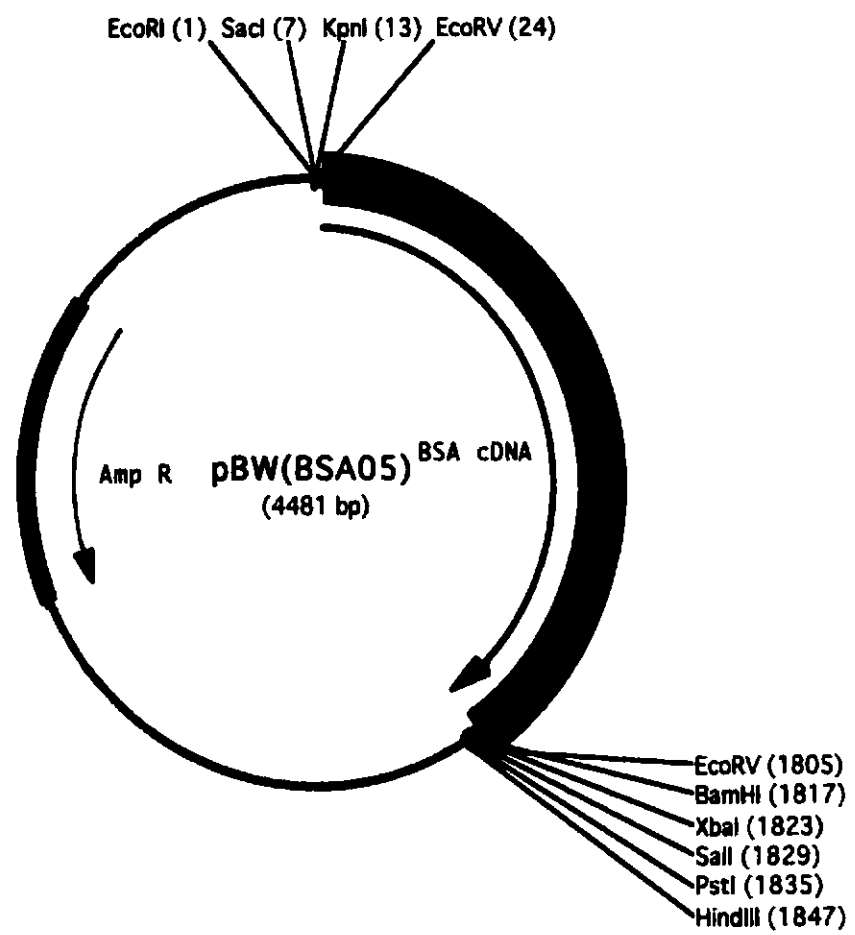


Figure 47. Sequence alignment for a putative *BSA05* gene in colony 1 against the known BSA cDNA sequence. Identical nucleotides are highlighted in yellow. The engineered generic bases designed for cutting efficiency are in pink, *EcoRV* sites are in blue, additional Gs are in green and the engineered start codon is in red.

FUTATIVE BSA05 BSA	GCOCGATATCGGATGAGGGGTGTGTTTCGTCCGAGATACACACAAGAGTGAGATTG AGGGGTGTGTTTCGTCCGAGATACACACAAGAGTGAGATTG	55 40
FUTATIVE BSA05 BSA	CTCATCGGTTTAAAGATTTGGGAGAAGAACATTTTAAAGGCTGGTACTGATTGC CTCATCGGTTTAAAGATTTGGGAGAAGAACATTTTAAAGGCTGGTACTGATTGC	110 95
FUTATIVE BSA05 BSA	CFTTTCAGTATCTCCAGCAGTGTCCATTTGATGAGCATGTAAAATTAGTGAAC CFTTTCAGTATCTCCAGCAGTGTCCATTTGATGAGCATGTAAAATTAGTGAAC	165 150
FUTATIVE BSA05 BSA	GAACTAACTGAGTTTGCAAAAACATGTGTTGCTGATGAGTCCCATGCCGGCTGTG GAACTAACTGAGTTTGCAAAAACATGTGTTGCTGATGAGTCCCATGCCGGCTGTG	220 205
FUTATIVE BSA05 BSA	AAAAGTCACTTCACACTCTCTTTGGAGATGAATTGTGTAAGTGCATCCCTTCG AAAAGTCACTTCACACTCTCTTTGGAGATGAATTGTGTAAGTGCATCCCTTCG	275 260
FUTATIVE BSA05 BSA	TGAAACCTATGGTGACATGGCTGACTGCTGTGAGAAACAAAGAGCCTGAAAGAAAT TGAAACCTATGGTGACATGGCTGACTGCTGTGAGAAACAAAGAGCCTGAAAGAAAT	330 315
FUTATIVE BSA05 BSA	GAATGCTTCCTGAGCCACAAGATGATAGCCAGACCTCCCTAAATTGAAACCAG GAATGCTTCCTGAGCCACAAGATGATAGCCAGACCTCCCTAAATTGAAACCAG	385 370
FUTATIVE BSA05 BSA	ACCCCAATACTTTGTGTGATGAGTTTAAAGGCAGATGAAAAGAAGTTTGGGGAAA ACCCCAATACTTTGTGTGATGAGTTTAAAGGCAGATGAAAAGAAGTTTGGGGAAA	440 425
FUTATIVE BSA05 BSA	ATACCTATACGAAATGCTAGAAAGACATCCCTACTTTTATGCACCAGAACTCCTT ATACCTATACGAAATGCTAGAAAGACATCCCTACTTTTATGCACCAGAACTCCTT	495 480
FUTATIVE BSA05 BSA	TACTATGCTAATAAATAAATGAGTTTTCAGAAATGCTGCCAAGCTGAAGATA TACTATGCTAATAAATAAATGAGTTTTCAGAAATGCTGCCAAGCTGAAGATA	550 535
FUTATIVE BSA05 BSA	AAGTGCCCTGCCTGCTACCAAAGATTGAAACTATGAGAGAAAAAGTACTGACTTC AAGTGCCCTGCCTGCTACCAAAGATTGAAACTATGAGAGAAAAAGTACTGACTTC	605 590
FUTATIVE BSA05 BSA	ATCTGCCAGACAGAGACTCAGGTGTGCCAGTATTCAAAAATTTGGAGAAGAGCT ATCTGCCAGACAGAGACTCAGGTGTGCCAGTATTCAAAAATTTGGAGAAGAGCT	660 645
FUTATIVE BSA05 BSA	TAAAAGCATGGTCAGTAGCTCGCTGAGCCAGAAATTTCCCAAGGCTGAGTTTG TAAAAGCATGGTCAGTAGCTCGCTGAGCCAGAAATTTCCCAAGGCTGAGTTTG	715 700
FUTATIVE BSA05 BSA	TAGAAGTTACCAAGCTAGTGACAGATCTCACAAAAGTCCACAAGGAATGCTGCCA TAGAAGTTACCAAGCTAGTGACAGATCTCACAAAAGTCCACAAGGAATGCTGCCA	770 755
FUTATIVE BSA05 BSA	TGGTGACCTACTTGAATGCCAGATGACAGGGCAGATCTTGCCAAGTACATATGT TGGTGACCTACTTGAATGCCAGATGACAGGGCAGATCTTGCCAAGTACATATGT	825 810
FUTATIVE BSA05 BSA	GATAATCAAGATACAATCTCCAGTAACTGAAGGAATGCTGTGATAAGCCTTTGT GATAATCAAGATACAATCTCCAGTAACTGAAGGAATGCTGTGATAAGCCTTTGT	880 865
FUTATIVE BSA05 BSA	TGGAAAAATCCCACTGCATTGCTGAGGTAGAAAAGATGCCATACCTGAAAACCT TGGAAAAATCCCACTGCATTGCTGAGGTAGAAAAGATGCCATACCTGAAAACCT	935 920
FUTATIVE BSA05 BSA	GCCCCATTAACTGCTGACTTTGCTGAAGATAAGGATGTTTGCAAAAACCTATCAG GCCCCATTAACTGCTGACTTTGCTGAAGATAAGGATGTTTGCAAAAACCTATCAG	990 975
FUTATIVE BSA05 BSA	GAAGCAAAGATGCCCTCCTGGGCTCGTTTTTGTATGAATATCAAGAAGGCATC GAAGCAAAGATGCCCTCCTGGGCTCGTTTTTGTATGAATATCAAGAAGGCATC	1045 1030
FUTATIVE BSA05 BSA	CTGAATATGCTGTCTCAGTGTCTATTGAGACTTGCCAAGGAATATGAAGCCACACT CTGAATATGCTGTCTCAGTGTCTATTGAGACTTGCCAAGGAATATGAAGCCACACT	1100 1085
FUTATIVE BSA05 BSA	GGAGGAATGCTGTGCCAAGATGATCCACATGCATGCTATTCCACAGTGTGTTGAC GGAGGAATGCTGTGCCAAGATGATCCACATGCATGCTATTCCACAGTGTGTTGAC	1155 1140
FUTATIVE BSA05 BSA	AAACTTAAGCATCTTGTGGATGAGCCTCAGAAATTAATCAAACAAAACCTGTGACC AAACTTAAGCATCTTGTGGATGAGCCTCAGAAATTAATCAAACAAAACCTGTGACC	1210 1195

POTATIVE BSA05	AATTCGAAAACTTGGAGAGTATGGATTCCAAAATGCGCTCATAGTTCGTTACAC	1265
BSA	AATTCGAAAACTTGGAGAGTATGGATTCCAAAATGCGCTCATAGTTCGTTACAC	1250
POTATIVE BSA05	CAGGAAGTACCCCAAGTGTCAACTCCAACCTCTCGTGGAGGTTTCAAGAAGCCTA	1320
BSA	CAGGAAGTACCCCAAGTGTCAACTCCAACCTCTCGTGGAGGTTTCAAGAAGCCTA	1305
POTATIVE BSA05	GGAAAAGTGGGTACTAGGTGTGTACAAAGCCGGAATCAGAAAGAATGCCCTGTA	1375
BSA	GGAAAAGTGGGTACTAGGTGTGTACAAAGCCGGAATCAGAAAGAATGCCCTGTA	1360
POTATIVE BSA05	CTGAAGACTATCTGAGCTTGATCCTGAACCGGTTGTGCGTGCATGAGAAGAC	1430
BSA	CTGAAGACTATCTGAGCTTGATCCTGAACCGGTTGTGCGTGCATGAGAAGAC	1415
POTATIVE BSA05	ACCAAGTGAAGTAAAAAGTCAACCAAGTGTGCACAGAGTCATTGGTGAACAGACGG	1485
BSA	ACCAAGTGAAGTAAAAAGTCAACCAAGTGTGCACAGAGTCATTGGTGAACAGACGG	1470
POTATIVE BSA05	CCAIGTTTCTCTGCTCTGACACCTGATGAAACATATGTACCCAAAGCCTTTGATG	1540
BSA	CCAIGTTTCTCTGCTCTGACACCTGATGAAACATATGTACCCAAAGCCTTTGATG	1525
POTATIVE BSA05	AGAAATGTTTACCTTCCATGCAGATATATGCACACTTCCCGATACTGAGAAACA	1595
BSA	AGAAATGTTTACCTTCCATGCAGATATATGCACACTTCCCGATACTGAGAAACA	1580
POTATIVE BSA05	AATCAAGAAACAACTGCACCTTGTGAGCTGTTGAAACACAAGCCCAAGGCAACA	1650
BSA	AATCAAGAAACAACTGCACCTTGTGAGCTGTTGAAACACAAGCCCAAGGCAACA	1635
POTATIVE BSA05	GAGGAACAACAGAAAACCGTCATGGAGAATTTGTGGCTTTTGTAGACAAGTGCT	1705
BSA	GAGGAACAACAGAAAACCGTCATGGAGAATTTGTGGCTTTTGTAGACAAGTGCT	1690
POTATIVE BSA05	GTGCAGCTGATGACAAAGAGGCCCTGCTTTGCTGTGGAGGTCCTAAAACCTTGTGT	1760
BSA	GTGCAGCTGATGACAAAGAGGCCCTGCTTTGCTGTGGAGGTCCTAAAACCTTGTGT	1745
POTATIVE BSA05	TTCAACTCAAACAGCCTTAGCCTAAGATATCCGGC	1795
BSA	TTCAACTCAAACAGCCTTAGCCTAA	1770

Figure 48. Plasmid map for pBW(BSA05). The vector contains an ampicillin resistance gene (solid black arrow). The BSA cDNA (purple arrow) was inserted into the multiple cloning site of pUC19 (green) via the blunt ended *SmaI* restriction site. The detailed sequence of the insert is shown along with some flanking sequences on the page following the plasmid map.



SacI (7)
 EcoRI (1) KpnI (13) EcoRV (24)
 GAATTCGAGCTCGGTACCCGCGGATATCGGATGAGGGGTGTGTTTCGTCGAGATACACACAAGAGTGAG
 ATTGCTCATCGGTTTAAAGATTTGGGAGAAGAACATTTTAAAGGCCTGGTACTGATTGCCTTTTCTCAGT
 ATCTCCAGCAGTGTCCATTTGATGAGCATGTAAATTAAGTGAACGAACTAACTGAGTTTGCAAAAAATG
 TGTGCTGATGAGTCCCATGCCGGCTGTGAAAAGTCACTTCACACTCTCTTTGGAGATGAATTGTGAAA
 GTTGATCCCTTCGTGAAACCTATGGTGACATGGCTGACTGCTGTGAGAAACAAGAGCCTGAAAGAAATG
 AATGCTTCTGAGCCACAAAGATGATAGCCAGACCTCCCTAAATTGAAACCAGACCCCAATACTTTGTG
 TGATGAGTTTAAAGCAGATGAAAAGAAGTTTTGGGAAAATACCTATACGAAATTGCTAGAAGACATCCC
 TACTTTTATGCACCAGAACTCCTTTACTATGCTAATAAATAATGGAGTTTTTCAAGAATGCTGCCAAG
 CTGAAGATAAAGGTGCCTGCCTGCTACCAAAGATTGAACTATGAGAGAAAAAGTACTGACTTCATCTGC
 CAGACAGAGACTCAGGTGTGCCAGTATCAAAAATTTGGAGAAAGAGCTTAAAAGCATGGTCAGTAGCT
 CGCCTGAGCCAGAAATTTCCAAGGCTGAGTTTGTAGAAGTTACCAAGCTAGTGACAGATCTCACAAAAG
 TCCACAAGGAATGCTGCCATGGTGACCTACTTGAATGCGCAGATGACAGGGCAGATCTTGCCAAGTACAT
 ATGTGATAATCAAGATACAATCTCCAGTAACTGAAGGAATGCTGTGATAAGCCTTTGTTGGAAAAATCC
 CACTGCATTGCTGAGGTAGAAAAGATGCCATACCTGAAAACCTGCCCCATTAAGTCTGACTTTGCTG
 AAGATAAGGATGTTTGCAAAAATATCAGGAAGCAAAAAGATGCCTTCCTGGGCTCGTTTTTGTATGAATA
 TTCAAGAAGGCATCCTGAATATGCTGTCTCAGTCTATTGAGACTTGCCAAGGAATATGAAGCCACACTG
 GAGGAATGCTGTGCCAAAGATGATCCACATGCATGCTATTCCACAGTGTGACAAACTTAAGCATCTTG
 TGGATGAGCCTCAGAATTAATCAAACAAAATGTGACCAATTCGAAAACCTTGAGAGTATGGATTCCA
 AAATGCGCTCATAGTTCGTTACACCAGGAAAGTACCCCAAGTGTCAACTCCAATCTCGTGAGGTTTCA
 AGAAGCCTAGGAAAAGTGGTACTAGGTGTTGTACAAAGCCGGAATCAGAAAGAATGCCCTGTACTGAAG
 ACTATCTGAGCTTGATCCTGAACCGGTTGTGCGTGCTGCATGAGAAGACCCAGTGAGTAAAAAGTAC
 CAAGTGCTGCACAGAGTCATTGGTGAACAGACGGCCATGTTTCTCTGCTCTGACACCTGATGAAACATAT
 GTACCCAAAGCCTTTGATGAGAAATTGTTACCTTCCATGCAGATATATGCACACTCCCGATACTGAGA
 AACAAATCAAGAAACAACTGCACTTGTGAGCTGTTGAAACACAAGCCCAAGGCAACAGAGGAACA
 GAAAACCGTCATGGAGAATTTTGTGGCTTTGTAGACAAGTGTGCTGTGAGCTGATGACAAAAGGGCCTGC
 TTTGCTGTGGAGGTCCTCAAACTGTTGTTTCAACTCAAACAGCCTTAGCCTAAGATATCCGCGGGGAT
 EcoRV (1805) BamHI (

Sall (1829)
 XbaI (1823) PstI (1835) HindIII (1847)
 CCTCTAGAGTCGACCTGCAGGCATGCAAGCTT

Figure 49. Restriction analysis using *Pst*I on plasmid DNA from putative pBW(BSA06) clones. DNA (1.5 µg) was restricted for 3 hours at 37°C and run on a 0.8% (w/v) agarose gel. The contents of each lane are as follows: 1, λ DNA/*Eco*RI+*Hind*III Ladder (MBI Fermentas, Flamborough, ON); 2, uncut pSBS2050; 3, uncut colony 1 plasmid DNA; 4, uncut colony 3 plasmid DNA; 5, uncut colony 5 plasmid DNA; 6, uncut colony 7 plasmid DNA; 7, restricted pSBS2050; 8, restricted colony 1 plasmid DNA; 9, restricted colony 3 plasmid DNA; 10, restricted colony 5 plasmid DNA; 11, restricted colony 7 plasmid DNA.

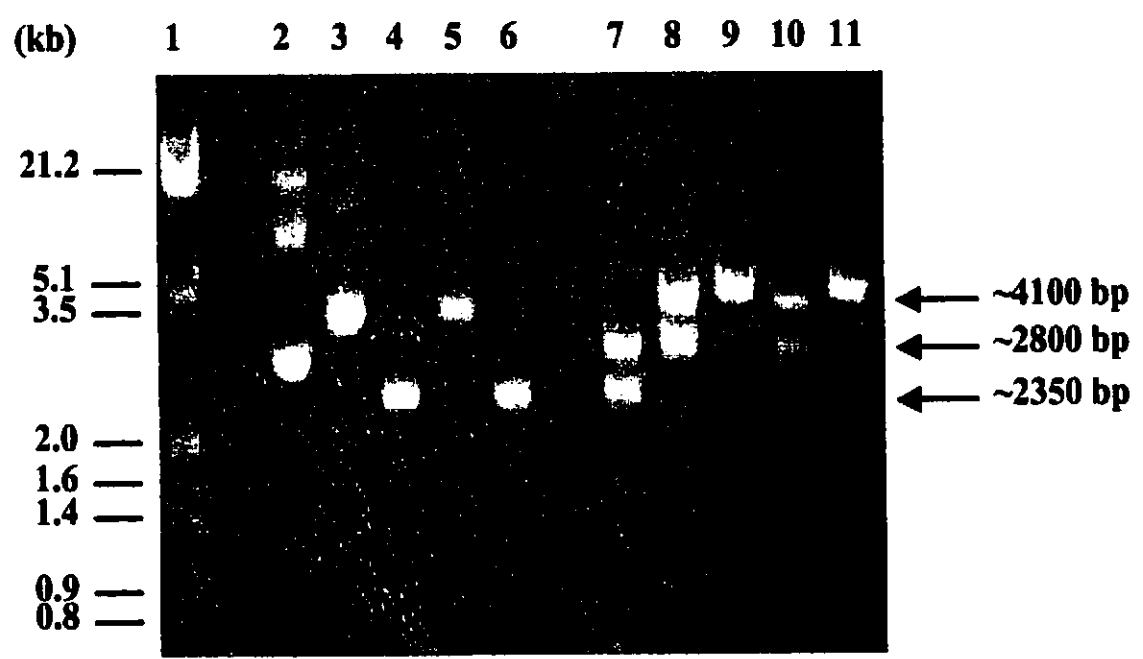
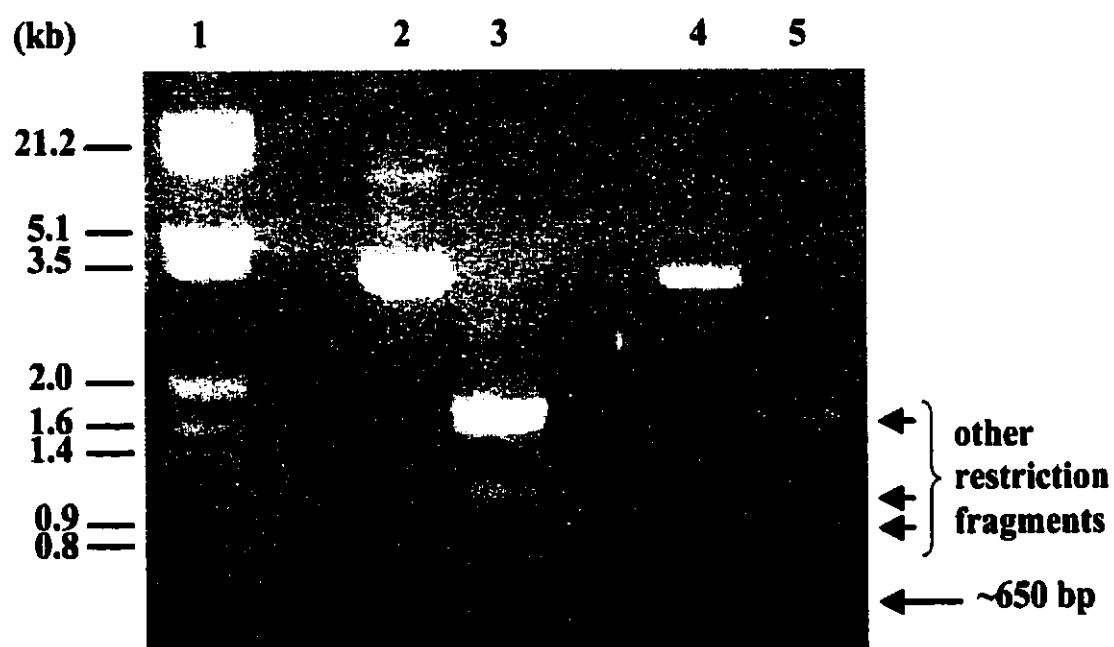


Figure 50. Orientation analysis using *Pst*I and *Sca*I on plasmid DNA from putative pBW(BSA06) clones. DNA (1.5 μ g) was restricted for 3 hours at 37°C and run on a 0.8% (w/v) agarose gel. The contents of each lane are as follows: 1, λ DNA/*Eco*RI+*Hind*III Ladder (MBI Fermentas, Flamborough, ON); 2, uncut colony 1 plasmid DNA; 3, restricted colony 1 plasmid DNA; 4, uncut colony 5 plasmid DNA; 5, restricted colony 5 plasmid DNA.



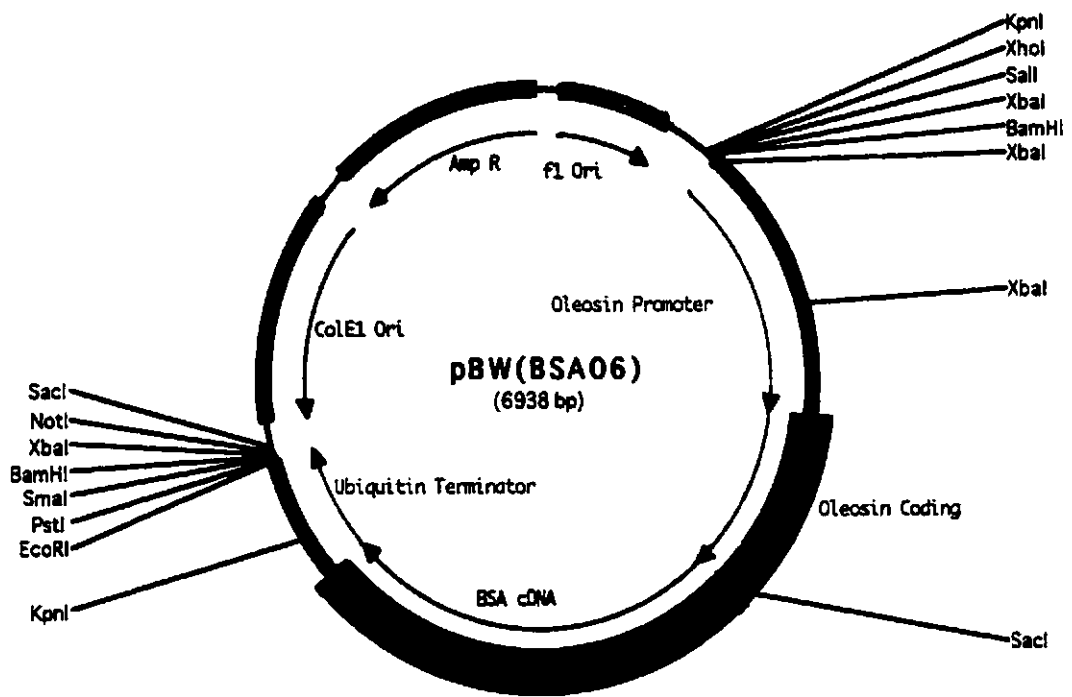
positive orientation was ~650 bp. Therefore, colony 1 was not only positive for the insert, but also had it in the correct orientation. The new plasmid containing the *Arabidopsis* oleosin promoter, DNA encoding oleosin protein, BSA cDNA and ubiquitin terminator was designated pBW(BSA06) (Figure 51). These DNA sequences are now ready to be inserted into the binary vector, pCGN1559 via the restriction site *Pst*I. There are no internal *Pst*I sites in the *BSA06* gene and this restriction enzyme is compatible with the multiple cloning site of pCGN1559.

4.4. Bacterial Expression of BSA and a Truncation of the Protein

The stimulatory effect of BSA on microsomal DGAT has been proposed to result from interactions with acyl-CoA that may promote more effective substrate delivery (Hershenson and Ernst-Fonberg, 1983). Since native BSA has a stimulatory effect on DGAT activity *in vitro* (Little *et al.*, 1994), testing the stimulatory nature of synthetic mature BSA and a truncation of synthetic BSA with high FA binding capacity was desirable. Vectors were engineered such that recombinant BSA or a truncated form of BSA could be expressed in *E. coli* to provide protein for future *in vitro* studies on stimulation of microsomal DGAT. *E. coli* carrying expression vectors containing the cDNA for BSA and truncated BSA could be used to produce these recombinant polypeptides.

With respect to selecting the appropriate truncation of BSA for bacterial expression studies, observations by other researchers were noted. Palmitic acid binds to lysine residues located at positions 116, 349, and 473 of the BSA amino acid sequence,

Figure 51. Plasmid map for pBW(BSA06). The vector contains an ampicillin resistance gene (solid black section and solid black arrow) as well as the *BSA06* gene, containing a 1.1 Kb *Arabidopsis* oleosin promoter (first light blue arrow), oleosin cDNA (green arrow), BSA cDNA (purple arrow) and ubiquitin terminator (second light blue arrow), that was inserted into blunt-ended *SwaI* restriction site of pSBS2050 via restricting the *BSA05* gene with the blunt-ending restriction enzyme *EcoRV*. The detailed sequence of the insert is shown along with some flanking sequences on the page following the plasmid map.



KpnI XbaI SfiI XbaI BamHI XbaI
 GGTACCGGGCCGCCCTCGAGTCCACTCTAGAGGATCCCCCTCTATTGATTCAAATACGATCTGATACTGATAACGCTAGA
 TTTTAGGGTTAAGCAATCAATCACTGACGATCAAGGTGGTTGGATCAGGATTCAGAAAAACAAGCAAGCTCTCA
 AAGCTAGACTCTTTGGATCATACTGAACTCTAACAACCTCTTATGTCGGTACTGCCAGTACAGAACTCTCGTAACCTCGGA
 TTGTGACGATGCAATGGTAFACCCAACTCGGCTTGGTCAACAGAACTCTCTGGTAAGCTAGCTCACTCCACAGAAA
 CAACCGGGCCAAATGGCGAATTGCTGACTGAAGACGGAACTATCTGGGCTCTTGGCGAATGGCGGGGAAAGATGGG
 TCACTTGGGCTTGGAGCAGACCCGAACTCCGACTGTTCAAAAGGTCTTCATTGGGATTTGTAACGGAAATGGTCTC
 CAGAGTTTCAGGAAAGGCAAAATGGTTTGGCTCTGGGAAGAGAGTCCGGCTTATAGAGAGAAATTGAGAGCTTTAGAGA
 CAGATGGCGGGGATGAAGGGGAGAGAGACGACGAGCACTGATTATCAAAAGCAAGTGGGGAATTTGAACTTTTAA

XbaI
 GAGGCAGATAGATTATTATTGATGCAATTTCTTCAATTTCTAGAAATCGCGGAACAATTTTAAACTAAATCTCAAAAT
 TTTCTAAATTTGTTCCAAATAGTGATATGTTGGCGTATAGAAAGCAATCTATTAAGGCCCAACCCATACTGACGAGCCCA
 AAGTTCTTTGCTTTTATGTTTGGTTTCAAGCCCAACTTCGAGCTAGGCAAAAACAACGTCTTTGAATAG
 ACTCTCTCTTAAGCAGATGCAAGCTGCAATGGTCAAGCCCAACTTAAACAGTGGCTAGCAATGGCATGATCTCCATTGACAG
 TGACTTCTGCTCTCTTTAATAATATTAACAACAACCTCTACTCTCTCAAAATATAACAGATCTTTTGAATCAATCTCT
 CATTCAAAATCTCATTCTCTAGTAAACAAAGAACAAAATAATGGCGATACAGTAGAGAACCCATCAGGATCATCGGCA
 GAGCCAGTACCAGATGGCGGAGAGCCAGACCACTACAGATGTCGGGACGAGGATCTGACTACTCCAACTGAGCCAGA
 TTGCTAAAGCTGCAACTGCTGCAAGCTGGTGGTTCCCTCTGTTCTCTCAGCTTACCTTGTGGAACTGCTAAGCTT
 TGACTTTGCAACACTCTGCTGTTATCTTCAGCCCAACTCTTGTCCGGCTCTCAGTCAAGTGGACTCTGATCAGCCGTT
 TTCTTCTCTGAGGGTTGGCATGCGCGTATAACGGTTTCTCTTGAATTAAGTAAAGCAGCATTTATCATCTTACTT
 CATAAATTTGTCATATGTCATGCAATGTTGAGCCAGTACTTTGATCAATTTTTTGGTGAATTAACAAATGAACAAT
 AAGAAATTCGAAATTCAGGAACTTTGGTTAATTAATACGAAATTTGACTAGCTAGCTTGAATGTGTGTTATATCAT
 CTATATAGGTAATAATGCTTGGTATGATCTATGATTTGAATAGTACGCAAGGGAGGACCCCAAGGATCAGCAAGT

SacI
 TGGACATGCAAGATGAATTTGGAAAGCAAGCTCAGGATCTGAAGACACAGCTCACTACTAGGAGACCAACTACTGTTG
 GGAACATGACCGTCAAGTACTGTTGGTGGCAGCAGCACTACTATTGGATGAGGGGTGTGTTCTGCGAGATACACACAAG
 GTGAGTGTCTCATCGTTTAAAGATTGGGGAAGCAATTTAAAGGCTGTACTGATTGCTTTCTGATGATCTCCAGC
 AGTGTCAATTTGATGAGCATGTAAATTAGTGAAGCAACTAATGAGTTTCAAAAACATGTCTTCTGATGCTCCATGCTC
 CCTGTGAAAGTCACTCAGCACTCTCTTGGAGATGAATGTGTAAGTTGATCCCTCTGTAAGCATATGTTGATGCTG
 ACTCTGTGGAAGCAAGACTGAAAGAAATGAATGCTTCTGAGCCCAAGATGATAGCCAGACTCCCTAAATGAAAC
 CAGACCCCAACTTTGTTGATGATTAAAGGAGTGAAGAAAGTTTGGGAAATACCTATACAAATTTCTGAAAGC
 ATCTCTACTTTATGACAGCAACTCTTTACTATGCTAATTAATATAATGAGTTTTCAGAAATGCTGCAAGCTGAAATA
 AAGTGGCTGCTGCTAECGAAAGATTGAACCTATGAGGAAAGGACTACTGATTCATCTGCGAGCAGAGACTCAGGTGCTCA
 GTATTCAAAATTTGGAAGAAAGCTTTAAAGCATGGTCACTGCTGCTGAGCCAGAAATTTCCAAAGCTGAGTTTGTAG
 AAGTACCAAGTACTGACAGATCTCAAAAAGTCCCAAGGAATGCTGATGCTGACTTGAATGGCAGATGACAGGG
 CAGATCTGCAAGTACATATGTAATCAAGATACAATCTCAGTAACTGAAGAAATGCTGATGAAGCTTTGTTGAAA
 AATCCCACTGCTGCTGAGGTGAAGAAAGTCCATACCTGAAGACTGCCCCCAATTAACTGCTGCTTTGCTGAAGTAAAG
 ATGTTTGAAGAACTATAGGAAAGCAAGATGCTTCTGAGGCTGTTTGTATGAATTTCAAGAGGCACTCGTAATATG
 CTGCTCAGTCTATGAGACTTGCAGGAAATGAAGCCAGACTGAGGAAATGCTGTGCAAGATGATCCAGTCTGCT
 ATTCCAGTGTTTGAAGCTTAAAGCATTTGCTGATGAGCTCAAAATTAATCAAAACAAGCTGCAACTGCAAAATC
 TTGAGAGTATGATTCAAAATGCGCTCATACTTGGTACAGCAGGAAATACCCCAAGTGTCAACTGCAACTCTGTTGGAG
 TTTCAAGAACCTTAGGAAAGTGGTACTAGGTTTGTACAAGCCGGAATCAGAAAGAAATGCTTACTGAAAGTATCTGA
 CCTGATCTGCAAGCTGTTGCTGCTGCTGATGAGAACACCACTGAGTGAAGAAATCAGCAAGTCTGCAAGATGATG
 TGACAGAGGCGCAATTTCTGCTCTGACACTGATGAACATATGATCCAAAGCTTGTATGAAATTTGTTCACTCTCC
 ATGCAATATATGCAACTCCCGTACTGAGAAACAATCAAGAAACAAGTCACTTGTGAGCTGTGAAAGCAGAGCCCA
 AGCCACAGAGAACTCAAAAGCTGATGGAATTTGTTGCTTTTATAGCAAGTCTGTTGAGCTGATGCAAGAGG
 CCTGCTTTGCTGAGGGTCAAAACTGTTGTTTCAACTCAAGAGCTTGGCTAAGATAAATCCAAAGCTTTTACCCCA
 TGATGCTATCTGATGCTCAATACTCCAATCTCGGGAGTATGTTATGAGAAATGAAGTGTTAAGAAATTTGATCAGGGCA

KpnI
 GATAAATAAGCCGAGTTTGAATTTTTGTTAAGTAACTTTATGTTGTTTCTATAATGTTGCAATGGTACCATGTTT
 TTTCCCTCTTTTGAATTCGAAGTGTGTTGTTACTTATTGCTTCTTTGTAAGTGTGAACCTGGCTATATATGG
 AAAAGCTCTGTTTGTAACTTATGTTAATGCTGCTTAAACCAAAAATTTCAATAGCTACAATTTAA
 CAGCAAGCGATGCACTATTAGTACATATTTATTCAAGTATTACATGCAACCCAAACTCAAAAAGTATGCTCT

EcoRI PstI SmaI BamHI XbaI NotI
 CCATTAATACTGAAATGCTCTGATTCTAGTGGATCCCATGCAATTTCTGAGCCCGGGGATCCACTAGTTCTAGAGCC
 SacI
 CCGCCACCGGGTGGAGCTC

suggesting that the regions surrounding these residues may be responsible for FA binding (Reed, 1986). The sequence of amino acid residues at the presumed opening of the regions where FA bind to BSA were identified (Cistola *et al.*, 1987). The three binding sites consisted of two clusters of His-Arg-Arg (amino acids 143-145 and 334-336), and one of Lys-His-Lys (amino acids 532-534). In addition, the amino acid residue tyrosine is located at or near FA binding sites on BSA (Parks *et al.*, 1983). This was verified when the ionization of tyrosine residues was hindered by the binding of FA. Based on the aforementioned observations, the DNA sequence chosen to perform this experiment encoded amino acid residues 377 to 581. Two FA binding sites would be found within this region (Reed, 1986; Hamilton *et al.*, 1991; Cistola *et al.*, 1987).

4.4.1. Genetic Construct to Facilitate Bacterial Expression of Mature BSA

PCR was performed using *Pfu* polymerase on the DNA sequence encoding BSA. The primers BSA975 and BSA976 (Figure 52) were selected so that the mature BSA cDNA would be amplified. A *Bam*HI restriction site was engineered on the 5' end of the fragment and a *Xho*I restriction site was engineered on the 3' end of the fragment by incorporating the necessary base pairs into the primers. The amplified product was analyzed by agarose gel electrophoresis (Figure 53). A DNA fragment of ~1800 bp DNA fragment was produced, corresponding to the size of the mature BSA cDNA. These restriction sites were added to the DNA sequence so that the amplified product, upon restriction, would be compatible with the multiple cloning site of pET26b(+) (Figure 54). A start codon was also added following the engineered *Bam*HI restriction site on the

Figure 52. Primers designed to amplify the mature BSA cDNA for insertion into pET26b(+). A *Bam*HI restriction site was incorporated into the BSA975 primer and an *Xho*I restriction site was incorporated into the BSA976 primer so that these sites would be present at the 5' and 3' ends of the sequence for compatibility with the multiple cloning site of pET26b(+). A start codon was engineered following the *Bam*HI restriction site for translational purposes.

Forward Primer: BSA975

5' - GCGCGGATCCATGAGGGGTGTGTTTCGTCGAGAT - 3'

| | | |
 Generic Bases Added for Cutting Efficiency

| | | |
*Bam*HI Restriction Site

| |
 Start Codon

|
 Bases Complementary to Lagging Strand of BSA
 cDNA Following Signal Peptide

Reverse Primer: BSA976

5' - GCGCCTCGAGGGCTAAGGCTGTTTGAGT - 3'

| | |
 Generic Bases Added for Cutting Efficiency

| | |
*Xho*I Restriction Site

|

Bases Complementary to Leading Strand of BSA
 cDNA

Figure 53. Agarose gel electrophoresis analysis of PCR amplification of BSA cDNA using *Pfu* polymerase and primers BSA975 and BSA976. The reaction conditions were 94°C for 1 minute (hot start) and 35 subsequent PCR cycles at 94°C for 45 seconds, 55°C for 1 minute and 72°C for 2 minutes. Following the 35 cycles, a final extension of 72°C for 5 minutes was performed. The amplified DNA was run on a 0.8% (w/v) agarose gel. Lane 1 contains λ DNA/ *EcoRI*+*HindIII* Ladder (MBI Fermentas, Flamborough, ON) and lane 2 contains 5 μ L of the PCR reaction.

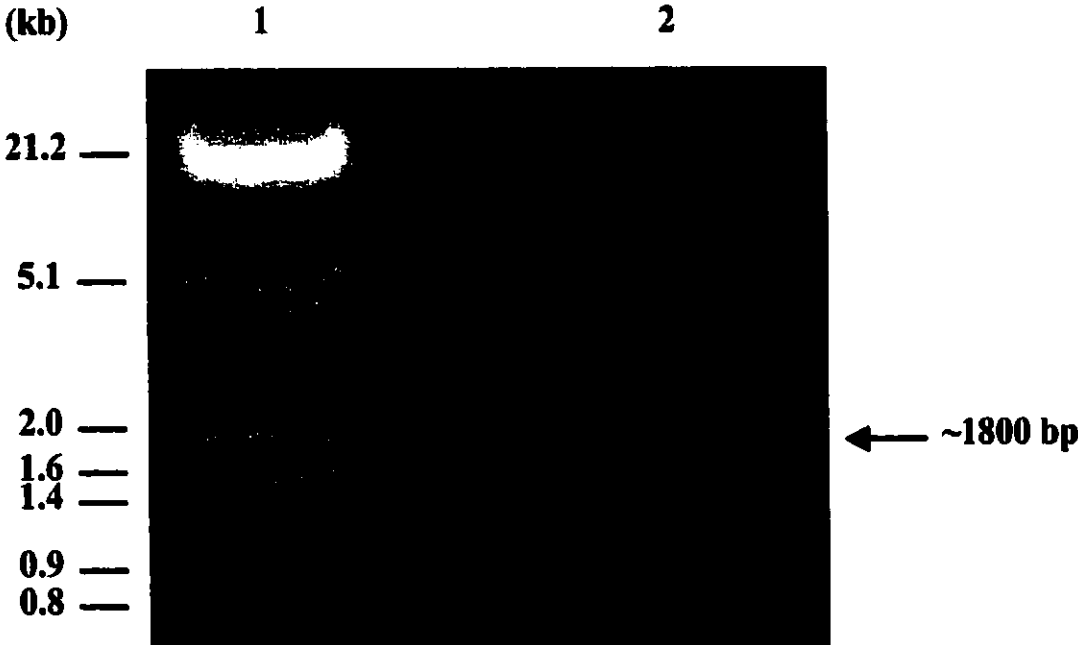
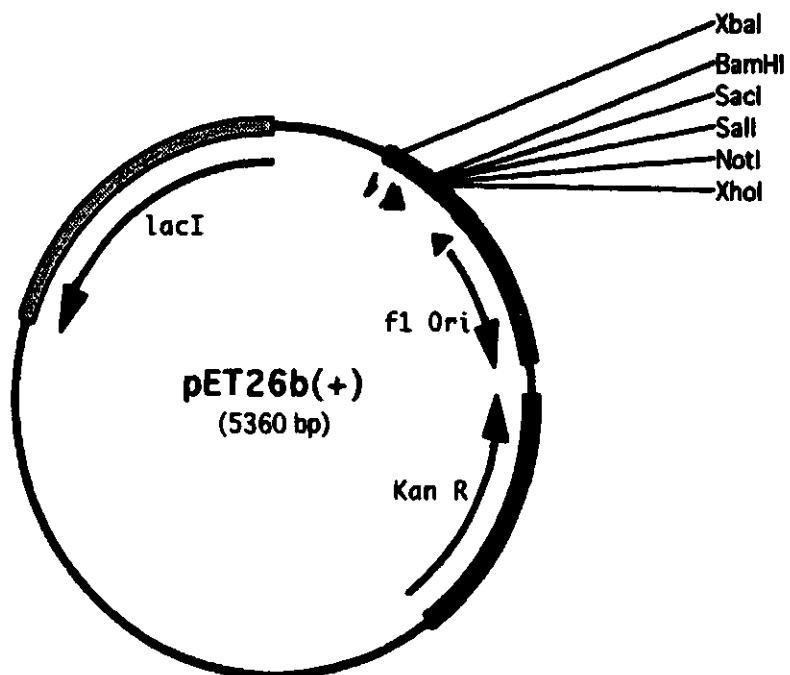


Figure 54. Plasmid map for the bacterial expression vector, pET26b(+). The vector contains a kanamycin resistance gene (solid black section and solid black arrow) as well as a pelB leader sequence (light blue arrow), T7 promoter (black arrow), ribosomal binding site (black arrow), multiple cloning site (green), 6x His tag (black) as well as a terminator (black reverse arrow). The detailed sequence of the multiple cloning site and flanking sequences are shown.



```

                                XbaI
TAATACGACTCACTATAGGGGAATTGTGAGCGGATAACAATTCCCCTCTAGAAATAATTTTGT
----->
TTAACTTTAAGAAGGAGATATACATATGAAATACCTGCTGCCGACCGTGCTGCTGGTCTGCT
      >-----
                                BamHI      SacI
GCTCCTCGCTGCCAGCCGGCGATGGCCATGGATATCGGAATTAATTCGGATCCGAATTCGAG
----->
      Sall      NotI      XhoI
CTCCGTCGACAAGCTTGCGGCCGCACTCGAGCACCACCACCACCACCTGAGATCCGGCTGC
TAACAAAGCCC GAAAGGAAGCTGAGTTGGCTGCTGCCACCGCTGAGCAATAACTAGCATAACC
-----<
CCTTGGGGCCTCTAAACGGGTCTTGAGGGGTTTTTTG
----->
    
```

forward primer so that the sequence could be properly translated once transcribed. In addition, GCGC was added external to the restrictions sites of each primer so that the amplified fragment could be directly restricted instead of ligating it into a generic plasmid like pUC19 or pBlueScript for efficient restriction.

The PCR amplification was restricted with *Bam*HI and *Xho*I, as was the pET26b(+) plasmid DNA. Following restrictions, a ligation reaction was set up and transformed into competent *E. coli* the following day. Putative clones were first identified using α -complementation. A patch plate was constructed with the white colonies and colony PCR was performed. The amplified product was analyzed by agarose gel electrophoresis (Figure 55). A DNA fragment of ~1800 bp was produced, corresponding to the size of the mature BSA cDNA. To ensure that the restriction sites were intact, a restriction analysis was performed. The digests were run on an agarose gel (Figure 56). In addition to the restriction fragments present when pET26b(+) was cut, the plasmid DNA from the three putative clones revealed a DNA fragment of ~1800 bp. This DNA fragment corresponded to the *BSA07* gene. The plasmid DNA from one of the putative clones was sent for sequencing and compared to the known cDNA sequence encoding BSA (Figure 57). The sequence for this plasmid was correct when compared to cDNA encoding BSA and it was inserted correctly in the *Bam*HI and *Xho*I restriction sites of pET26b(+). This new plasmid containing the mature BSA cDNA in the parent pET26b(+) plasmid was named pPB(Ex-BSA07) (Figure 58).

Figure 55. Electrophoretic analysis of DNA following colony PCR using *Taq* polymerase and primers BSA975 and BSA976. The reaction conditions were 94°C for 1 minute (hot start) and 35 subsequent PCR cycles at 94°C for 45 seconds, 55°C for 1 minute and 72°C for 1 minute. Following the 35 cycles, a final extension of 72°C for 5 minutes was performed. The amplified DNA was run on a 0.8% (w/v) agarose gel. Lane 1 contains λ DNA/ *EcoRI*+*HindIII* Ladder (MBI Fermentas, Flamborough, ON) and lanes 2 through 6 contain 5 μ L of the PCR reaction from colonies 1 through 5, respectively.

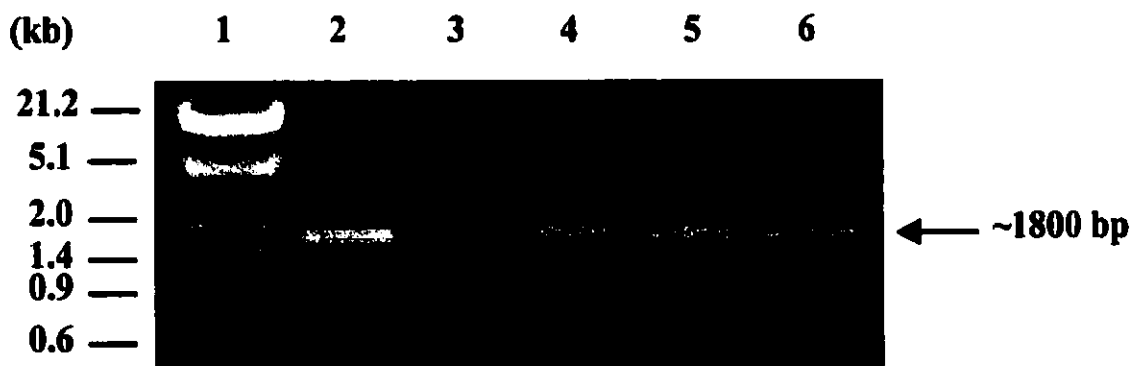


Figure 56. Restriction analysis using *Bam*HI and *Xho*I on plasmid DNA from putative pPB(Ex-BSA07) clones. DNA (1 µg) was restricted for 3 hours at 37°C and run on a 0.8% (w/v) agarose gel. The contents of each lane are as follows: 1, λ DNA/*Eco*RI+*Hind*III Ladder (MBI Fermentas, Flamborough, ON); 2, restricted colony 1 plasmid DNA; 3, restricted colony 2 plasmid DNA; 4, restricted colony 3 plasmid DNA; 5, restricted pET26b(+).

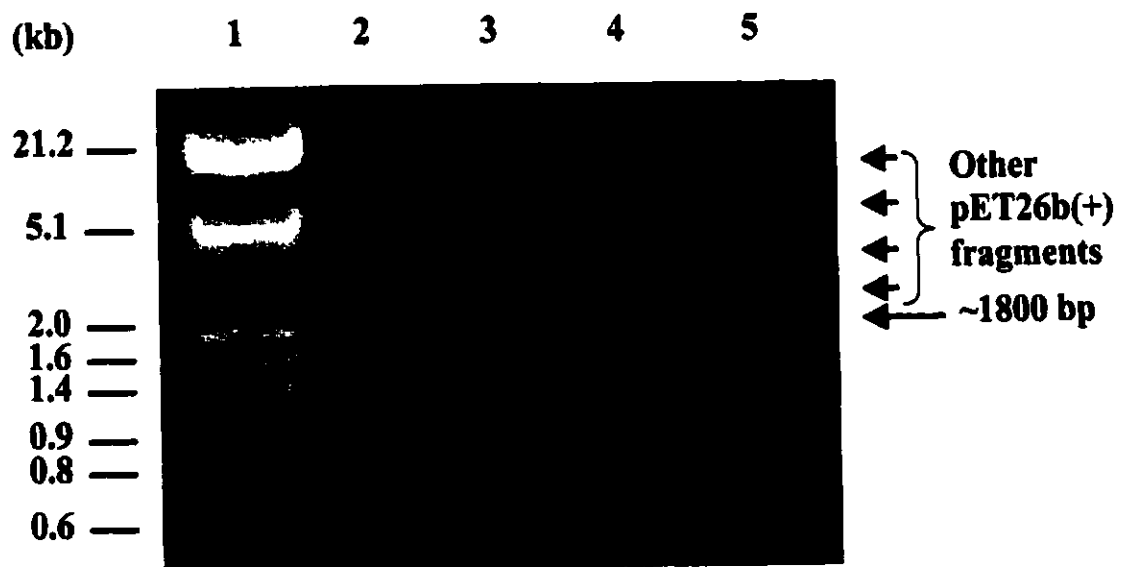
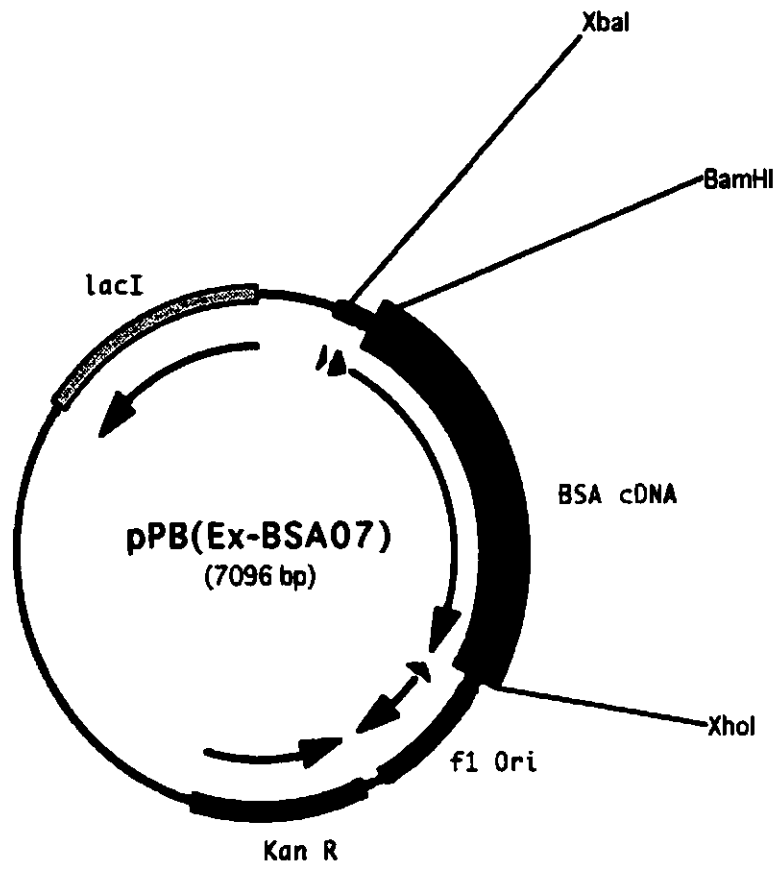


Figure 57. Sequence alignment for a putative *BSA07* gene in colony 1 against the known BSA cDNA sequence. Identical nucleotides are highlighted in yellow. The engineered *Bam*HI and *Xho*I restriction sites are shown in blue and the engineered start codon is shown in red.

PUTATIVE BSA07 pBT26B(+)	TGGTCTGCTGCTCCTCGCTGCCAGCCGGCGATGGCCATGGATATCGGAATTAAT TGGTCTGCTGCTCCTCGCTGCCAGCCGGCGATGGCCATGGATATCGGAATTAAT	55 55
PUTATIVE BSA07 pBT26B(+)/BSA	TCGGATCCATGAGGGGTGTGTTTCGTGAGATACACACAAGAGTGAGATTGCTCA TC AGGGGTGTGTTTCGTGAGATACACACAAGAGTGAGATTGCTCA	110 57/44
PUTATIVE BSA07 BSA	TCGGTTTAAAGATTGGGAGAAGAACATTTTAAAGCCTGGTACTGATTGCCTTT TCGGTTTAAAGATTGGGAGAAGAACATTTTAAAGCCTGGTACTGATTGCCTTT	165 99
PUTATIVE BSA07 BSA	TCTCAGTATCTCCAGCAGTGTCCATTTGATGAGCATGTAAAATTAGTGAACGAAC TCTCAGTATCTCCAGCAGTGTCCATTTGATGAGCATGTAAAATTAGTGAACGAAC	220 154
PUTATIVE BSA07 BSA	TAACTGAGTTTGCAAAAACATGTGTGCTGATGAGTCCCATGCCGGCTGTGAAAA TAACTGAGTTTGCAAAAACATGTGTGCTGATGAGTCCCATGCCGGCTGTGAAAA	275 209
PUTATIVE BSA07 BSA	GTCAC TTCACACTCTCTTTGGAGATGAATTGTGTAAGTTGCATCCCTTCGTGAA GTCAC TTCACACTCTCTTTGGAGATGAATTGTGTAAGTTGCATCCCTTCGTGAA	330 264
PUTATIVE BSA07 BSA	ACCTATGGTGACATGGCTGACTGCTGTGAGAAACAAGAGCCTGAAAGAAATGAAT ACCTATGGTGACATGGCTGACTGCTGTGAGAAACAAGAGCCTGAAAGAAATGAAT	385 319
PUTATIVE BSA07 BSA	GCTTCCTGAGCCACAAGATGATAGCCAGACCTCCCTAAATTGAAACCAGACCC GCTTCCTGAGCCACAAGATGATAGCCAGACCTCCCTAAATTGAAACCAGACCC	440 374
PUTATIVE BSA07 BSA	CAATACTTTGTGTGATGAGTTTAAAGGCAGATGAAAAGAAGTTTGGGGAAAATAC CAATACTTTGTGTGATGAGTTTAAAGGCAGATGAAAAGAAGTTTGGGGAAAATAC	495 429
PUTATIVE BSA07 BSA	CTATACGAAATTGCTAGAAGACATCCCTACTTTTATGCACCAGAACTCCTTTACT CTATACGAAATTGCTAGAAGACATCCCTACTTTTATGCACCAGAACTCCTTTACT	550 484
PUTATIVE BSA07 BSA	ATGCTAATAAATATAATGGAGTTTTTCAAGAAATGCTGCCAAGCTGAAGATAAAGG ATGCTAATAAATATAATGGAGTTTTTCAAGAAATGCTGCCAAGCTGAAGATAAAGG	605 539
PUTATIVE BSA07 BSA	TGCCTGCCTGCTACCAAGATTGAAACTATGAGAGAAAAGTACTGACTTCATCT TGCCTGCCTGCTACCAAGATTGAAACTATGAGAGAAAAGTACTGACTTCATCT	660 594
PUTATIVE BSA07 BSA	GCCAGACAGAGACTCAGGTGTGCCAGTATCAAAAATTTGGAGAAAGAGCTTTAA GCCAGACAGAGACTCAGGTGTGCCAGTATCAAAAATTTGGAGAAAGAGCTTTAA	715 649
PUTATIVE BSA07 BSA	AAGCATGGTCAGTAGCTCGCCTGAGCCAGAAATTTCCAAGGCTGAGTTGTAGA AAGCATGGTCAGTAGCTCGCCTGAGCCAGAAATTTCCAAGGCTGAGTTGTAGA	770 704
PUTATIVE BSA07 BSA	AGTTACCAAGCTAGTGACAGATCTCACAAAAGTCCACAAGGAATGCTGCATGGT AGTTACCAAGCTAGTGACAGATCTCACAAAAGTCCACAAGGAATGCTGCATGGT	825 759
PUTATIVE BSA07 BSA	GACCTACTTGAATGCCAGATGACAGGGCAGATCTTGCCAAGTACATATGTGATA GACCTACTTGAATGCCAGATGACAGGGCAGATCTTGCCAAGTACATATGTGATA	880 814
PUTATIVE BSA07 BSA	ATCAAGATACAATCTCCAGTAACTGAAGGAATGCTGTGATAAGCCTTTGTTGGA ATCAAGATACAATCTCCAGTAACTGAAGGAATGCTGTGATAAGCCTTTGTTGGA	935 869
PUTATIVE BSA07 BSA	AAAAATCCCACTGCATTGCTGAGGTAGAAAAGATGCCATACCTGAAAACCTGCC AAAAATCCCACTGCATTGCTGAGGTAGAAAAGATGCCATACCTGAAAACCTGCC	990 924
PUTATIVE BSA07 BSA	CCATTAACCTGCTGACTTTGCTGAAGATAAGGATGTTTGCAAAAACCTATCAGGAAG CCATTAACCTGCTGACTTTGCTGAAGATAAGGATGTTTGCAAAAACCTATCAGGAAG	1045 979
PUTATIVE BSA07 BSA	CAAAAGATGCCTTCCCTGGGCTCGTTTTTGTATGAATATTCAAGAAGGCATCCTGA CAAAAGATGCCTTCCCTGGGCTCGTTTTTGTATGAATATTCAAGAAGGCATCCTGA	1100 1034
PUTATIVE BSA07 BSA	ATATGCTGTCTCAGTGTATTGAGACTTGCCAAGGAATATGAAGCCACACTGGAG ATATGCTGTCTCAGTGTATTGAGACTTGCCAAGGAATATGAAGCCACACTGGAG	1155 1089
PUTATIVE BSA07 BSA	GAATGCTGTGCCAAGATGATCCACATGCATGCTATTCCACAGTGTGACAAAC GAATGCTGTGCCAAGATGATCCACATGCATGCTATTCCACAGTGTGACAAAC	1210 1144

POTATIVE BSA07 BSA	TTAAGCATCTTGEGGATGAGCCTCAGRATTAAATCAAACAAAACCTGTGACCAATT TTAAGCATCTTGTGGATGAGCCTCAGRATTAAATCAAACAAAACCTGTGACCAATT	1265 1199
POTATIVE BSA07 BSA	CGAAAAACTTGGAGAGTATGGATTCCAAAATGCGCTCATAGTTCGTTACACCAGG CGAAAAACTTGGAGAGTATGGATTCCAAAATGCGCTCATAGTTCGTTACACCAGG	1320 1254
POTATIVE BSA07 BSA	AAAGTACCCCAAGTGTCAACTCCAACCTCTCGTGGAGGTTTCAAGAAGCCTAGGAA AAAGTACCCCAAGTGTCAACTCCAACCTCTCGTGGAGGTTTCAAGAAGCCTAGGAA	1375 1309
POTATIVE BSA07 BSA	AAGTGGTACTAGGTGTTGTACAAAGCCGGAATCAGAAGAATGCCCTGTACTGA AAGTGGTACTAGGTGTTGTACAAAGCCGGAATCAGAAGAATGCCCTGTACTGA	1430 1364
POTATIVE BSA07 BSA	AGACTATCTGAGCTTGATCCTGAACCGGTTGTGGCTGCTGCATGAGAAGACACCA AGACTATCTGAGCTTGATCCTGAACCGGTTGTGGCTGCTGCATGAGAAGACACCA	1485 1419
POTATIVE BSA07 BSA	GTGAGTGAAAAGTCACCAAGTGTGACAGAGTCAATGGTGAACAGACGGCCAT GTGAGTGAAAAGTCACCAAGTGTGACAGAGTCAATGGTGAACAGACGGCCAT	1540 1474
POTATIVE BSA07 BSA	GTTTCTCTGCTCTGACACCTGATGAACATATGTACCCAAAGCCTTTGATGAGAA GTTTCTCTGCTCTGACACCTGATGAACATATGTACCCAAAGCCTTTGATGAGAA	1595 1529
POTATIVE BSA07 BSA	ATTGTTACCTTCCATGCAGATATATGCACACTTCCCGATACTGAGAAACAAATC ATTGTTACCTTCCATGCAGATATATGCACACTTCCCGATACTGAGAAACAAATC	1650 1584
POTATIVE BSA07 BSA	AAGAAACAAACTGCACTTGTGAGCTGTTGAAACACAAGCCCAAGGCAACAGAGG AAGAAACAAACTGCACTTGTGAGCTGTTGAAACACAAGCCCAAGGCAACAGAGG	1705 1639
POTATIVE BSA07 BSA	AACAACGAAAACCGTCAATGGAGAATTTGTGGCTTTTGTAGACAAGTGTGTGC AACAACGAAAACCGTCAATGGAGAATTTGTGGCTTTTGTAGACAAGTGTGTGC	1760 1694
POTATIVE BSA07 BSA	AGCTGATGACAAAGAGGCCTGCTTTGCTGTGGAGGGTCCAAAACCTTGTGTTTCA AGCTGATGACAAAGAGGCCTGCTTTGCTGTGGAGGGTCCAAAACCTTGTGTTTCA	1815 1749
POTATIVE BSA07 BSA/pET26B(+)	ACTCAAACAGCCTTAGCCCTCGAGCACCACCACCACCACCCTGA ACTCAAACAGCCTTAGCC CACCACCACCACCACCCTGA	1860 1767/21

Figure 58. Plasmid map for pPB(Ex-BSA07). The vector contains a kanamycin resistance gene (solid black section and solid black arrow) as well as the mature BSA cDNA (purple arrow) that was inserted into the multiple cloning site of pET26b(+) via the *Bam*HI and *Xho*I restriction sites that were engineered on the 5' and 3' ends of the DNA sequence, respectively. The vector also contains a *pelB* leader sequence (light blue arrow), T7 promoter (black arrow) and a terminator (black reverse arrow). The detailed sequence of the insert is shown along with some flanking sequences on the page following the plasmid map.



BamHI

GGA TCCATGAGGGGTGTGTTTCGTGCGAGATACACACAAGAGTGAGATTGCTCATCGGTTTAAAG
 A TTTGGGAGAAGAACATTTTAAAGGCCTGGTACTGATTGCCTTTCTCAGTATCTCCAGCAGTG
 TCCATTTGATGAGCATGTAAAATTAGTGAACGAACTAAGTGTGTTGCAAAAACATGTGTTGCT
 GATGAGTCCCATGCCGGCTGTGAAAAGTCACTTCACTCTCTTTGGAGATGAATTGTGTTAAAG
 TTGCATCCCTTCGTGAAACCTATGGTGACATGGCTGACTGCTGTGAGAAAACAAGAGCCTGAAAG
 AAATGAATGCTTCTGAGCCACAAAGATGATAGCCAGACCTCCCTAAATTGAAACCAGACCCC
 AATACTTTGTGTGATGAGTTTAAAGGCAGATGAAAAGAAGTTTGGGGAAAATACCTATACGAAA
 TTGCTAGAAGACATCCCTACTTTTATGCACCAGAACTCCTTTACTATGCTAATAAATAAATGG
 AGTTTTTCAAGAATGCTGCCAAGCTGAAGATAAAGGTGCCTGCCTGCTACCAAAGATTGAAACT
 ATGAGAGAAAAGTACTGACTTCACTGCCAGACAGAGACTCAGGTGTGCCAGTATTCAAAAAT
 TTGGAGAAAAGACTTTAAAAGCATGGTCAGTAGCTCGCCTGAGCCAGAAATTTCCCAAGGCTGA
 GTTTGTAGAAGTTACCAAGCTAGTGACAGATCTCAAAAAGTCCACAAGGAATGCTGCCATGGT
 GACCTACTTGAATGCCGAGATGACAGGGCAGATCTTGCCAAGTACATATGTGATAATCAAGATA
 CAATCTCCAGTAACTGAAGGAATGCTGTGATAAGCCTTTGTTGGAAAAATCCCACTGCATTGC
 TGAGGTAGAAAAGATGCCATACCTGAAAACCTGCCCCATTAAGTCTGACTTTGCTGAAGAT
 AAGGATGTTTGCAAAACATAACAGGAAGCAAAGATGCCTTCTGGGCTCGTTTTTGTATGAAT
 ATTCAGAAGGCATCCTGAATATGCTGTCTCAGTGCTATTGAGACTTGCCAAGGAATATGAAGC
 CACTGAGGAATGCTGTGCCAAAGATGATCCACATGCATGCTATTCCACAGTGTGTTGACAAA
 CTTAAGCATCTTGTGGATGAGCCTCAGAAATTAATCAAACTGACCAATTGAAAAAC
 TTGGAGATGGAATCCAAAATGCGCTCATAGTTCGTTACACCAGGAAAGTACCCCAAGTGTG
 AACTCCAACCTCTCGTGGAGTTTCAAGAAGCCTAGGAAAAGTGGTACTAGGTGTTGTACAAAG
 CCGGAATCAGAAAGATGCCCTGACTGAAGACTATCTGAGCTTGATCCTGAACCGTTGTGGC
 TGCTGCATGAGAAGACACCAGTGTGAAAAGTCAACCAAGTGTGCAAGAGTCAATTGGTGAA
 CAGACGGCCATGTTTCTGCTCTGACACCTGATGAAACATATGTACCCAAAGCCTTTGATGAG
 AAATGTTACCTTCCATGCAGATATATGCACACTCCCGATACTGAGAAAACAAATCAAGAAAC
 AAATGCACTTGTGAGCTGTTGAAACACAAGCCCAAGGCAACAGAGGAACAACTGAAAACCGT
 CATGGAGAATTTGTGGCTTTGTAGACAAGTGTGTGCACTGATGACAAAGAGGCCTGCTTT

XhoI

GCTGTGGAGGGTCCAAAACCTGTTGTTTCAACTCAAACAGCCTTAGCCCTCGAGCACCACCACC
 ACCACCACTGAGATCCGGCTGCTAACAAAGCCC GAAAAGGAGCTGAGTTGGCTGCTGCCACCGC
 TGAGCAATAACTAGCATAACCCCTTGGGGCCTCAAACGGGTCTTGAGGGGTTTTTTG

4.4.2. Genetic Construct to Facilitate Bacterial Expression of a Truncation of BSA

PCR was performed using *Pfu* polymerase on the DNA sequence encoding BSA. The primers BSA842 and BSA843 (Figure 59) were selected so that the truncated BSA cDNA encompassed the DNA sequence encoding amino acids 377 through 581. The amplified product was analyzed by agarose gel electrophoresis (Figure 60). A DNA fragment of ~600 bp DNA fragment was produced, corresponding to the size of the BSA cDNA truncation. A *Bam*HI restriction site was engineered on the 5' end of the fragment and a *Xho*I restriction site was engineered on the 3' end of the fragment by incorporating the necessary base pairs into the primers. Restriction sites were added to the DNA sequence so that the amplified product, upon restriction, would be compatible with the multiple cloning site of pET26b(+) (Figure 54). A start codon was also added following the engineered *Bam*HI restriction site on the forward primer so that the sequence could be properly translated once transcribed. In addition, GCGC was added external to the restrictions sites of each primer so that the amplified fragment could be directly restricted instead of ligating it into a generic plasmid like pUC19 or pBlueScript for efficient restriction.

The PCR amplification was restricted with *Bam*HI and *Xho*I, as was the pET26b(+) plasmid DNA. Following restrictions, a ligation reaction was set up and transformed into competent *E. coli* the following day. Putative clones were first identified using α -complementation. A patch plate was constructed with the white colonies and colony PCR was performed. The amplified product was analyzed by agarose gel electrophoresis (Figure 61). A DNA fragment of ~600 bp was produced for

Figure 59. Primers designed to amplify a truncation of the BSA cDNA for insertion into pET26b(+). A *Bam*HI restriction site was incorporated into the BSA842 primer and an *Xho*I restriction site was incorporated into the BSA843 primer so that these sites would be present at the 5' and 3' ends of the sequence for compatibility with the multiple cloning site of pET26b(+). A start codon was engineered following the *Bam*HI restriction site for translational purposes.

Forward Primer: BSA842

5' - GCGCGGATCCATGGAAGCCACACTGGAGGAA - 3'

| | | |
 Generic Bases Added for Cutting Efficiency

| | |
*Bam*HI Restriction Site

| |
 Start Codon

|
 Bases Complementary to Lagging Strand of BSA
 cDNA Starting at Amino Acid 377

Reverse Primer: BSA843

5' - GCGCCTCGAGGCACTTGTCTACAAAAG - 3'

| | |
 Generic Bases Added for Cutting Efficiency

| |
*Xho*I Restriction Site

|
 Bases Complementary to Leading Strand of BSA
 cDNA Ending at Amino Acid 581

Figure 60. Agarose gel electrophoresis analysis of PCR amplification of BSA cDNA using *Pfu* polymerase and primers BSA842 and BSA843. The reaction conditions were 94°C for 1 minute (hot start) and 35 subsequent PCR cycles at 94°C for 45 seconds, 55°C for 1 minute and 72°C for 1 minute. Following the 35 cycles, a final extension of 72°C for 5 minutes was performed. The amplified DNA was run on a 0.8% (w/v) agarose gel. Lane 1 contains λ DNA/ *EcoRI*+*HindIII* Ladder (MBI Fermentas, Flamborough, ON) and lane 2 contains 5 μ L of the PCR reaction.

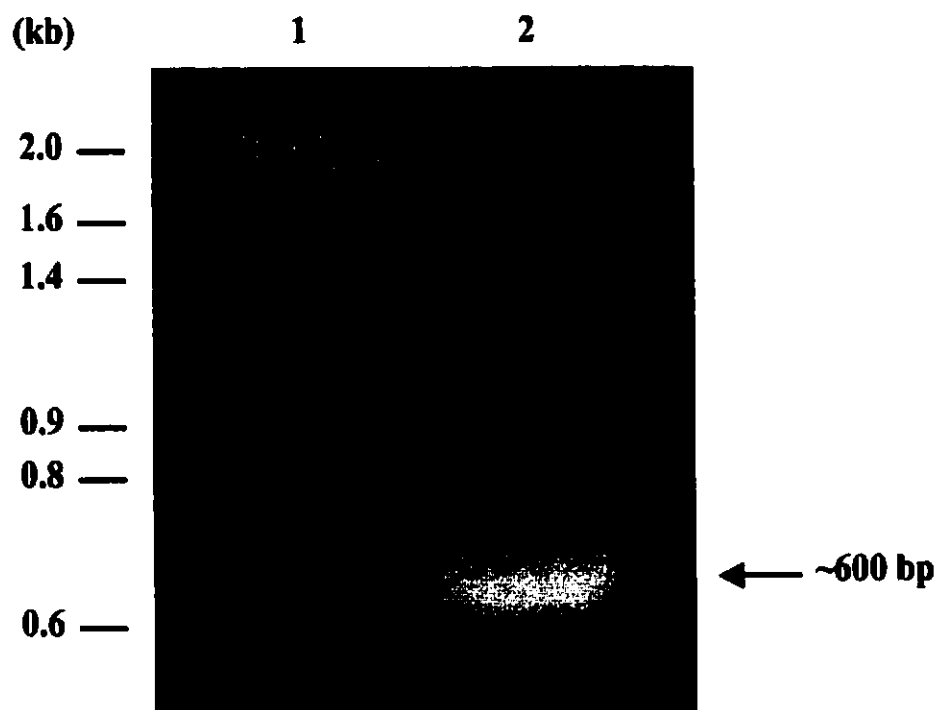
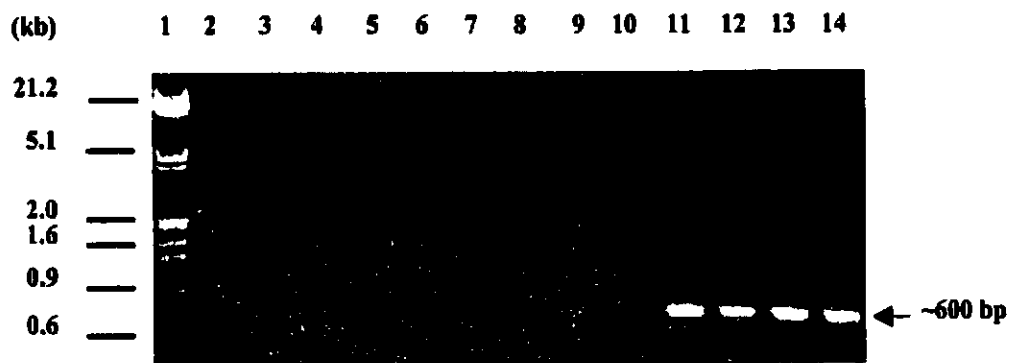


Figure 61. Electrophoretic analysis of DNA following colony PCR using *Taq* polymerase and primers BSA842 and BSA843. The reaction conditions were 94°C for 1 minute (hot start) and 35 subsequent PCR cycles at 94°C for 45 seconds, 55°C for 1 minute and 72°C for 1 minute. Following the 35 cycles, a final extension of 72°C for 5 minutes was performed. The amplified DNA was run on a 0.8% (w/v) agarose gel. Lane 1 contains λ DNA/ *EcoRI*+*HindIII* Ladder (MBI Fermentas, Flamborough, ON) and lanes 2 through 14 contain 5 μ L of the PCR reaction from colonies 1 through 13, respectively.

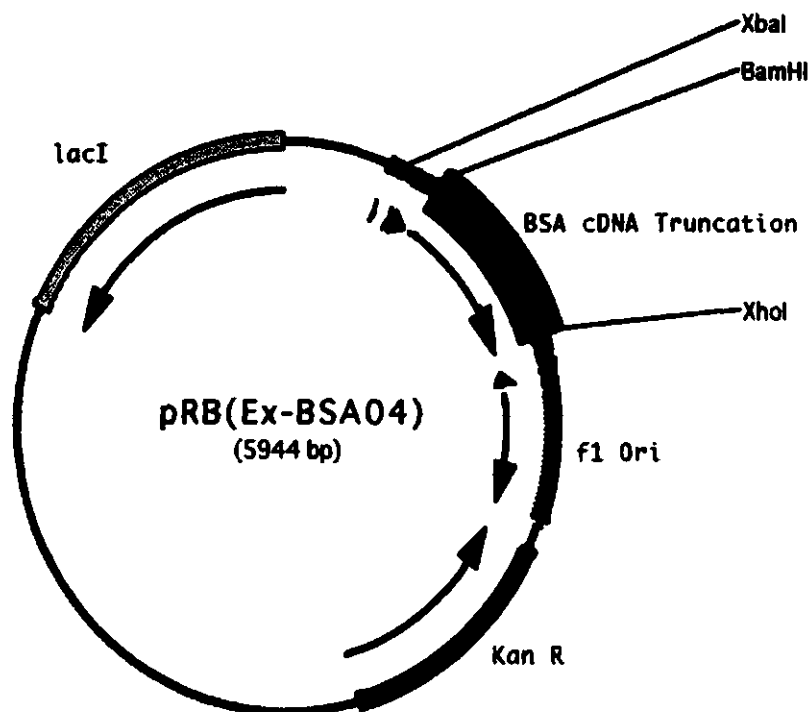


colonies 10, 11, 12 and 13. This fragment corresponded to the size of the truncation of BSA cDNA. The plasmid DNA from one of the putative clones was sent for sequencing and compared to the known cDNA sequence encoding BSA (Figure 62). The sequence for this plasmid was correct when compared to truncated DNA encoding BSA and was inserted correctly in the *Bam*HI and *Xho*I restriction sites of pET26b(+). This new plasmid containing the BSA cDNA truncated in the parent pET26b(+) plasmid was named pRB(Ex-BSA04) (Figure 63).

Figure 62. Sequence alignment for a putative *BSA04* gene in colony 10 against the known BSA cDNA sequence. Identical nucleotides are highlighted in yellow. The engineered *Bam*HI and *Xho*I restriction sites are shown in blue and the engineered start codon is shown in red.

POTATIVE BSA04 pBT26b(+)	TGGTCTGCTGCTCCTCGCTGCCAGCCGGCGATGGCCATGGATATCGGAATTAAT TGGTCTGCTGCTCCTCGCTGCCAGCCGGCGATGGCCATGGATATCGGAATTAAT	55 55
POTATIVE BSA04 pBT26b(+)/BSA	TCGGATCCATGGAGCCCACTGGAGGAATGCTGTGCCAAAGATGATCCACATGC TC GAAGCCCACTGGAGGAATGCTGTGCCAAAGATGATCCACATGC	110 57 / 44
POTATIVE BSA04 BSA	ATGCTATCCACAGTGTGTTGACAACTTAAGCATCTTGTGGATGAGCCTCAGAAT ATGCTATCCACAGTGTGTTGACAACTTAAGCATCTTGTGGATGAGCCTCAGAAT	165 99
POTATIVE BSA04 BSA	TTAATCAAACAAAACGTGACCAATTCGAAAACTTGGAGAGTATGGATTCCAAA TTAATCAAACAAAACGTGACCAATTCGAAAACTTGGAGAGTATGGATTCCAAA	220 154
POTATIVE BSA04 BSA	ATGCGCTCATAGTTCGTTACACCAGGAAAGTACCCCAAGTGTCAACTCCAACCTCT ATGCGCTCATAGTTCGTTACACCAGGAAAGTACCCCAAGTGTCAACTCCAACCTCT	275 209
POTATIVE BSA04 BSA	CGTGGAGGTTTCAAGAAGCCTAGGAAAAGTGGTACTAGGTGTTGTACAAAGCCG CGTGGAGGTTTCAAGAAGCCTAGGAAAAGTGGTACTAGGTGTTGTACAAAGCCG	330 264
POTATIVE BSA04 BSA	GAATCAGAAAGAATGCCCTGTACTGAAGACTATCTGAGCTTGATCCTGAACCGGT GAATCAGAAAGAATGCCCTGTACTGAAGACTATCTGAGCTTGATCCTGAACCGGT	385 319
POTATIVE BSA04 BSA	TGTGCGTGTGCTGATGAGAAGACACCAGTGAAGTAAAAAGTACCAAGTGTGCAC TGTGCGTGTGCTGATGAGAAGACACCAGTGAAGTAAAAAGTACCAAGTGTGCAC	440 374
POTATIVE BSA04 BSA	AGAGTCATTGGTGAACAGACAGCCCATGTTTCTCTGCTCTGACACCTGATGAAACA AGAGTCATTGGTGAACAGACAGCCCATGTTTCTCTGCTCTGACACCTGATGAAACA	495 429
POTATIVE BSA04 BSA	TATGTACCCAAAGCCTTTGATGAGAAATGTTTACCTTCCATGCAGATATATGCA TATGTACCCAAAGCCTTTGATGAGAAATGTTTACCTTCCATGCAGATATATGCA	550 484
POTATIVE BSA04 BSA	CACTCCCGTACTGAGAAACAAATCAAGAAACAACTGCACTTGTGAGCTGTT CACTCCCGTACTGAGAAACAAATCAAGAAACAACTGCACTTGTGAGCTGTT	605 539
POTATIVE BSA04 BSA	GAAACACAAGCCCAAGGCAACAGAGGAACAACCTGAAAACCGTCATGGAGAATTTT GAAACACAAGCCCAAGGCAACAGAGGAACAACCTGAAAACCGTCATGGAGAATTTT	660 594
POTATIVE BSA04 BSA/pBT26b(+)	GTGGCTTTTGTAGACAAGCTGAGCACCCACCACCACCACCCTGA GTGGCTTTTGTAGACAAG CACCACCACCACCACCCTGA	705 612 / 21

Figure 63. Plasmid map for pRB(Ex-BSA04). The vector contains a kanamycin resistance gene (solid black section and solid black arrow) as well as the BSA cDNA truncation (purple arrow) that was inserted into the multiple cloning site of pET26b(+) via the *Bam*HI and *Xho*I restriction sites that were engineered on the 5' and 3' ends of the DNA sequence, respectively. The vector also contains a *pelB* leader sequence (light blue arrow), T7 promoter (black arrow) and a terminator (black reverse arrow). The detailed sequence of the insert is shown along with some flanking sequences.



BamHI
 GGATCCATGGAAGCCACACTGGAGGAATGCTGTGCCAAAGATGATCCACATGCATGCTATTCCACAGT
 GTTTGACAAACTTAAGCATCTTGTGGATGAGCCTCAGAATTTAATCAAACAAAAGTGTGACCAATTGG
 AAAAACTTGGAGAGTATGGATTCCAAAATGCGCTCATAGTTCGTTACACCAGGAAAGTACCCCAAGTG
 TCAACTCCAACCTCTCGTGGAGGTTTCAAGAAGCCTAGGAAAAGTGGGTACTAGGTGTTGTACAAAGCC
 GGAATCAGAAAGAATGCCCTGTAAGACTATCTGAGCTTGATCCTGAACCGGTTGTGCGTGCTGC
 ATGAGAAGACACCAGTGAGTGAAAAAGTCACCAAGTCTGCACAGAGTCATTGGTGAACAGACGGCCA
 TGTTTCTCTGCTCTGACACCTGATGAAACATATGTACCCAAAGCCTTTGATGAGAAATTGTTACCTT
 CCATGCAGATATATGCACACTTCCCGATACTGAGAAACAAATCAAGAAACAAACTGCACCTGTTGAGC
 TGTTGAAACACAAGCCCAAGGCAACAGAGGAACAACTGAAAACCGTCATGGAGAATTTTGTGGCTTTT
 GTAGACAAGTGCCTCGAGCACCACCACCACCCTGAGATCCGGCTGCTAACAAAGCCCGAAAGGA
 AGCTGAGTTGGCTGCTGCCACCGCTGAGCAATAACTAGCATAACCCCTTGGGGCCTCTAAACGGGTCT
 TGAGGGGTTTTTTG

XhoI

5. SUMMARY

Genetic constructs were engineered to facilitate the expression of ASP or BSA in the cytosol of developing seeds and other genetic constructs were engineered to facilitate the expression of ASP or BSA in the ER of developing seeds. Following transformation of *B. napus* using *A. tumefaciens* and tissue culture, the first generation data revealed that the total lipid within the mature seeds of ASP and BSA plants was not significantly different than the total lipid of negative control plants using both gravimetric and LR-NMR methods of analysis. There was a correlation, however, that was highly significant between the two methods of analysis when measuring the total lipid from mature seeds of all plants ($r^2 = 0.70$, $P < 0.0001$). This correlation between the two methods has been identified in previous studies (Gunstone and Wolff, 1996; Hutton *et al.*, 1999). Upon observation of the data on a per seed basis, it was found that ASP 8 and ASP 10 plants did produce significantly more lipid per seed than negative control plants ($P = 0.04$ and $P = 0.03$, respectively). The seeds from BSA plants when measured on a per seed basis, however, were not significantly different than those obtained from negative control plants. ASP 3 and ASP 14 had significant differences in the FA composition of total acyl lipids as compared to control plants. ASP 3 had significantly lower levels of C18:2 ($P = 0.05$) and ASP 14 had significantly lower levels of C18:3 ($P = 0.04$). ASP 14 had the DNA sequence encoding ASP incorporated into the genome and transcripts were detected. ASP protein, however, was not detected immunochemically in mature seed tissue. ASP 3 also had the DNA sequence encoding ASP incorporated into the plant genome but transcript and protein were not detected due to a shortage of seeds for

analysis. The FA composition of the total acyl lipids within mature seeds of BSA 11 was significantly different than the profile obtained from the seeds of control plants. BSA 11 had significantly higher levels of C18:2 ($P = 0.04$) and C18:3 ($P = 0.02$). BSA 11 had the BSA cDNA incorporated into the genome and also produced transcript. BSA protein, however, was not immunochemically detected.

6. FUTURE DIRECTIONS

6.1. Transgenic Plants Carrying ASP or BSA for Cytosolic Expression

Further investigation is required for these plants. The next logical step would be to grow a second generation to ensure that the ASP or BSA gene is integrated in a stable manner into the *B. napus* genome. Identification of the polypeptides within seeds from these transgenic plants is crucial to linking phenotypic effects with bioactivity of the mammalian proteins.

6.2. Genetic Constructs to Facilitate the Expression of ASP or BSA in the ER

The next step that must be performed with these genetic constructs is to insert the ASP and BSA genes into a binary vector that can be electroporated into *A. tumefaciens*. In doing this, transformation can be performed using the tissue culture technique, the same as was used when transforming plants with the constructs to facilitate cytosolic expression of ASP or BSA. Upon engineering these plants, seeds analysis should be performed and compared to the data sets for negative control plants as well as for the plants putatively expressing ASP or BSA in the cytoplasm of seeds.

6.3. Crossing ASP Plants With BSA Plants

BSA-producing plants could be crossed with ASP-producing plants as a means of examining possible synergistic effects of the two genes on oil accumulation. Another interesting study would be to perform a series of crosses with these plants producing mammalian proteins, some expressing the proteins in the ER and others in the cytosol, and observing optimal combinations. This would require that the genetic constructs to facilitate the expression of ASP or BSA in the ER were taken through tissue culture and transgenic plants were obtained, identified and characterized.

6.4. Bacterial Expression of Mature BSA and a Truncation of BSA

Now that the constructs have been engineered to facilitate the expression of recombinant BSA and a truncation of BSA in *E. coli*, the expression and purification work should be carried out. Upon obtaining relatively pure polypeptides, *in vitro* experimentation should be performed to investigate the stimulatory nature of these proteins on DGAT.

6.5. Biophysical Studies With ASP or BSA and Recombinant DGAT

A recombinant N-terminal truncation of the DGAT enzyme is available (Weselake *et al.*, 2000b). *In vitro* experimentation involving potential physical interactions between ASP or BSA with this recombinant DGAT truncation would be beneficial to understanding their stimulatory nature. These studies may give insight into potential binding sites on DGAT where the mammalian proteins may act.

6.6. Expression of Truncated BSA in Developing Seeds

Depending on the results obtained with *in vitro* stimulation of DGAT by bacterially expressed recombinant BSA and the truncation of BSA, genetic constructs could be engineered to express the BSA truncation in the cytoplasm or ER of developing seeds of *B. napus*. These experiments would provide a comparison between the whole BSA polypeptide to the truncation having high acyl-CoA binding capacity on a variety of traits such as oil content and seed size in developing plants.

LITERATURE CITED

- Abell, B.M., Holbrook, L.A., Abenes, M., Murphy, D.J., Hills, M.J. and Moloney, M.M. 1997. Role of the proline know motif in oleosin endoplasmic reticulum topology and oil body targeting. *Plant Cell* 9: 1481-1493.
- Ahmad, N. and Qasim, M.A. 1995. Fatty acid binding to bovine serum albumin prevents formation of intermediate during denaturation. *European Journal of Biochemistry* 227: 563-565.
- Al-Malah, K.I., Azzam, M.O.J. and Omari, R.M. 2000. Emulsifying properties of BSA in different vegetable oil emulsions using conductivity technique. *Food Hydrocolloids* 14: 485-490.
- Aronel, V., Lemieux, B., Hwang, I., Gibson, S., Goodman, H. and Somerville, C. 1992. Map-based cloning of a gene controlling omega-3 fatty acid desaturation in *Arabidopsis*. *Science* 258: 1353-1355.
- Bafor, M., Stobart, A.K. and Stymne, S. 1990. Properties of the glycerol acylating enzymes in microsomal preparations from the developing seeds of safflower (*Carthamus tinctorius*) and turnip rape (*Brassica campestris*) and their ability to assemble cocoa-butter type fats. *Journal of the American Oil Chemists' Society* 67: 217-225.
- Baldo, A., Dardano, N., Sniderman, A.D. and Cianflone, K. 1996. Evidence for a novel key regulatory step in the triglyceride synthetic pathway of human adipose tissue: Partial purification of diacylglycerol acyltransferase kinase. *McGill Journal of Medicine* 2: 12-19.
- Baldo, A., Sniderman, A.D., St-Luce, S., Avramoglu, R.K., Maslowska, M., Hoang, B., Monge, J.C., Bell, A., Mulay, S. and Cianflone, K. 1993. The adipsin-acylation stimulating protein system and regulation of intracellular triglyceride synthesis. *Journal of Clinical Investigation* 92: 1543-1557.
- Baldo, A., Sniderman, A.D., St-Luce, S., Zhang, X-J. and Cianflone, K. 1995. Signal transduction pathway of acylation stimulating protein: Involvement of protein kinase C. *Journal of Lipid Research* 36: 1415-1426.
- Baneyx, F. 1999. Recombinant protein expression in *Escherichia coli*. *Current Opinion in Biotechnology* 10: 411-421.
- Barandiaran, X., DiPietro, A. and Martin, J. 1998. Biolistic transfer and expression of a uidA reporter gene in different tissues of *Allium sativum* L. *Plant Cell Reports* 17: 737-741.

- Beaudoin, F., Lacey, D.J. and Napier, J.A. 1999. The biogenesis of the plant seed oil body: oleosin protein is synthesized by ER-bound ribosomes. *Plant Physiology and Biochemistry* 37: 481-490.
- Bernerth, B. and Frentzen, M. 1990. Utilization of erucoyl-CoA by acyltransferases from developing seeds of *Brassica napus* (L.) involved in triacylglycerol biosynthesis. *Plant Science* 67: 21-28.
- Bommineni, V.R., Jauhar, P.P. and Peterson, T.S. 1997. Transgenic durum wheat by microprojectile bombardment of isolated scutella. *The Journal of Heredity* 88: 475-481.
- Booth, J.G., Saponja, J.A. and Parmenter, D.L. 1997. Molecular farming in plants: oilseeds as vehicles for the production of pharmaceutical proteins. *Drug Development Research* 42: 172-181.
- Bouvier-Navé, P., Benveniste, P., Oelkers, P., Sturley, S.L. and Schaller, H. 2000. Expression in yeast and tobacco of plant cDNAs encoding acyl CoA:diacylglycerol acyltransferase. *European Journal of Biochemistry* 267: 85-96.
- Brennan, S.O., Owen, M.C., Boswell, D.R., Lewis, J.H. and Carrell, R.W. 1984. Circulating proalbumin associated with a variant proteinase inhibitor. *Biochimica et Biophysica Acta* 802: 24-28.
- Brough, C.L., Coventry, J.M., Christie, W.W., Kroon, J.T.M., Brown, A.P., Barsby, T.L. and Slabas, A.R. 1996. Towards genetic engineering of triacylglycerols of defined fatty acid composition: major changes in erucic acid content at the *sn*-2 position affected by the introduction of a 1-acyl-*sn*-glycerol-3-phosphate acyltransferase from *Limnanthes douglasii* into oil seed rape. *Molecular Breeding* 2: 133-142.
- Carter, D.C. and Ho, J.X. 1994. Structure of Serum Albumin. *Advances in Protein Chemistry* 45: 153-203.
- Champ, P.J. and Randall, D.D. 1985. Purification and characterization of the pea chloroplast pyruvate dehydrogenase complex. *Plant Physiology* 77: 571-577.
- Chaudhary, S., Parmenter, D.L. and Moloney, M.M. 1998. Transgenic *Brassica carinata* as a vehicle for the production of recombinant proteins in seeds. *Plant Cell Reports* 17: 195-200.
- Cho, M.J., Jiang, W. and Lemaux, P.G. 1998. Transformation of recalcitrant barley cultivars through improvement of regenerability and decreased albinism. *Plant Science* 138: 229-244.

- Choudhary, M.L., Chin, C-K., Polashock, J.J. and Martin, C.E. 1994. *Agrobacterium* mediated transformation of *Petunia hybrida* with yeast Δ -9 fatty acid desaturase. *Plant Growth Regulation* 15: 113-116.
- Choy, L.N., Rosen, B.S. and Spiegelman, B.M. 1992. Adipsin and an endogenous pathway of complement from adipose cells. *The Journal of Biological Chemistry* 267: 12736-12741.
- Christie, W.W. 1992. Preparation of fatty acid methyl esters. *INFORM* 3: 1031-1034.
- Chung, M.H., Chen, M.K. and Pan, S.M. 2000. Floral spray transformation can efficiently generate *Arabidopsis* transgenic plants. *Transgenic Research* 9: 471-476.
- Cianflone, K., Kwiterovich, P.O., Walsh, M., Rodriguez, M.A. and Sniderman, A.D. 1987. Stimulation of fatty acid uptake and triglyceride synthesis in human cultured skin fibroblasts and adipocytes by a serum protein. *Biochemical and Biophysical Research Communications* 144: 94-100.
- Cianflone, K. and Maslowska, M. 1995. Differentiation induced production of ASP in human adipocytes. *European Journal of Clinical Investigation* 25: 817-825.
- Cianflone, K., Roncari, D.A.K., Maslowska, M., Baldo, A., Forden, J. and Sniderman, A.D. 1994. The adipsin-acylation stimulating protein system in human adipocytes: Regulation of triacylglycerol synthesis. *Biochemistry* 33: 9489-9495.
- Cianflone, K., Sniderman, A.D., Walsh, M.J., Vu, H., Gagnon, J. and Rodriguez, M.A. 1989. Purification and characterization of acylation-stimulating protein. *The Journal of Biological Chemistry* 264: 426-430.
- Cistola, D.P., Small, D.M. and Hamilton, J.A. 1987. Carbon 13 NMR studies of saturated fatty acids bound to bovine serum albumin. *The Journal of Biological Chemistry* 262: 10971-10979.
- Clough, S.J. and Bent, A.F. 1998. Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. *Plant Journal* 16: 735-743.
- Cummins, I., Hills, M.J., Ross, J.H.E., Hobbs, D.H., Watson, M.D., and Murphy, D.J. 1993. Differential, temporal and spatial expression of genes involved in storage oil and oleosin accumulation in developing rapeseed embryos: implications for the role of oleosins and the mechanisms of oil-body formation. *Plant Molecular Biology* 23: 1015-1027.

- Davies, H.M., Hawking, D.J. and Nelsen, J.S. 1995. Lysophosphatidic acid acyltransferase from immature coconut endosperm having medium chain length substrate specificity. *Phytochemistry* 39: 989-996.
- Dubendorff, J.W. and Studier, F.W. 1991. Controlling basal expression in an inducible T7 Expression system by blocking the target T7 promoter with *lac* repressor. *Journal of Molecular Biology* 219: 45-59.
- Etscheid, B.G., Albert, K.A., Villereal, M.L. and Palfrey, H.C. 1991. Transduction of the bradykinin response in human fibroblasts: Prolonged elevation of diacylglycerol level and its correlation with protein kinase C activation. *Cell Regulation* 3: 229-239.
- Fehling, E. and Mukherjee, K.D. 1991. Acyl-CoA elongase from a higher plant (*Lunaria annua*): metabolic intermediates of very-long-chain acyl-CoA products and substrate specificity. *Biochimica et Biophysica Acta* 1082: 239-246.
- Fox, B.G., Shanklin, J., Somerville, C.R. and Munck, E. 1993. Stearoyl-acyl carrier protein desaturase from *Ricinus communis* is a diiron-oxo protein. *Proceedings of the National Academy of Science USA* 90: 2486-2490.
- Frentzen, M., Heinz, E., McKeon, T.A. and Stumpf, P.K. 1983. Specificities and selectivities of glycerol-3-phosphate acyltransferase and monoacylglycerol-3-phosphate acyltransferase from pea and spinach chloroplasts. *European Journal of Biochemistry* 129: 629-636.
- Fuhrmann, J., Johnen, T. and Heise, K-P. 1994. Compartmentation of fatty acid metabolism in zygotic rape embryos. *Journal of Plant Physiology* 143: 565-569.
- Galili, G., Sengupta-Gopalan, C. and Ceriotti, A. 1998. The endoplasmic reticulum of plant cells and its role in protein maturation and biogenesis of oil bodies. *Plant Molecular Biology* 38: 1-29.
- Germinario, R., Sniderman, A.D., Manuel, S., Pratt, S., Baldo, A. and Cianflone, K. 1993. Coordinate regulation of triacylglycerol synthesis and glucose transport by acylation stimulating protein. *Metabolism* 40: 574-580.
- Giancola, C., De Sena, C., Fessas, D., Graziano, G. and Barone, G. 1997. DSC studies on bovine serum albumin denaturation - Effects of ionic strength and SDS concentration. *International Journal of Biological Macromolecules* 20: 193-204.
- Griffiths, G., Stobart, A.K. and Stymne, S. 1988a. Δ_6 and Δ_{12} desaturase activities and phosphatidic acid formation in microsomal preparations from the developing cotyledons of common borage (*Borago officinalis*). *Biochemical Journal* 252: 641-647.

Griffiths, G., Stymne, S. and Stobart, A.K. 1988b. The utilisation of fatty-acid substrates in triacylglycerol biosynthesis by tissue-slices of developing safflower (*Carthamus tinctorius* L.) and sunflower (*Helianthus annuus* L.) cotyledons. *Planta* 173: 309-316.

Guerche, P., Jouanin, L., Tepfer, D. and Pelletier, G. 1987. Genetic transformation of oilseed rape (*Brassica napus*) by the Ri T-DNA of *Agrobacterium rhizogenes* and analysis of inheritance of the transformed phenotype. *Molecular Genetics* 206: 382-386.

Gunstone, F.D. 1986. Fatty acid structure. In Gunstone, F.D., Harwood, J.L. and Padley, F.B. (Eds.). *The Lipid Handbook*. Chapman and Hall, London: 1-24.

Gunstone, F.D. and Wolff, R.L. 1996. Conifer seed oils: distribution of delta 5 acids between alpha and beta chains by C-13 nuclear magnetic resonance spectroscopy. *Journal of the American Oil Chemists Society* 73: 1611-1613.

Hagio, T., Blowers, A.D. and Earle, E.D. 1991. Stable transformation of sorghum cell cultures after bombardment with DNA-coated microprojectiles. *Plant Cell Reports* 10: 260-264.

Hamilton, J.A., Era, S., Bhamidipati, S.P. and Reed, R.G. 1991. Locations of three primary binding sites for long-chain fatty acids on bovine serum albumin. *Proceedings of the National Academy of Science USA* 88: 2051-2054.

Han, S.J., Cho, H.S., You, J.S., Nam, Y.W., Park, E.K., Shin, J.S., Park, Y.I., Park, W.M. and Paek, K.H. 1999. Gene silencing-mediated resistance in transgenic tobacco plants carrying potato virus Y coat protein gene. *Molecules and Cells* 9: 376-383.

Hang, Q., Woods, L., Feiss, M. and Catalano, C.E. 1999. Cloning, expression, and biochemical characterization of hexahistidine-tagged terminase proteins. *The Journal of Biological Chemistry* 274: 15305-15314.

Harwood, J.L. 1988. Fatty acid metabolism. *Annual Review of Plant Physiology and Molecular Biology* 39: 101-138.

Hatzopoulos, P., Franz, G., Choy, L. and Sung, R.Z. 1990. Interaction of nuclear factors with upstream sequences of a lipid body membrane protein gene from carrot. *Plant Cell* 2: 457-467.

Hershenson, S. and Ernst-Fonberg, M.L. 1983. Kinetic studies of the glycerophosphate acyltransferase from *Euglena* microsomes, including the effects of serum albumin. *Biochimica et Biophysica Acta* 751: 412-421.

Hills, M.J., Watson, M.D. and Murphy, D.L. 1993. Targeting of oleosins to the oil bodies of oilseed rape (*Brassica napus* L.). *Planta* 189: 24-29.

- Hoekema, A., Hirsch, P.R., Hooykaas, P.J.J. and Schilperoort, R.A. 1983. A binary vector strategy based on separation of vir- and T-region of the *Agrobacterium tumefaciens* Ti-plasmid. *Nature* 303: 179-180.
- Holbrook, L.H., van Rooijen, G.J.H., Wilen, R.W. and Moloney, M.M. 1991. Oilbody proteins in microspore derived embryos of *Brassica napus*: hormonal, osmotic and developmental regulation of synthesis. *Plant Physiology* 97: 1051-1058.
- Hooykaas, P.J.J. and Beijersbergen, A.G.M. 1994. The virulence system of *Agrobacterium tumefaciens*. *Annual Review of Phytopathology* 32: 157-179.
- Huang, A.H.C. 1996. Oleosins and oil bodies in seeds and other organs. *Plant Physiology* 110: 1055-1061.
- Huang, A.H.C. 1994. Structure of plant seed oil bodies. *Current Opinion in Structural Biology* 4: 493-498.
- Huang, A.H.C. 1992. Oil bodies and oleosins in seeds. *Annual Review of Plant Physiology and Plant Molecular Biology* 43: 177-200.
- Hutton, W.C., Garbow, J.R. and Hayes, T.R. 1999. Nondestructive NMR determination of oil composition in transformed canola seeds. *Lipids* 34: 1339-1346.
- Ichihara, K. 1984. *sn*-glycerol-3-phosphate acyltransferase in a particulate fraction from maturing safflower seeds. *Archives in Biochemistry and Biophysics* 2: 685-698.
- Ishimaru, K., Ichikawa, H., Matsuoka, M. and Ohsugi, R. 1997. Analysis of a C4 maize pyruvate, orthophosphate dikinase expressed in C3 transgenic *Arabidopsis* plants. *Plant Science* 129: 57-64.
- Ishizuka, T., Hoffman, J., Cooper, D.R., Watson, J.E., Pushkin, D.B. and Farese, R.V. 1989. Glucose-induced synthesis of diacylglycerol de novo is associated with translocation (activation) of protein kinase C in rat adipocytes. *FEBS Letter* 249: 234-238.
- Janknecht, R., De Martynoff, G., Lou, J., Hipskind, R.A., Nordheim, A. and Stunnenberg, H.G. 1991. Rapid and efficient purification of native histidine-tagged protein expressed by recombinant vaccinia virus. *Proceedings of the National Academy of Science USA* 88: 8972-8976.
- Jaworski, J.G., Clough, R.C. and Barnum, S.R. 1989. A cerulenin insensitive short chain 3-keto-acyl carrier protein synthase in *Spinacia oleracea* leaves. *Plant Physiology* 90: 41-44.

Judah, J.D., Gamble, M. and Steadman, J.H. 1973. Biosynthesis of serum albumin in rat liver, evidence for the existence of 'proalbumin'. *Biochemistry Journal* 134: 1083-1091

Kalant, D., Phélis, S., Fielding, B.A., Frayn, K.N., Cianflone, K. and Sniderman, A.D. 2000. Increased postprandial fatty acid trapping in subcutaneous adipose tissue in obese women. *Journal of Lipid Research* 41: 1963-1968.

Kang, F. and Rawsthorne, S. 1994. Starch and fatty acid synthesis in plastids from developing embryos of oilseed rape (*Brassica napus* L.). *Plant Journal* 6: 795-805.

Kennedy, E.P. 1961. Biosynthesis of complex lipids. *Federal Proceedings of the American Society of Experimental Biology* 20: 934-940.

Kermouni, A., Mahmoud, S.S., Moloney, M.M. and Habibi, H.R. 1998. Cloning of a full-length goldfish insulin-like growth factor-I complementary DNA and development of a quantitative PCR method for its measurement. *General and Comparative Endocrinology* 111: 51-60.

Khan, M.Y., Agarwal, S.K. and Hangloo, S. 1987. Urea-induced structural transformations in bovine serum albumin. *Journal of Biochemistry* 102: 313-317.

Kim, L.H., Lopez-Casillas, F., Bai, D.H., Lou, X. and Pape, M.E. 1989. Role of reversible phosphorylation of acetyl-CoA carboxylase in long-chain fatty acid synthesis. *Federal Proceedings of the Federal American Society of Experimental Biology* 3: 2250-2254.

Knutzon, D.S., Hayes, T.R., Wyrick, A., Xiong, H., Davies, H.M. and Voelker, T.A. 1999. Lysophosphatidic acid acyltransferase from coconut endosperm mediates the insertion of laurate at the *sn*-2 position of triacylglycerols in lauric rapeseed oil and can increase total laurate levels. *Plant Physiology* 120: 739-746.

Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227: 680-685.

Latta, M., Knapp, M., Sarmientos, P., Bréfort, G., Becquart, J., Guerrier, L., Jung, G. and Mayaux, J-F. 1987. Synthesis and purification of mature human serum albumin from *E. coli*. *Bio/Technology* 5: 1309-1314.

Laurent, P. and Huang, A.H.C. 1992. Organ- and development-specific acyl Coenzyme A lysophosphatidate acyltransferases in palm and meadowfoam. *Plant Physiology* 99: 1711-1715.

- Little, D., Weselake, R., Pomeroy, K., Furukawa-Stoffer, T. and Bagu, J. 1994. Solubilization and characterization of diacylglycerol acyltransferase from microspore-derived cultures of oilseed rape. *Biochemistry Journal* 304: 951-958.
- Liu, J.H., Selinger, L.B., Cheng, K.-J., Beauchemin, K.A. and Moloney, M.M. 1998. Plant seed oil-bodies as immobilization matrix for recombinant xylanase from the rumen fungus *Neocallimastix patriciarum*. *Molecular Breeding* 3: 463-470.
- Liu, W., Torisky, R.S., McAllister, K.P., Avdiushko, S., Hildebrand, D. and Collins, G.B. 1996. Somatic embryo cycling: evaluation of a novel transformation and assay system for seed-specific gene expression in soybean. *Plant Cell, Tissue and Organ Culture* 47: 33-42.
- Löhden, I. and Frentzen, M. 1992. Triacylglycerol biosynthesis in developing seeds of *Tropaeolum majus* L. and *Limnanthes douglasii* R. Br. *Planta* 188: 215-224.
- Maslowska, M., Sniderman, A.D., Germinario, R. and Cianflone, K. 1997. ASP stimulates glucose transport in cultured human adipocytes. *Journal of Obesity* 21: 261-266.
- Matthey, B., Engert, A., Klimka, A., Diehl, V. and Barth, S. 1999. A new series of pET-derived vectors for high efficiency expression of *Pseudomonas* exotoxin-based fusion proteins. *Gene* 229: 145-153.
- McDonald, B.E. 1990. Canola oil nutritional properties. Canola Council of Canada, Winnipeg, Canada.
- Moloney, M.M., Walker, J.M. and Sharma, K.K. 1989. High efficiency transformation of *Brassica napus* using *Agrobacterium* vectors. *Plant Cell Reports* 8: 238-242.
- Murphy, D.J. 1990. Storage lipid bodies in plants and other organisms. *Progress in Lipid Research* 29: 299-324.
- Murphy, D.J. 1993. Structure, function and biogenesis of storage lipid bodies and oleosins in plants. *Progress in Lipid Research* 32: 247-280.
- Murphy, D.J. and Cummins, I. 1989. Biosynthesis of seed storage products during embryogenesis in rapeseed, *Brassica napus*. *Journal of Plant Physiology* 135: 63-69.
- Murray, I., Kohl, J. and Cianflone, K. 1999a. Acylation-stimulating protein (ASP): structure-function determinants of cell surface binding and triacylglycerol synthetic activity. *Biochemistry Journal* 342: 41-48.

- Murray, I., Parker, R.A., Kirchgessner, T.G., Tran, J., Zhang, Z.J., Westerlund, J. and Cianflone, K. 1997. Functional bioactive recombinant acylation stimulating protein is distinct from C3a anaphylatoxin. *Journal of Lipid Research* 38: 2492-2501.
- Murphy, D.J. Rawsthorne, S. and Hills M.J. 1993. Storage lipid formation in seeds. *Seed Science Research* 3: 79-95.
- Murray, I., Sniderman, A.D. and Cianflone, K. 1999b. Mice lacking acylation stimulating protein (ASP) have delayed postprandial triglyceride clearance. *Journal of Lipid Research* 40: 1671-1676.
- Napier, J.A., Stobart, A.K. and Shewry, P.R. 1996. The structure and biogenesis of plant oil bodies: the role of the ER membrane and the oleosin class of proteins. *Plant Molecular Biology* 31: 945-956.
- Nossal, N.G. and Heppel, L.A. 1966. The release of enzymes by osmotic shock from *Escherichia coli* in exponential phase. *The Journal of Biological Chemistry* 241: 3055-3062.
- Nykiforuk, C.L. and Johnson-Flanagan, A.M. 1999. Storage reserve mobilization during low temperature germination and early seedling growth in *Brassica napus*. *Plant Physiology and Biochemistry* 37: 939-947.
- Nykiforuk, C.L., Laroche, A. and Weselake, R.J. 1999. Isolation and characterization of a cDNA encoding a second putative diacylglycerol acyltransferase from a microspore-derived cell suspension culture of *Brassica napus* L. cv Jet Neuf (Accession No. AF164434). (PGR99-158) *Plant Physiology* 121: 1057.
- Ohlrogge, J., Jaworski, J., Post-Beittenmiller, D., Roughan, G., Roessler, P. and Nakahira, K. 1993. Regulation of flux through the fatty acid biosynthesis pathway. In *Biochemistry and Molecular Biology of Membrane and Storage Lipids of Plants*. Murata, N. and Somerville, C. (eds.). American Society of Plant Physiologists, Rockville, MD: 102-112.
- Olins, P.O. and Rangwala, S.H. 1989. A novel sequence element derived from bacteriophage T7 mRNA acts as an enhancer of translation of the *lacZ* gene in *Escherichia coli*. *The Journal of Biological Chemistry* 264: 16973-16976.
- Oo, K-C. and Chew, Y-H. 1992. Diacylglycerol acyltransferase in microsomes and oil bodies of oil palm mesocarp. *Plant Cell Physiology* 33: 189-195.
- Oo, K.C. and Huang, A.H.C. 1989. Lysophosphatidate acyltransferase activities in the microsomes from palm endosperm, maize scutellum and rapeseed cotyledon of maturing seeds. *Plant Physiology* 91: 1288-1295.

- Ooms, G., Bains, A., Burrell, M., Karp, A., Twell, D. and Wilcox, E. 1985. Genetic manipulation in cultivars of oilseed rape (*Brassica napus*) using *Agrobacterium*. *Theoretical Applied Genetics* 71: 325-329.
- Parks, J., Cistola D., Small, D. and Hamilton, J. 1983. Interactions of the carboxyl group of oleic acid with bovine serum albumin: A ^{13}C NMR study. *The Journal of Biological Chemistry* 262: 10980-10985.
- Parmenter, D.L., Booth, J.G., van Rooijen, G.J.H., Yeung, E.C. and Moloney, M.M. 1995. Production of biologically active hirudin in plant seeds. *Plant Molecular Biology* 29: 1167-1180.
- Perry, H.J., Bligny, R., Gout, E. and Harwood, J.L. 1999. Changes in Kennedy pathway intermediates associated with increased triacylglycerol synthesis in oilseed rape. *Phytochemistry* 52: 799-804.
- Perry, H.J. and Harwood, J.L. 1993. Changes in the lipid content of developing seeds of *Brassica napus*. *Phytochemistry* 32: 1411-1415.
- Poirier, Y., Ventre, G. and Nawrath, C. 2000. High-frequency linkage of co-expressing T-DNA in transgenic *Arabidopsis thaliana* transformed by vacuum infiltration of *Agrobacterium tumefaciens*. *Theoretical and Applied Genetics* 100: 487-493.
- Pollard, M.R., Anderson, L., Fan, C., Hawkins, D.J. and Daview, H.M. 1991. A specific acyl-ACP thioesterase implicated in medium-chain fatty acid production in immature cotyledons of *Umbellularia californica*. *Archives of Biochemistry and Biophysics* 284: 306-312.
- Pollard, M. and Ohlrogge, J. 1999. Testing models of fatty acid transfer and lipid synthesis in spinach leaf using in vivo oxygen-18 labeling. *Plant Physiology* 121: 1217-1226.
- Porath, J., Carlsson, J., Ingmar, O. and Belfrage, G. 1975. Metal chelate affinity chromatography, a new approach to protein fractionation. *Nature* 258: 598-599.
- Post-Beittenmiller, M.A., Jaworski, J. and Ohlrogge, J.B. 1991. *In vivo* pools of free and acylated acyl carrier protein in spinach: evidence for sites of regulation of fatty acid biosynthesis. *The Journal of Biological Chemistry* 266: 1858-1865.
- Post-Beittenmiller, D., Roughan, P.G. and Ohlrogge, J.B. 1992. Regulation of plant fatty acid biosynthesis: analysis of acyl-CoA and acyl-ACP substrate pools in spinach and pea chloroplasts. *Plant Physiology* 100: 923-930.

- Powell, D.J., Friedman, J.M., Oulette, A.J., Krauter, K.S. and Darnell Jr., J.E. 1984. Transcriptional and post-translational control of specific messenger RNAs in adult and embryonic liver. *Journal of Molecular Biology* 179: 21-35.
- Pugsley, A.P. and Schwartz, M. 1985. Export and secretion of protein by bacteria. *FEMS Microbiology Review* 32: 3-38.
- Qing, H., Yanlin, H., Fenlin, S. and Zuyi, T. 1996. Effects of pH and metal ions on the conformation of bovine serum albumin in aqueous solution - An attenuated total reflection (ATR) FTIR spectroscopic study. *Spectrochimica Acta Part A* 52: 1795-1800.
- Qu, R. and Huang, A.H.C. 1990. Oleosin KD on the surface of oil bodies in maize. *The Journal of Biological Chemistry* 272: 3699-3706.
- Radke, S.E., Andrews, B.M., Moloney, M.M., Crouch, M.L., Kridl, J.C. and Knauf, V.C. 1988. Transformation of *Brassica napus* L. using *Agrobacterium tumefaciens*: developmentally regulated expression of a reintroduced napin gene. *Theoretical and Applied Genetics* 75: 685-694.
- Reed, R.G. 1986. Location of long chain fatty acid binding sites of bovine serum albumin by affinity labeling. *The Journal of Biological Chemistry* 261: 15619-15624.
- Richards, E.W., Hamm, M.W., Fletcher, J.E. and Otto, D.A. 1990. The binding of palmitoyl-CoA to bovine serum albumin. *Biochimica et Biophysica Acta* 1044: 361-367.
- Rijven, A.H.G.C. and Gifford, R.M. 1983. Accumulation and conversion of sugars by developing wheat grains. Non-diffusional uptake of sucrose, the substrate preferred by endosperm slices. *Plant Cell and Environment* 6: 417-425.
- Roughan, P.G. and Ohlrogge, J.B. 1994. On the assay of acetyl-CoA synthetase in chloroplasts and leaf extracts. *Analytical Biochemistry* 216: 77-82.
- Rousler, K., Shintani, D., Savage, L., Buddupalli, S. and Ohlrogge, J. 1997. Targeting of the *Arabidopsis* homomeric acetyl-coenzyme A carboxylase to plastids of rapeseeds. *Plant Physiology* 113: 75-81.
- Saleh, J., Summers, L.K.M., Cianflone, K., Fielding, B.A., Sniderman, A.D. and Frayn, K.N. 1998. Coordinated release of acylation stimulating protein (ASP) and triacylglycerol clearance by human adipose tissue in vivo in the postprandial period. *Journal of Lipid Research* 39: 884-891.
- Sall, J. and Lehman, A. 1996. JMP[®] Start Statistics User's Guide. SAS Inst. Inc., Cary, NC.

- Scarth, R., McVetty, P.B.E., Rimmer, S.R. and Daun, J. 1992. Breeding for special oil quality in Canola/rapeseed: The University of Manitoba Program In: Seed Oils for the Future. Mackenzie, S.L. and Taylor, D.C. (eds.). AOCS Press, Champaign, Illinois: 171-176.
- Schussler, J.R., Brenner, M.L. and Brun, W.A. 1984. Abscisic acid and its relationship to seed filling in soybeans. *Plant Physiology* 76: 301-306.
- Shanklin, J. and Cahoon, E.B. 1998. Desaturation and related modifications of fatty acids. *Annual Review of Plant Physiology and Molecular Biology* 49: 611-341.
- Sheng, J. and Citovsky, V. 1996. Agrobacterium-plant cell DNA transport: have virulence proteins, will travel. *The Plant Cell* 8: 1699-1710.
- Smith, M.C., Furman, T.C., Ingolia, T.D. and Pidgeon, C. 1988. Chelating peptide-immobilized metal ion affinity chromatography. *The Journal of Biological Chemistry* 263: 7211-7215.
- Smith, R.G., Gauthier, D.A., Dennis, D.T. and Turpin, D.H. 1992. Malate- and pyruvate-dependent fatty acid synthesis in leucoplasts from developing castor endosperm. *Plant Physiology* 98: 1233-1238.
- Sniderman, A.D., Cianflone, K.M. and Eckel, R.H. 1991. Levels of acylation stimulating protein in obese women before and after moderate weight loss. *International Journal of Obesity* 15: 333-336.
- Sniderman, A.D., Cianflone, K., Summers, L., Fielding, B. and Frayn, K. 1997. The acylation-stimulating protein pathway and regulation of postprandial metabolism. *Proceedings of the Nutrition Society* 56: 703-712.
- Sniderman, A.D., Masłowska, M. and Cianflone, K. 2000. Of mice and men (and women) and the acylation-stimulating protein pathway. *Current Opinion in Lipidology* 11: 291-296.
- Sokal, R.R. and Rohlf, F.J. 1995. *Biometry* (3rd Ed.). W.H. Freeman and Company, New York, NY: 227-229.
- Stobart, A.K. and Stymne, S. 1990. Triacylglycerol biosynthesis. *Methods in Plant Biochemistry* 4: 19-46.
- Studier, F.W. and Moffatt, B.A. 1986. Use of bacteriophage T7 RNA polymerase to direct selective high-level expression of cloned genes. *Journal of Molecular Biology* 189: 113-130.

- Stymne, S. and Glad, G. 1981. Acyl exchange between oleoyl-CoA and phosphatidylcholine in microsomes of developing soya bean cotyledons and its role in fatty acid desaturation. *Lipids* 16: 298-305.
- Sun, C., Coa, Y.Z. and Huang, A.H.C. 1988. Acyl coenzyme A preference of the glycerol phosphate pathway in the microsomes from the maturing seeds of palm, maize and rapeseed. *Plant Physiology* 88: 56-60.
- Tao, Y., Cianflone, K., Sniderman, A.D., Colby-Germinario, S.P. and Germinario, R.J. 1997. Acylation-stimulating protein (ASP) regulates glucose transport in rat L6 muscle cell line. *Biochimica et Biophysica Acta* 1344: 221-229.
- Thumser, A.E.A., Buckland, A.G. and Wilton, D.C. 1998. Monoacylglycerol binding to human serum albumin: Evidence that monooleoylglycerol binds at the dansylsarcosine site. *Journal of Lipid Research* 39: 1033-1038.
- Ting, J.T.L., Balsamo, R.A., Ratnayake, C. and Huang, A.H.C. 1997. Oleosin of plant seed oil bodies is correctly targeted to the lipid bodies in transformed yeast. *The Journal of Biological Chemistry* 272: 3699-3706.
- Tjokrokusumo, D., Heinrich, T., Wylie, S., Potter, R. and McComb, J. 2000. Vacuum infiltration of *Petunia hybrida* pollen with *Agrobacterium tumefaciens* to achieve plant transformation. *Plant Cell Reports* 19: 792-797.
- Tocher, D.R., Leaver, M.J. and Hodgson, P.A. 1998. Recent advances in the biochemistry and molecular biology of fatty acyl desaturases. *Progress in Lipid Research* 27: 73-117.
- Trigatti, B.L. and Gerber, G.E. 1995. A direct role for serum albumin in the cellular uptake of long-chain fatty acids. *Biochemistry Journal* 308: 155-159.
- Tzen, J.T.C., Cao, Y-Z, Laurent, P., Ratnayake, C. and Huang, A.H.C. 1993. Lipids, proteins, and structure of seed oil bodies from diverse species. *Plant Physiology* 101: 267-276.
- Tzen, J.T.C. and Huang, A.H.C. 1992. Surface structure and properties of plant seed oil bodies. *The Journal of Cell Biology* 117: 327-335.
- Tzen, J.T.C., Lie, G.C. and Huang, A.H.C. 1992. Characterization of the charged components and their topology in the surface of plant seed oil bodies. *The Journal of Biological Chemistry* 267: 15626-15634.

- Valkama, M., Pehu, T., Santala, A., Valkonen, J.P.T., Koivu, K., Lehto, K. and Pehu, E. 2000. High level of resistance to potato virus Y by expressing P1 sequence in antisense orientation in transgenic potato. *Molecular Breeding* 6: 95-104.
- van Rooijen, G.J.H. and Moloney, M.M. 1995. Plant seed oil-bodies as carriers for foreign proteins. *Bio/Technology* 13: 72-77.
- van Rooijen, G.J.H., Terning, L.I. and Moloney, M.M. 1992. Nucleotide sequence of an *Arabidopsis thaliana* oleosin gene. *Plant Molecular Biology* 18: 1177-1179.
- Vance, V.B. and Huang, A.H.C. 1987. The major protein from lipid bodies of maize: characterization of structure based on cDNA cloning. *The Journal of Biological Chemistry* 262: 11275-11279.
- Vaucheret, H. and Fagard, M. 2001. Transcriptional gene silencing in plants: targets, inducers and regulators. *Trends in Genetics* 17: 29-35.
- Voelker, T.A., Hayes, T.R., Cranmer, A.C. and Davies, H.M. 1996. Genetic engineering of a quantitative trait: metabolic and genetic parameters influencing the accumulation of laurate in rapeseed. *The Plant Journal* 9: 229-241.
- Voelker, T.A., Worrell, A.C., Anderson, L, Bleibaum, J., Fan, C., Hawkins, D.J., Radke, S.E. and Daview, H.M. 1992. Fatty acid biosynthesis redirected to medium chains in transgenic oilseed plants. *Science* 257: 72-74.
- Vogelstein, B. and Gillespie, D. 1979. Preparative and analytical purification of DNA from agarose. *Proceedings of the National Academy of Science USA* 76: 615-619.
- Walsh, M.J., Sniderman, A.D., Cianflone, K., Vu, H., Rodriguez, M.A. and Forse, R.A. 1989. The effect of ASP on the adipocyte of the morbidly obese. *Journal of Surgical Research* 46: 470-473.
- Wang, S.M. and Huang, A.H.C. 1987. Biosynthesis of lipase in the scutellum of maize kernel. *The Journal of Biological Chemistry* 262: 2270-2274.
- Ward, K.A., Tung, P., Lamb, N., Abrams, S.R., Reid, D.M., Moloney, M.M. and Holbrook, L.A. 1999. Structural requirements for biologically active jasmonates: Induction of protease inhibitors and cotyledon senescence. *Plant Growth Regulation* 27: 49-56.
- Watson, B., Currier, T.C., Gordon, M.P., Chilton, M-D. and Nester, E.W. 1975. Plasmid required for virulence of *Agrobacterium tumefaciens*. *Journal of Bacteriology* 123: 255-264.

- Weselake, R.J. 2000. Lipid biosynthesis in cultures of oilseed rape. *In Vitro Cellular Development Biology - Plant* 36: 338-348.
- Weselake, R.J., Kazala, E.C., Cianflone, K., Boehr, D.D., Middleton, C.K., Rennie, C.D., Laroche, A. and Recnik, I. 2000a. Human acylation stimulating protein enhances triacylglycerol biosynthesis in plant microsomes. *FEBS Letters* 481: 189-192.
- Weselake, R.J., Nykiforuk, C.L., Laroche, A., Patterson, N.A., Wiehler, W.B., Szarka, S.J., Moloney, M.M., Tari, L.W. and Derekh, U. 2000b. Expression and properties of diacylglycerol acyltransferase from cell-suspension cultures of oilseed rape. *Biochemical Society Transactions* 28: 684-686.
- Weselake, R.J., Pomeroy, M.K., Furukawa, T.L., Golden, J.L., Little, D.B. and Laroche, A. 1993. Developmental profile of diacylglycerol acyltransferase in maturing seeds of oilseed rape and safflower and microspore-derived cultures of oilseed rape. *Plant Physiology* 102: 565-571.
- Yadav, N.S., Wierzbicki, A., Aegerter, M., Caster, C.S., Perez-Grau, L., Kinney, A.J., Hitz, W.D., Booth Jr, J.R., Schweiger, B. and Stecca, K.L. 1993. Cloning of higher plant ω -3 fatty acid desaturases. *Plant Physiology* 103: 467-476.
- Yasruel, Z., Cianflone, K., Sniderman, A.D., Rosenbloom, M., Walsh, M. and Rodrigues, M.A. 1991. Effect of acylation stimulating protein on the triacylglycerol synthetic pathway of human adipose tissue. *Lipids* 26: 495-499.
- Ye, G.N., Stone, D., Pang, S.Z., Creely, W., Gonzalez, K. and Hinchee, M. 1999. *Arabidopsis* ovule is the target for *Agrobacterium* in planta vacuum infiltration transformation. *Plant Journal* 19: 249-257.
- Zou, J., Katavic, V., Giblin, E.M., Barton, D.L., MacKenzie, S.L., Keller, W.A., Hu, X. and Taylor, D.C. 1997. Modification of seed oil content and acyl composition in Brassicaceae by expression of a yeast *sn-2* acyltransferase gene. *The Plant Cell* 9: 909-923.