MOLECULAR STRATEGIES FOR INCREASING SEED OIL CONTENT IN CANOLA

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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.

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List of Abbreviations

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

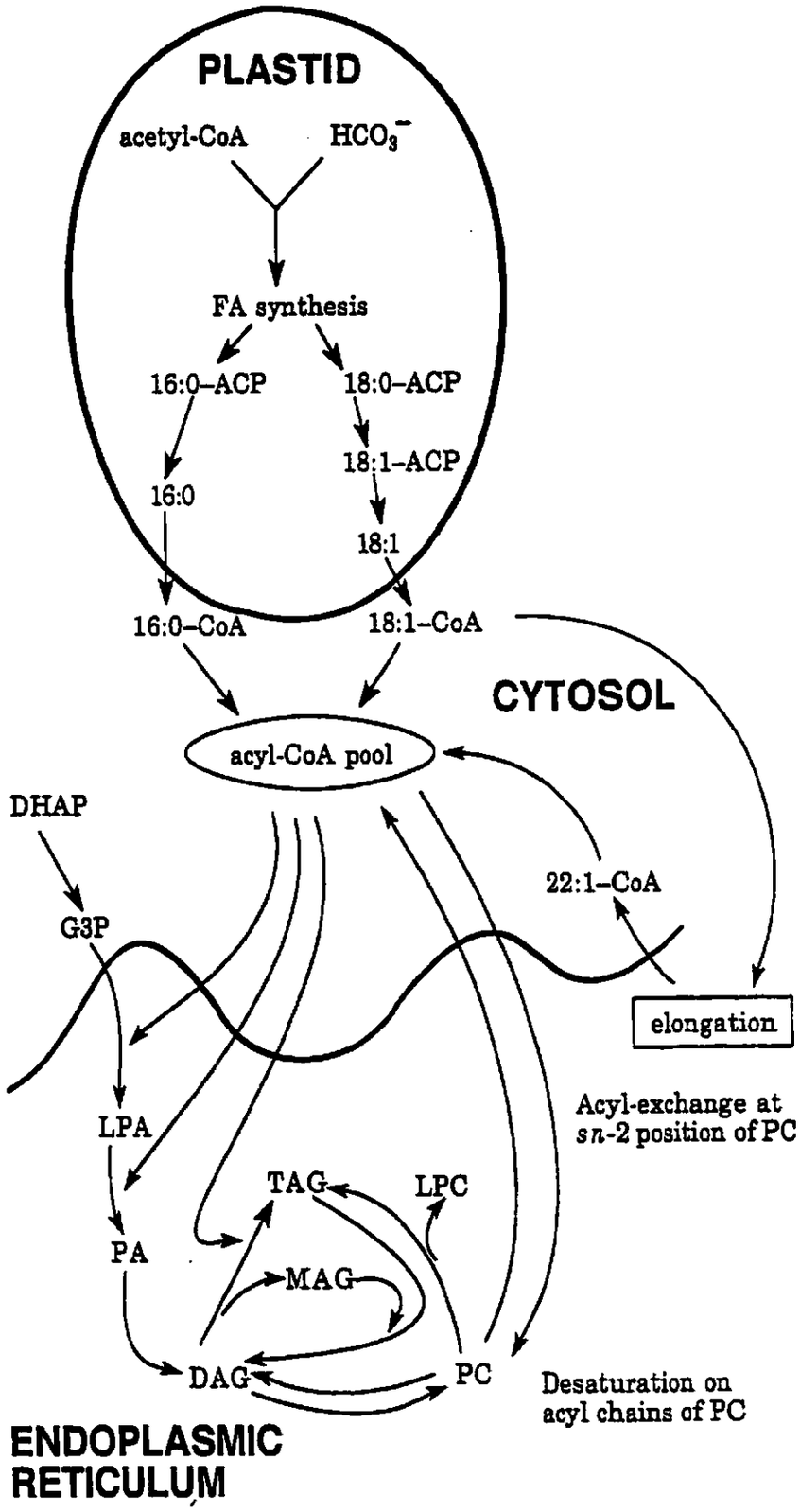
2. Literature Review

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.


PLASTID
acetyl-CoA
HCO_3^-
FA synthesis
16:0–ACP
18:0–ACP
18:1–ACP
16:0
18:1
16:0–CoA
18:1–CoA
CYTOSOL
acyl-CoA pool
DHAP
G3P
22:1–CoA
elongation
Acyl-exchange at
sn-2 position of PC
LPA
TAG
LPC
PA
MAG
DAG
PC
Desaturation on
acyl chains of PC
ENDOPLASMIC
RETICULUM

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 CO_2 (from HCO_3^-) 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 CO_2 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 CO_2 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 (Bernerth 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).

^{13}C NMR (Hamilton *et al.*, 1991; Cistola *et al*, 1987) and ^{14}C-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 DH5α, 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 (d^2H_2O).

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 d^2H_2O.

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 H_2O (or 12.0 μL if *Pfu* polymerase was used in place of *Taq* polymerase) and 2.0 μL DNA template (0.05 μg/μL). 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 x *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 d^2H_2O. The tube was incubated at 55°C for 5 minutes and centrifuged at 13,000 x *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 x *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 $CaCl_2$, 250 mM KCl, 55 mM $MnCl_2$), 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 µL aliquots, flash frozen in liquid nitrogen and stored at -80°C until required.

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

A 40 µL 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 µF 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 $MgCl_2$, 10 mM $MgSO_4$, 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 µL aliquot of the cell suspension was added to AB plates (Watson *et al.*, 1975) containing 25 µg gentamycin/mL and 50 µg 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 Na_2HPO_4 pH 7.0, 0.07% β-mercaptoethanol, 10 mM Na_2EDTA 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 [γ-^{32}P] ATP mix (15 μM cold ATP, 4 μM [γ-^{32}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 [γ-^{32}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-isopropylnaphthalene 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 μL 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 x *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 μL 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 μL of extraction buffer containing 4 mM 2-mercaptoethanol and 20 μg 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 Na_2HPO_4/NaH_2PO_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 x *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 ^{32}P-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-^{32}P]-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 cm^3) 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 H_2O 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. Bulked 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) Na_2SO_4 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' - GGATCCATGTCCGTTCAGCTGACT - 3'

*Bam*HI Restriction Site

Bases Complementary to Lagging Strand of DNA Sequence Encoding ASP

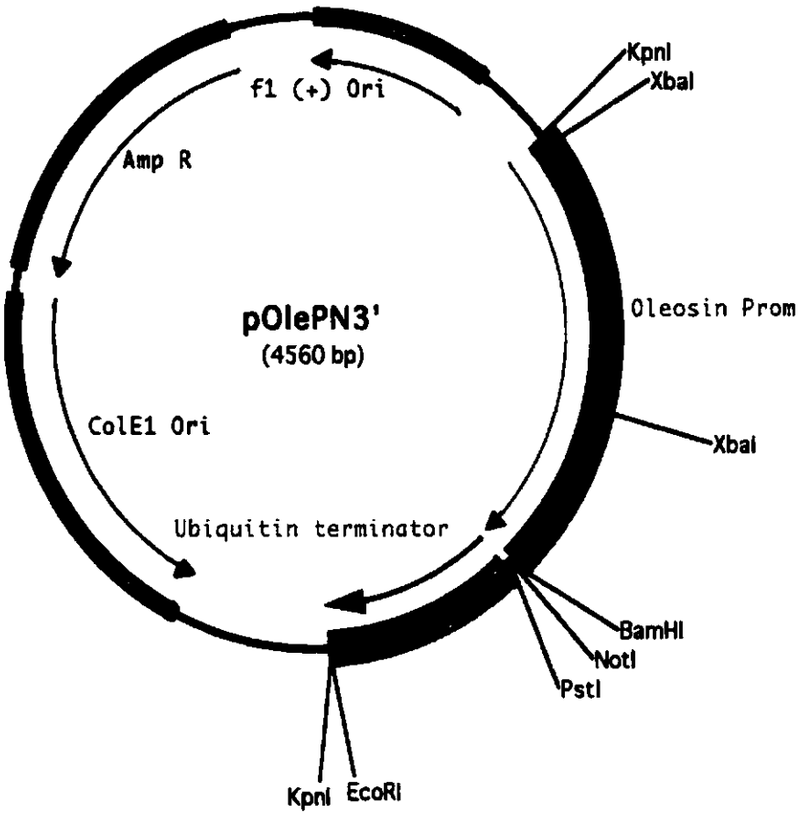
Reverse Primer: ASP743

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.


f1 (+) Ori
Amp R
pOlePN3'
(4560 bp)
ColE1 Ori
Ubiquitin terminator
KpnI
XbaI
Oleosin Prom
XbaI
BamHI
NotI
PstI
KpnI
EcoRI

KpnI XbaI
GGTACCCAAATACGATCTGATACTGATAACGTCTAGATTTTTAGGGTTAAAGCAATCAATCACCTG

ACGATTCAAGGTGGTTGGATCATGACGATTCCAGAAAACATCAAGCAAGCTCTCAAAGCTACACTC

TTTGGGATCATACTGAACTCTAACAACCTCGTTATGTCCCGTAGTGCCAGTACAGACATCCTCGTA

ACTCGGATTATGCACGATGCCATGGCTATACCCAACCTCGGTCTTGGTCACACCAGGAACTCTCTG

GTAAGCTAGCTCCACTCCCCAGAAACAACCGGCGCCAAATTGCCGGAATTGCTGACCTGAAGACGG

AACATCATCGTCGGGTCCTTGGGCGATTGCGGCGGAAGATGGGTCAGCTTGGGCTTGAGGACGAGA

CCCGAATCGAGTCTGTTGAAAGGTTGTTCATTGGGATTTGTATACGGAGATTGGTCGTCGAGAGGT

TTGAGGGAAAGGACAAATGGGTTTGGCTCTGGAGAAAGAGAGTGCGGCTTTAGAGAGAGAATTGAG

AGGTTTAGAGAGAGATGCGGCGGCGATGACGGGAGGAGAGACGACGAGGACCTGCATTATCAAAGC

AGTGACGTGGTGAAATTTGGAACTTTTAAGAGGCAGATAGATTTATTATTTGTATCCATTTTCTTC

XbaI
ATTGTTCTAGAATGTCGCGGAACAAATTTTAAAACTAAATCCTAAATTTTTCTAATTTTGTTGCCA

ATAGTGGATATGTGGGCCGTATAGAAGGAATCTATTGAAGGCCCAAACCCATACTGACGAGCCCAA

AGGTTCGTTTTGCGTTTTATGTTTCGGTTCGATGCCAACGCCACATTCTGAGCTAGGCAAAAAACA

AACGTGTCTTTGAATAGACTCCTCTCGTTAACACATGCAGCGGCTGCATGGTGACGCCATTAACAC

GTGGCCTACAATTGCATGATGTCTCCATTGACACGTGACTTCTCGTCTCCTTTCTTAATATATCTA

ACAAACACTCCTACCTCTTCCAAAATATATACACATCTTTTTGATCAATCTCTCATTCAAAATCTC

BamHI NotI PstI
ATTCTCTCTAGTAAACAGGATCCCCCTCGCGGCCGCAGGGCTGCAGAATGAGTTCCAAGATGGTTT

GTGACGAAGTTAGTTGGTTGTTTTTATGGAACTTTGTTTAAGCTTGTAATGTGGAAAGAACGTGTG

GCTTTGTGGTTTTTAAATGTTGGTGAATAAAGATGTTTCCTTTGGATTAACTAGTATTTTTCCTAT

TGGTTTCATGGTTTTAGCACACAACATTTTAAATATGCTGTTAGATGATATGCTGCCTGCTTTATT

ATTTACTTACCCCTCACCTTCAGTTTCAAAGTTGTTGCAATGACTCTGTGTAGTTTAAGATCGAGT

GAAAGTAGATTTTGTCTATATTTATTAGGGGTATTTGATATGCTAATGGTAAACATGGTTTATGAC

AGCGTACTTTTTTGGTTATGGTGTTGACGTTTCCTTTTAAACATTATAGTAGCGTCCTTGGTCTGT

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/ *Eco*RI+*Hin*dIII Ladder (MBI Fermentas, Flamborough, ON) and lanes 2 and 3 contain 5μL of the PCR reaction from two independent trials.

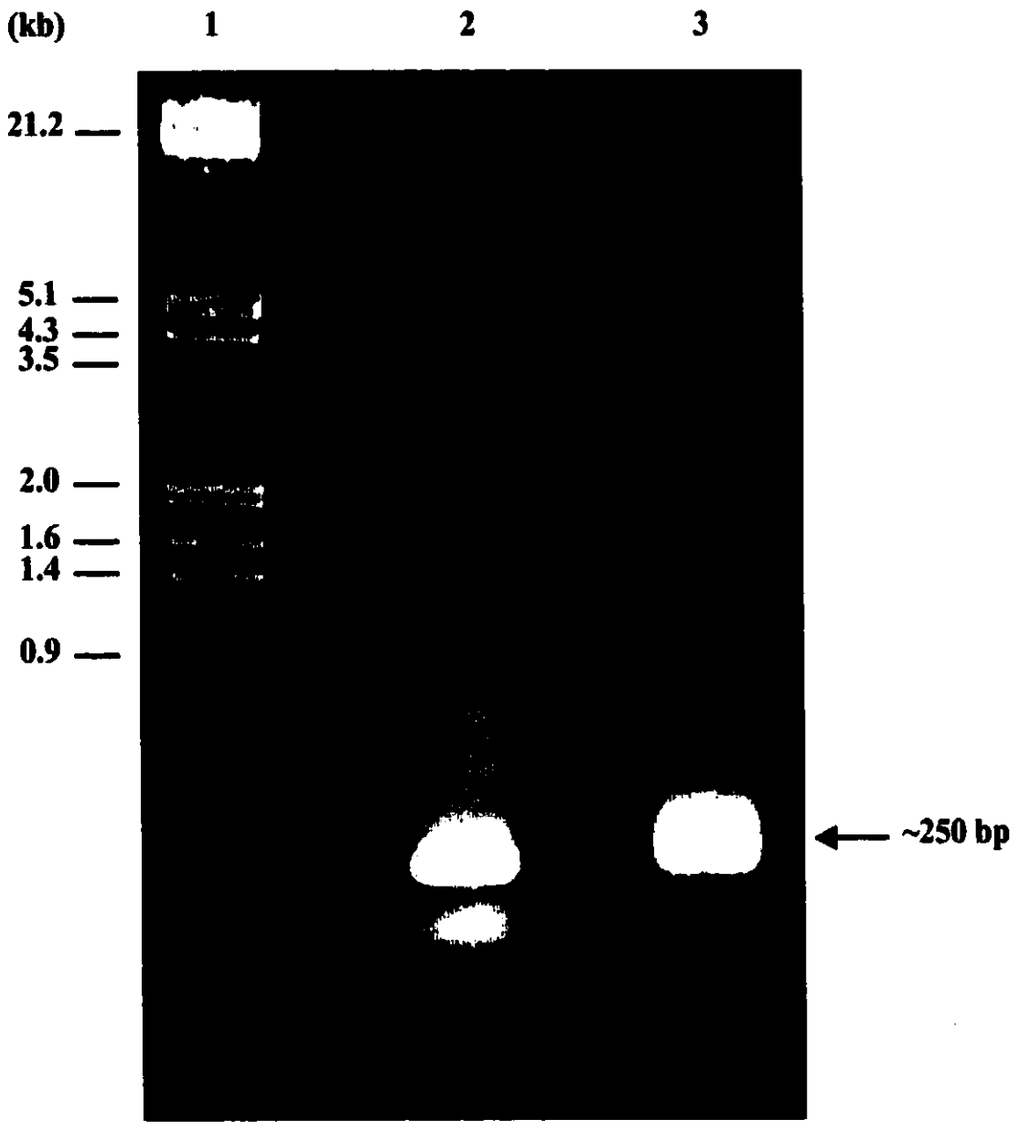

(kb)
1
2
3
21.2
5.1
4.3
3.5
2.0
1.6
1.4
0.9
~250 bp

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+*Hin*dIII Ladder (MBI Fermentas, Flamborough, ON); 2, uncut colony 1 plasmid DNA; 3, restricted colony 1 plasmid DNA; 4, uncut pUC19; 5, restricted pUC19.

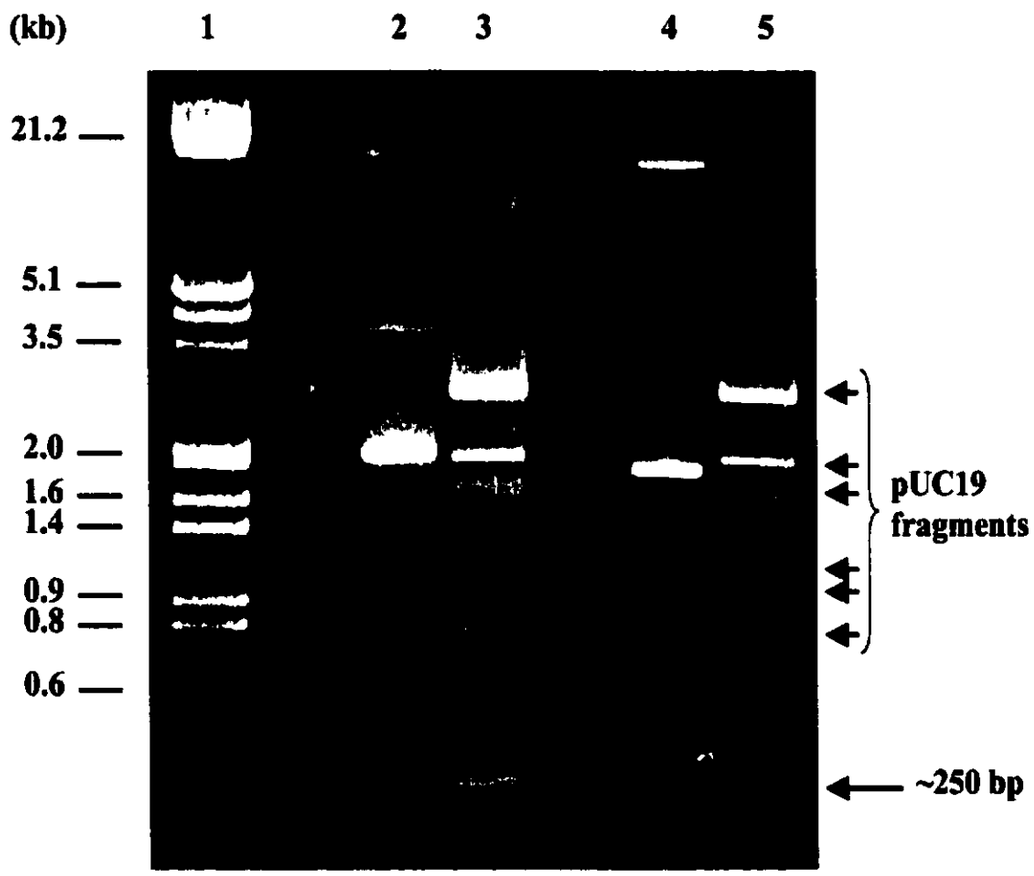

(kb)
1
2
3
4
5
21.2
5.1
3.5
2.0
1.6
1.4
0.9
0.8
0.6
pUC19
fragments
~250 bp

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    GGATCCATGTCCGTTCAGCTGACTGAAAAACGTATGGATAAAGTTGGTAAGTACC   55
ASP cDNA                ATGTCCGTTCAGCTGACTGAAAAACGTATGGATAAAGTTGGTAAGTACC   49

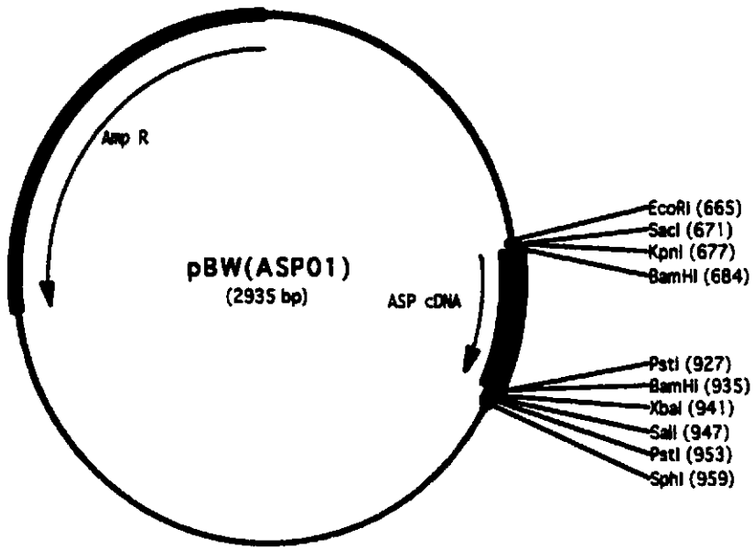
PUTATIVE ASP01    CGAAGGAACTGCGTAAATGTTGCGAAGACGGGATGCGTGAAAACCCGATGCGTTT  110
ASP cDNA          CGAAGGAACTGCGTAAATGTTGCGAAGACGGGATGCGTGAAAACCCGATGCGTTT  104

PUTATIVE ASP01    CTCTTGCCAACGTCGTACCCGCTTCATCTCCCTGGGCGAAGCTTGCAAAAAGGTT  165
ASP cDNA          CTCTTGCCAACGTCGTACCCGCTTCATCTCCCTGGGCGAAGCTTGCAAAAAGGTT  159

PUTATIVE ASP01    TTCCTGGACTGCTGCAACTACATCACTGAACTGCGTCGTCAGCACGCTCGTGCTT  220
ASP cDNA          TTCCTGGACTGCTGCAACTACATCACTGAACTGCGTCGTCAGCACGCTCGTGCTT  214

PUTATIVE ASP01    CTCACCTGGGTCTGGCTTAATGACTGCAG                            249
ASP cDNA          CTCACCTGGGTCTGGCTTAATGA                                  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)
GAATTCGAGCTCGGTACCCGGATCCATGTCCGTTCAGCTGACTGAAAAACGTATGGATAAAGTTGGTAAGTACCCGAAGGAACTGC

GTAAATGTTGCGAAGACGGGATGCGTGAAAACCCGATGCGTTTCTCTTGCCAACGTCGTACCCGCTTCATCTCCCTGGGCGAAGCT

TGCAAAAAGGTTTTCCTGGACTGCTGCAACTACATCACTGAACTGCGTCGTCAGCACGCTCGTGCTTCTCACCTGGGTCTGGCTTA

XbaI (941) PstI (953)
PstI (927) BamHI (935) SalI (947) SphI (959)
ATGACTGCAGGGGGATCCTCTAGAGTCGACCTGCAGGCATGCAA

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 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/*Hin*dIII 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'.

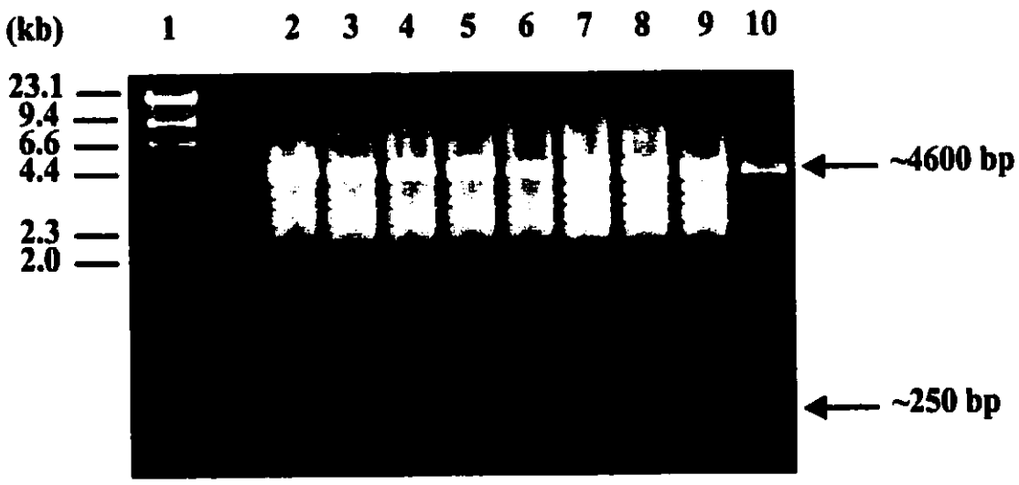
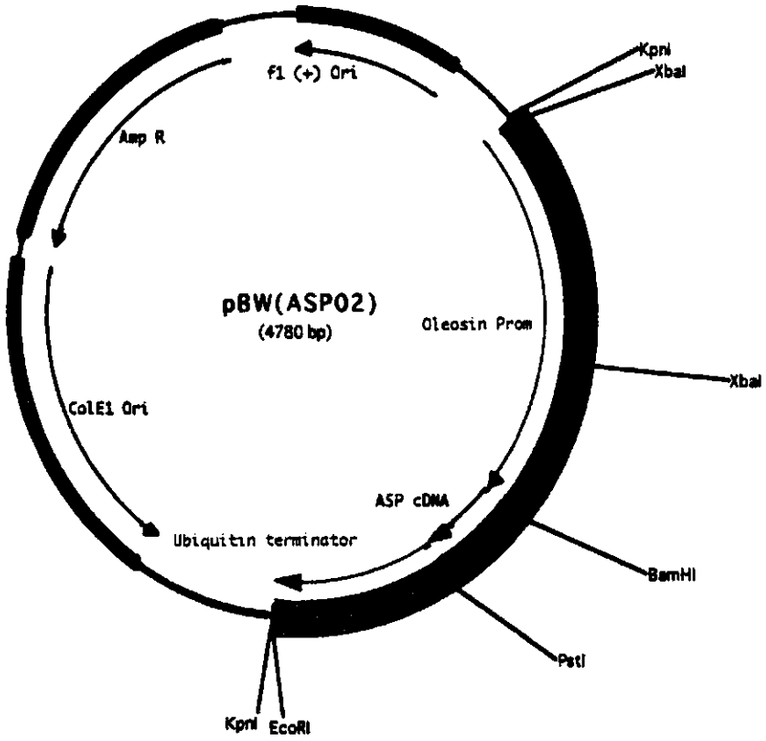

(kb)
1
2
3
4
5
6
7
8
9
10
23.1
9.4
6.6
4.4
2.3
2.0
~4600 bp
~250 bp

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.


f1 (+) Ori
KpnI
XbaI
Amp R
pBW(ASP02)
(4780 bp)
Oleosin Prom
XbaI
ColE1 Ori
ASP cDNA
Ubiquitin terminator
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PstI
KpnI
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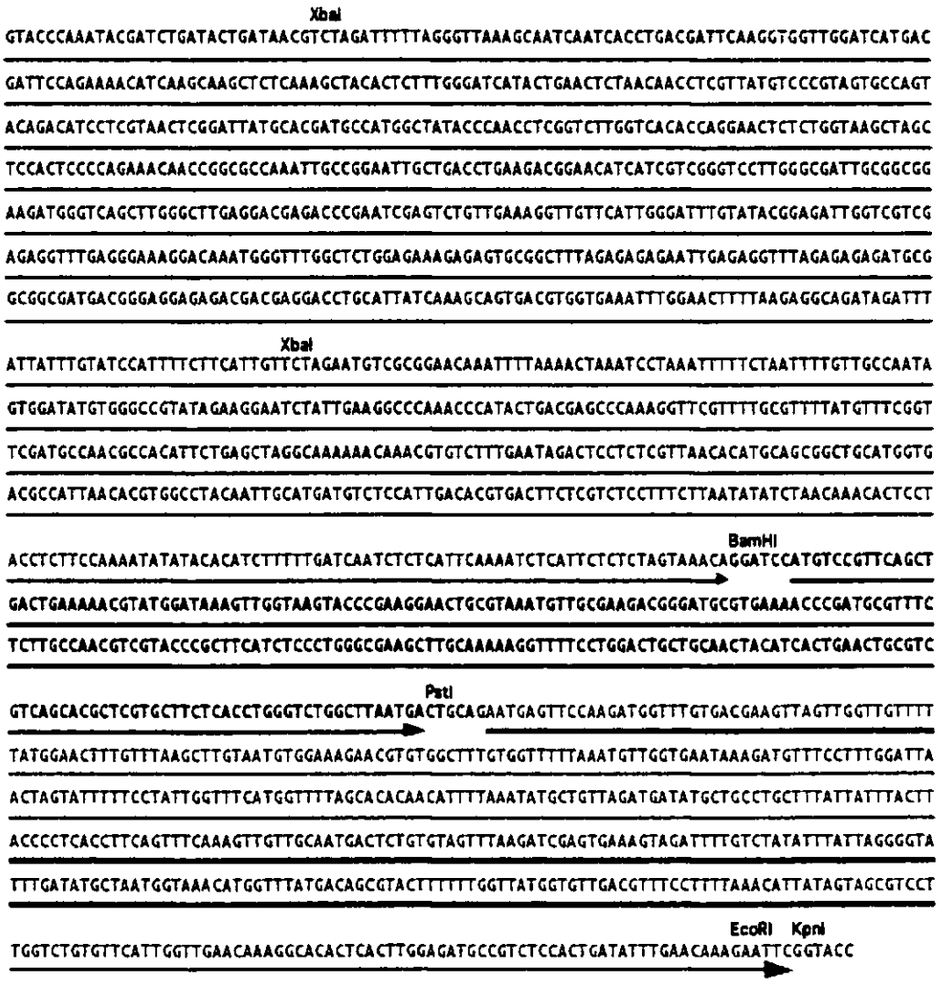
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XbaI
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BamHI
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PstI
GTCAGCACGCTCGTGCTTCTCACCTGGGTCTGGCTTAATGACTGCAGAATGAGTTCCAAGATGGTTTGTGACGAAGTTAGTTGGTTGTTTT
TATGGAACTTTGTTTAAGCTTGTAATGTGGAAAGAACGTGTGGCTTTGTGGTTTTTAAATGTTGGTGAATAAAGATGTTTCCTTTGGATTA
ACTAGTATTTTTCCTATTGGTTTCATGGTTTTAGCACACAACATTTTAAATATGCTGTTAGATGATATGCTGCCTGCTTTATTATTTACTT
ACCCCTCACCTTCAGTTTCAAAGTTGTTGCAATGACTCTGTGTAGTTTAAGATCGAGTGAAAGTAGATTTTGTCTATATTTATTAGGGGTA
TTTGATATGCTAATGGTAAACATGGTTTATGACAGCGTACTTTTTTGGTTATGGTGTTGACGTTTCCTTTTAAACATTATAGTAGCGTCCT
EcoRI KpnI
TGGTCTGTGTTCATTGGTTGAACAAAGGCACACTCACTTGGAGATGCCGTCTCCACTGATATTTGAACAAAGAATTCGGTACC

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).

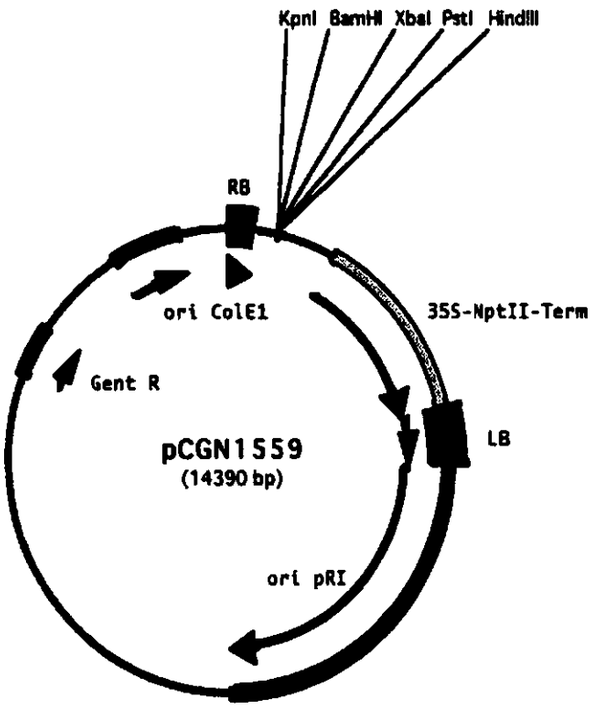
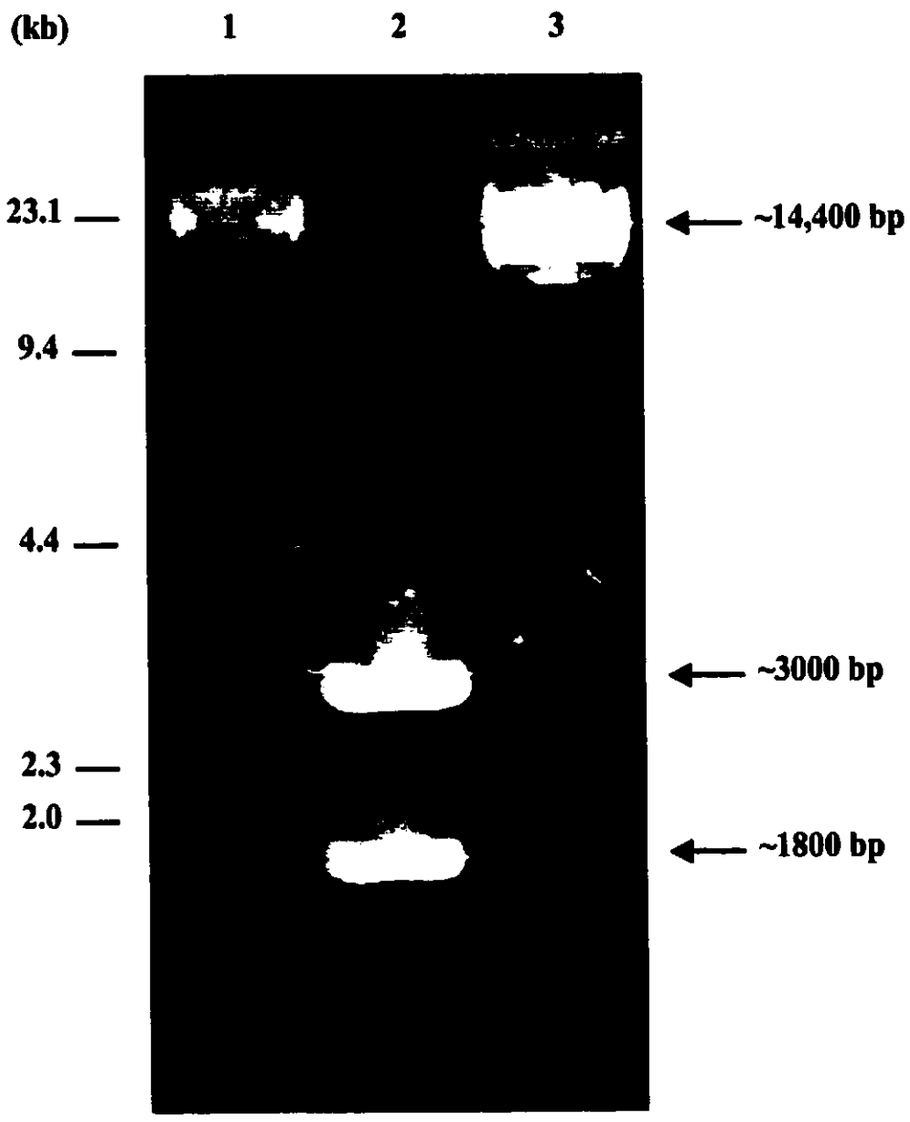

KpnI BamHI XbaI PstI HindIII
RB
ori ColE1
35S-NptII-Term
Gent R
LB
pCGN1559
(14390 bp)
ori pRI

Figure 11. Restriction analysis using *Kpn*I 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/*Hin*dIII Ladder, and lanes 2 and 3 contain restricted pBW(ASP02) and pCGN1559, respectively.


(kb)
1
2
3
23.1
9.4
4.4
2.3
2.0
~14,400 bp
~3000 bp
~1800 bp

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/*Hin*dIII Ladder and lane 2 contains restricted plasmid DNA from the putative colony.

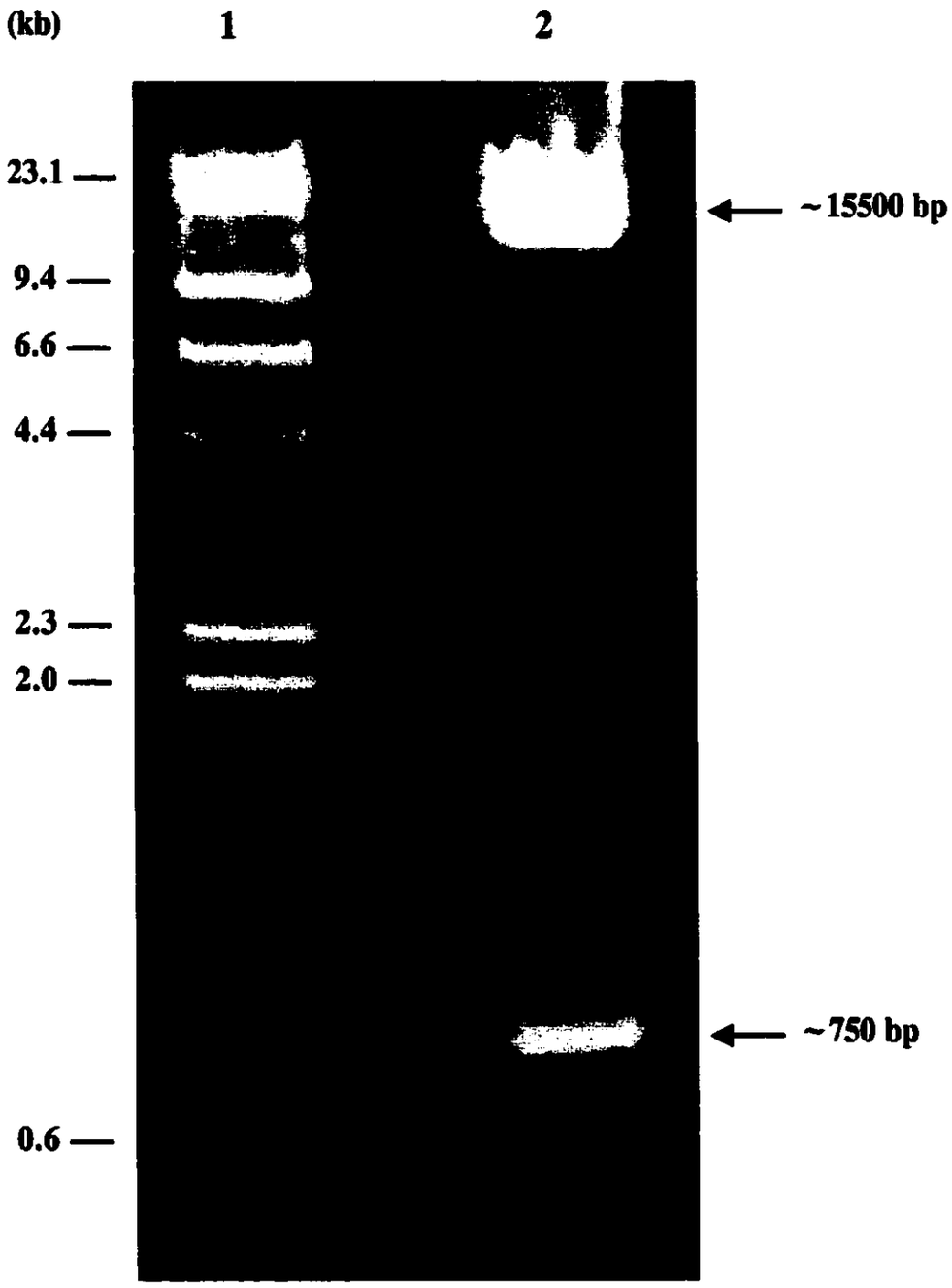
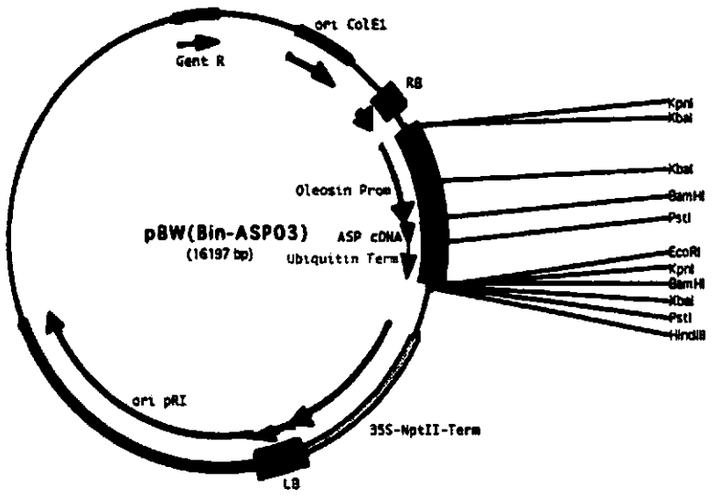

(kb)
1
2
23.1
9.4
6.6
4.4
2.3
2.0
0.6
~15500 bp
~750 bp

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.

PUTATIVE ASP03	TGGCTATACCCAACCTCGGTCTTGGTCACACCAGGAACTCTCTGGTAAGCTAGCT	55
OLEOSIN PROMOTER	TGGCTATACCCAACCTCGGTCTTGGTCACACCAGGAACTCTCTGGTAAGCTAGCT	55
PUTATIVE ASP03	CCACTCCCCAGAAACAACCGGCGCCAAATTGCCGGAATTGCTGACCTGAAGACGG	110
OLEOSIN PROMOTER	CCACTCCCCAGAAACAACCGGCGCCAAATTGCCGGAATTGCTGACCTGAAGACGG	110
PUTATIVE ASP03	AACATCATCGTCGGGTCCTTGGGCGATTGCGGCGGAAGATGGGTCAGCTTGGGCT	165
OLEOSIN PROMOTER	AACATCATCGTCGGGTCCTTGGGCGATTGCGGCGGAAGATGGGTCAGCTTGGGCT	165
PUTATIVE ASP03	TGAGGACGAGACCCGAATCGAGTCTGTTGAAAGGTTGTTCATTGGGATTTGTATA	220
OLEOSIN PROMOTER	TGAGGACGAGACCCGAATCGAGTCTGTTGAAAGGTTGTTCATTGGGATTTGTATA	220
PUTATIVE ASP03	CGGAGATTGGTCGTCGAGAGGTTTGAGGGAAAGGACAAATGGGTTTGGCTCTGGA	275
OLEOSIN PROMOTER	CGGAGATTGGTCGTCGAGAGGTTTGAGGGAAAGGACAAATGGGTTTGGCTCTGGA	275
PUTATIVE ASP03	GAAAGAGAGTGCGGCTTTAGAGAGAGAATTGAGAGGTTTAGAGAGAGATGCGGCG	330
OLEOSIN PROMOTER	GAAAGAGAGTGCGGCTTTAGAGAGAGAATTGAGAGGTTTAGAGAGAGATGCGGCG	330
PUTATIVE ASP03	GCGATGACGGGAGGAGAGACGACGAGGACCTGCATTATCAAAGCAGTGACGTGGT	385
OLEOSIN PROMOTER	GCGATGACGGGAGGAGAGACGACGAGGACCTGCATTATCAAAGCAGTGACGTGGT	385
PUTATIVE ASP03	GAAATTTGGAACTTTTAAGAGGCAGATAGATTTATTATTTGTATCCATTTTCTTC	440
OLEOSIN PROMOTER	GAAATTTGGAACTTTTAAGAGGCAGATAGATTTATTATTTGTATCCATTTTCTTC	440
PUTATIVE ASP03	ATTGTTCTAGAATGTCGCGGAACAAATTTTAAAACTAAATCCTAAATTTTTCTAA	495
OLEOSIN PROMOTER	ATTGTTCTAGAATGTCGCGGAACAAATTTTAAAACTAAATCCTAAATTTTTCTAA	495
PUTATIVE ASP03	TTTTGTTGCCAATAGTGGATATGTGGGCCGTATAGAAGGAATCTATTGAAGGCCC	550
OLEOSIN PROMOTER	TTTTGTTGCCAATAGTGGATATGTGGGCCGTATAGAAGGAATCTATTGAAGGCCC	550
PUTATIVE ASP03	AAACCCATACTGACGAGCCCAAAGGTTCGTTTTGCGTTTTATGTTTCGGTTCGAT	605
OLEOSIN PROMOTER	AAACCCATACTGACGAGCCCAAAGGTTCGTTTTGCGTTTTATGTTTCGGTTCGAT	605
PUTATIVE ASP03	GCCAACGCCACATTCTGAGCTAGGCAAAAAACAAACGTGTCTTTGAATAGACTCC	660
OLEOSIN PROMOTER	GCCAACGCCACATTCTGAGCTAGGCAAAAAACAAACGTGTCTTTGAATAGACTCC	660
PUTATIVE ASP03	TCTCGTTAACACATGCAGCGGCTGCATGGTGACGCCATTAACACGTGGCCTACAA	715
OLEOSIN PROMOTER	TCTCGTTAACACATGCAGCGGCTGCATGGTGACGCCATTAACACGTGGCCTACAA	715
PUTATIVE ASP03	TTGCATGATGTCTCCATTGACACGTGACTTCTCGTCTCCTTTCTTAATATATCTA	770
OLEOSIN PROMOTER	TTGCATGATGTCTCCATTGACACGTGACTTCTCGTCTCCTTTCTTAATATATCTA	770
PUTATIVE ASP03	ACAAACACTCCTACCTCTTCCAAAATATATACACATCTTTTTGATCAATCTCTCA	825
OLEOSIN PROMOTER	ACAAACACTCCTACCTCTTCCAAAATATATACACATCTTTTTGATCAATCTCTCA	825
PUTATIVE ASP03	TTCAAAATCTCATTCTCTCTAGTAAACAGGATCCATGTCCGTTCAGCTGACTGAA	880
OLEOSIN PROMOTER/ASP	TTCAAAATCTCATTCTCTCTAGTAAACA ATGTCCGTTCAGCTGACTGAA	853/21
PUTATIVE ASP03	AAACGTATGGATAAAGTTGGTAAGTACCCGAAGGAACTGCGTAAATGTTGCGAAG	935
ASP	AAACGTATGGATAAAGTTGGTAAGTACCCGAAGGAACTGCGTAAATGTTGCGAAG	76
PUTATIVE ASP03	ACGGGATGCGTGAAAACCCGATGCGTTTCTCTTGCCAACGTCGTACCCGCTTCAT	990
ASP	ACGGGATGCGTGAAAACCCGATGCGTTTCTCTTGCCAACGTCGTACCCGCTTCAT	131
PUTATIVE ASP03	CTCCCTGGGCGAAGCTTGCAAAAAGGTTTTCCTGGACTGCTGCAACTACATCACT	1045
ASP	CTCCCTGGGCGAAGCTTGCAAAAAGGTTTTCCTGGACTGCTGCAACTACATCACT	186
PUTATIVE ASP03	GAACTGCGTCGTCAGCACGCTCGTGCTTCTCACCTGGGTCTGGCTTAATGACTGC	1100
ASP	GAACTGCGTCGTCAGCACGCTCGTGCTTCTCACCTGGGTCTGGCTTAATGA	233
PUTATIVE ASP03	AGAATGAGTTCCAAGATGGTTTGTGACGAAGTTAGTTGGTTGTTTTTATGGAACT	1155
UBIQUITIN TERMINATOR	AATGAGTTCCAAGATGGTTTGTGACGAAGTTAGTTGGTTGTTTTTATGGAACT	53
PUTATIVE ASP03	TTGTTTAAGCTTGTAATGTGGAAAGAACGTGTGGCTTTGTGGTTTTTAA	1204
UBIQUITIN TERMINATOR	TTGTTTAAGCTTGTAATGTGGAAAGAACGTGTGGCTTTGTGGTTTTTAA	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 *Kpn*I 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 Xbal
GGTACCCAAATACGATCTGATACTGATAACGTCTAGATTTTTAGGGTTAAAGCAATCAATCACCTGACGATTCAAGGTGGTTGGATCATGACGATTCCAGAAAACA

TCAAGCAAGCTCTCAAAGCTACACTCTTTGGGATCATACTGAACTCTAACAACCTCGTTATGTCCCGTAGTGCCAGTACAGACATCCTCGTAACTCGGATTATGCA

CGATGCCATGGCTATACCCAACCTCGGTCTTGGTCACACCAGGAACTCTCTGGTAAGCTAGCTCCACTCCCCAGAAACAACCGGCGCCAAATTGCCGGAATTGCTG

ACCTGAAGACGGAACATCATCGTCGGGTCCTTGGGCGATTGCGGCCGGAAGATGGGTCAGCTTGGGCTTGAGGACGAGACCCGAATCGAGTCTGTTGAAAGGTTGTT

CATTGGGATTTGTATACGGAGATTGGTCGTCGAGAGGTTTGAGGGAAAGGACAAATGGGTTTGGCTCTGGAGAAAGAGAGTGCGGCTTTAGAGAGAGAATTGAGAG

GTTTAGAGAGAGATGCGGCGGCGATGACGGGAGGAGAGACGACGAGGACCTGCATTATCAAAGCAGTGACGTGGTGAAATTTGGAACTTTTAAGAGGCAGATAGAT

Xbal
TTATTATTTGTATCCATTTTCTTCATTGTTCTAGAATGTCGCGGAACAAATTTTAAAACTAAATCCTAAATTTTTCTAATTTTGTTGCCAATAGTGGATATGTGGG

CCGTATAGAAGGAATCTATTGAAGGCCCAAACCCATACTGACGAGCCCAAAGGTTCGTTTTGCGTTTTATGTTTCGGTTCGATGCCAACGCCACATTCTGAGCTAG

GCAAAAAACAAACGTGTCTTTGAATAGACTCCTCTCGTTAACACATGCAGCGGCTGCATGGTGACGCCATTAACACGTGGCCTACAATTGCATGATGTCTCCATTG

ACACGTGACTTCTCGTCTCCTTTCTTAATATATCTAACAAACACTCCTACCTCTTCCAAAATATATACACATCTTTTTGATCAATCTCTCATTCAAAATCTCATTC

BamHI
TCTCTAGTAAACAGGATCCATGTCCGTTCAGCTGACTGAAAAACGTATGGATAAAGTTGGTAAGTACCCGAAGGAACTGCGTAAATGTTGCGAAGACGGGATGCGT

GAAAACCCGATGCGTTTCTCTTGCCAACGTCGTACCCGCTTCATCTCCCTGGGCGAAGCTTGCAAAAAGGTTTTCCTGGACTGCTGCAACTACATCACTGAACTGC

PstI
GTCGTCAGCACGCTCGTGCTTCTCACCTGGGTCTGGCTTAATGACTGCAGAATGAGTTCCAAGATGGTTTGTGACGAAGTTAGTTGGTTGTTTTTATGGAACTTTG

TTTAAGCTTGTAATGTGGAAAGAACGTGTGGCTTTGTGGTTTTTAAATGTTGGTGAATAAAGATGTTTCCTTTGGATTAACTAGTATTTTTCCTATTGGTTTCATG

GTTTTAGCACACAACATTTTAAATATGCTGTTAGATGATATGCTGCCTGCTTTATTATTTACTTACCCCTCACCTTCAGTTTCAAAGTTGTTGCAATGACTCTGTG

TAGTTTAAGATCGAGTGAAAGTAGATTTTGTCTATATTTATTAGGGGTATTTGATATGCTAATGGTAAACATGGTTTATGACAGCGTACTTTTTTGGTTATGGTGT

Ec
TGACGTTTCCTTTTAAACATTATAGTAGCGTCCTTGGTCTGTGTTCATTGGTTGAACAAAGGCACACTCACTTGGAGATGCCGTCTCCACTGATATTTGAACAAAG

Kpni BamHI Xbal PstI HindIII
AATTCGGTACCGGATCCTCTAGACTGCAGGCATGCAAGCTT

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.

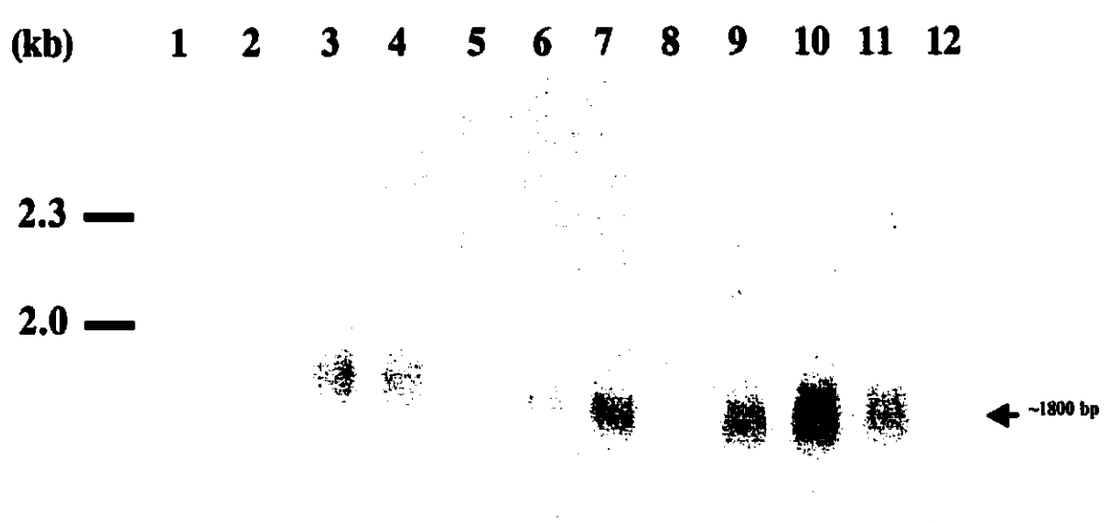
4.1.4. Integration of ASP cDNA into the Plant Genome

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 *Kpn*I 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 [^{32}P]-*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 [^{32}P]-*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.

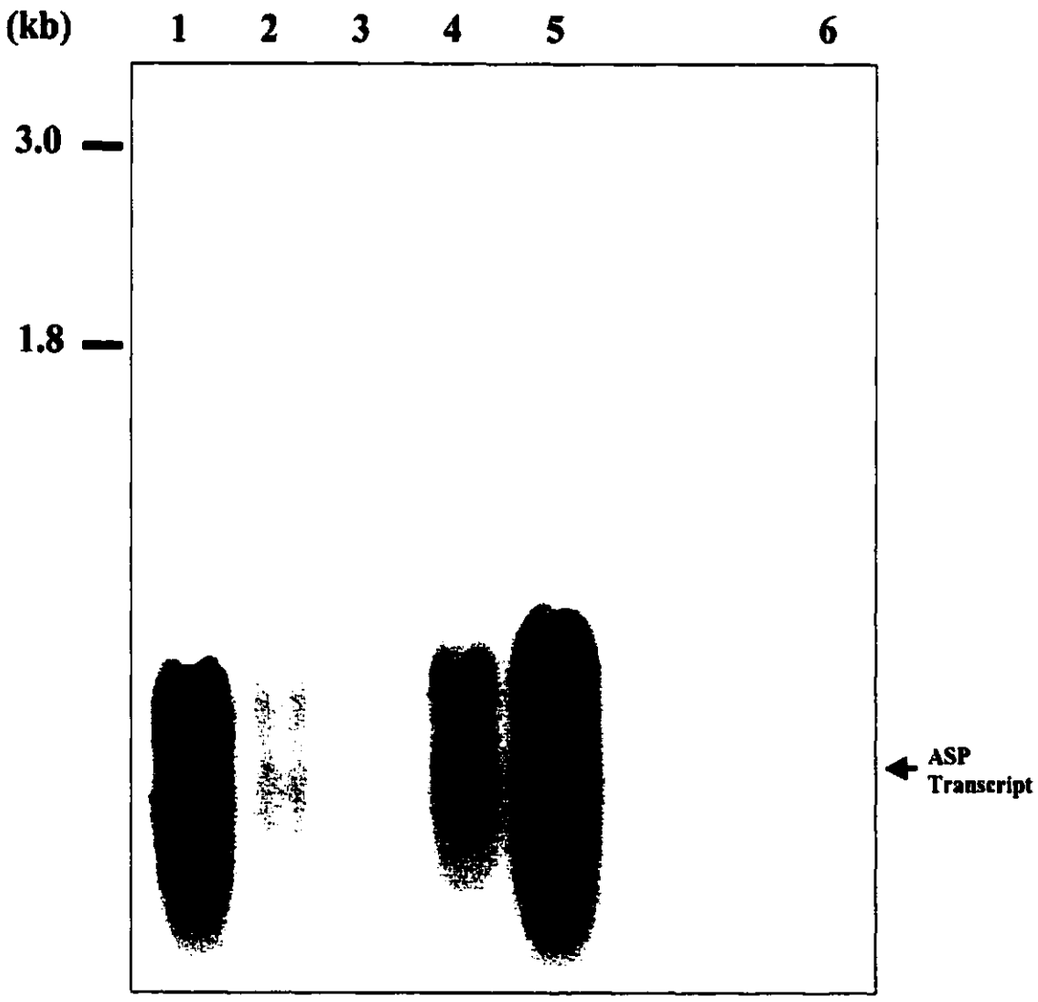

(kb)
1
2
3
4
5
6
7
8
9
10
11
12
2.3
2.0
~1800 bp

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 [^{32}P]-*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 μg 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 [^{32}P]-*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.

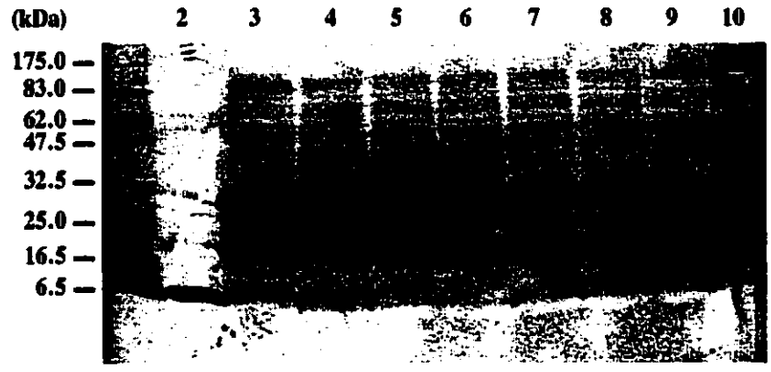

(kb)
1
2
3
4
5
6
3.0
1.8
ASP
Transcript

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 immunorcactive 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 μg negative control protein extract spiked with 100 ng ASP; 4, 200 μg negative control protein extract spiked with 75 ng ASP; 5, 200 μg negative control protein extract spiked with 50 ng ASP; 6, 200 μg negative control protein extract spiked with 25 ng ASP; 7, 200 μg negative control protein extract spiked with 10 ng ASP; 8, 200 μg negative control protein extract; 9, 200 μg ASP 1 protein extract; 10, 200 μg ASP 14 protein extract.


(kDa)
2
3
4
5
6
7
8
9
10
175.0
83.0
62.0
47.5
32.5
25.0
16.5
6.5

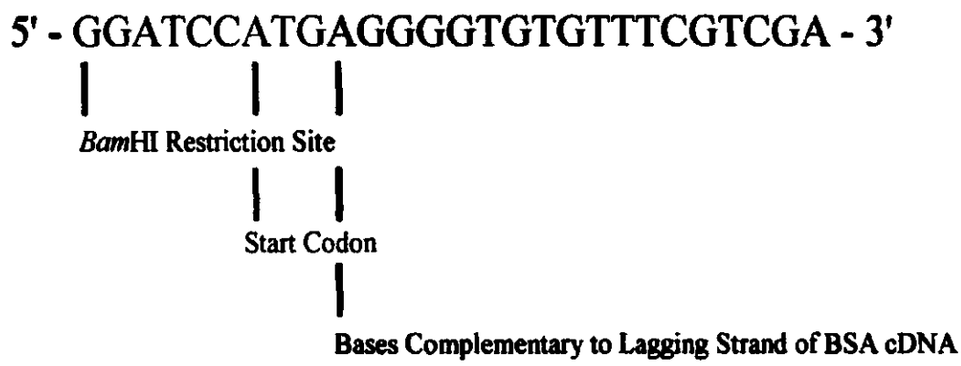
directed by homologous transgenes. Post-transcriptional gene silencing was also suggested when low levels of introduced P1 sequence of potato virus Y was found in transformed potato cultivar Pito (Valkama *et al.*, 2000). In addition, we may be facing a stability problem with the protein. Boothe *et 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



Reverse Primer: BSA747

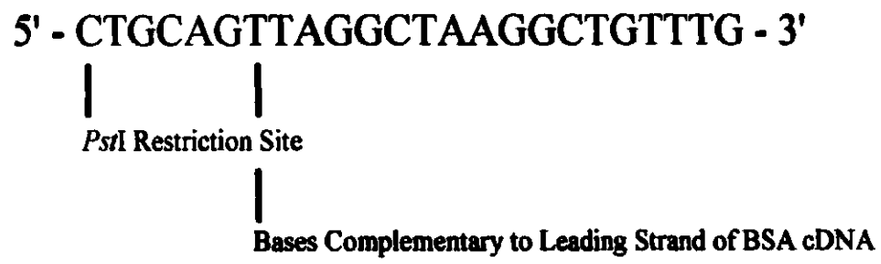
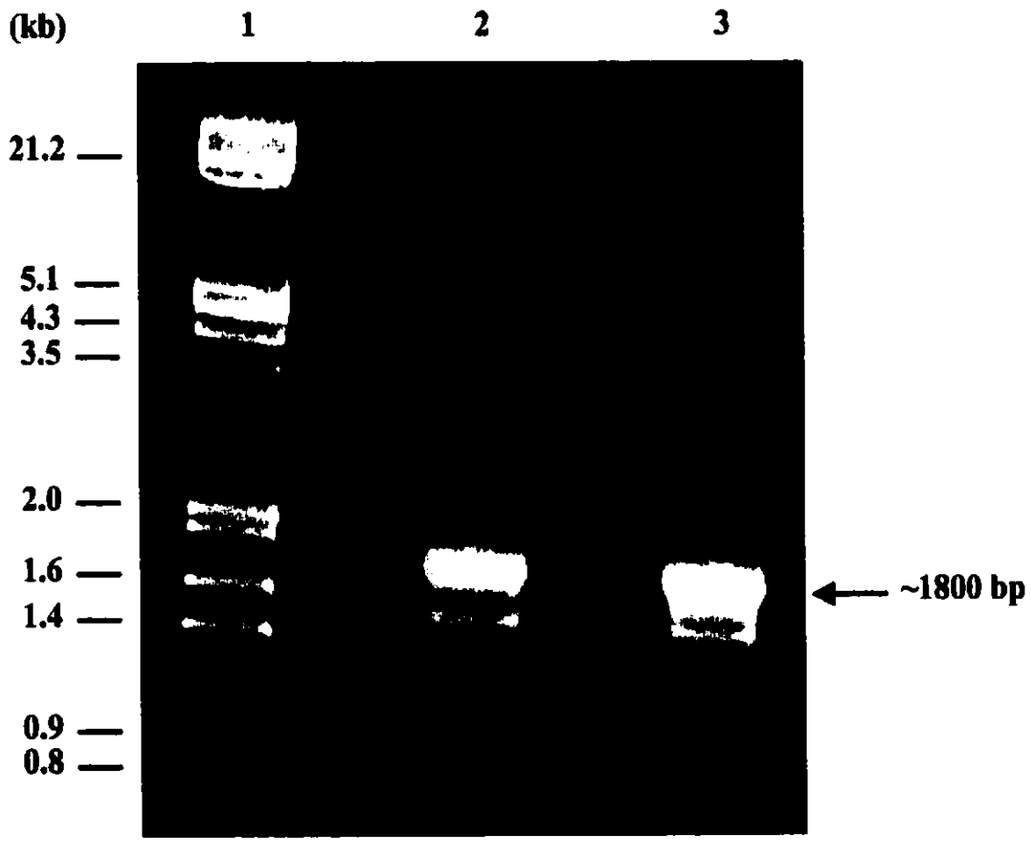


Figure 19. Agarose gel electrophoresis analysis of PCR of BSA cDNA using *Pfu* polymerase and primers BSA736 and BSA747. The reaction conditions were 94°C for 1 minute (hot start) and 35 subsequent PCR cycles at 94°C for 45 seconds, 55°C for 1 minute and 72°C for 2 minutes. Following the 35 cycles, a 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/ *Eco*RI+*Hin*dIII Ladder (MBI Fermentas, Flamborough, ON), and lanes 2 and 3 contain 5μL of the PCR reaction from two independent trials.


(kb)
1
2
3
21.2
5.1
4.3
3.5
2.0
1.6
1.4
0.9
0.8
~1800 bp

The blunt-ended PCR fragment was ligated into a BlueScript plasmid that was previously linearized with *Eco*RV. 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+*Hin*dIII 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.

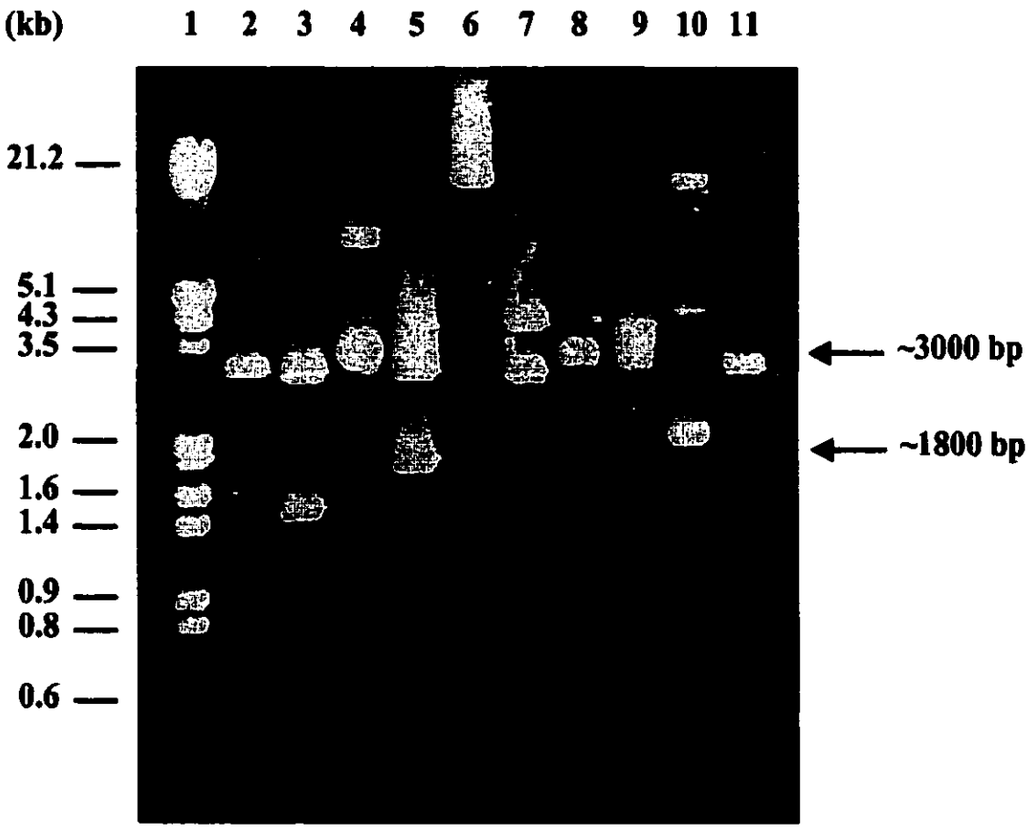

(kb)
1 2 3 4 5 6 7 8 9 10 11
21.2
5.1
4.3
3.5
2.0
1.6
1.4
0.9
0.8
0.6
~3000 bp
~1800 bp

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 BSAO1    GGATCCATGAGGGGTGTGTTTCGTCGAGATACACACAAGAGTGAGATTGCTCATC   55
BSA cDNA                AGGGGTGTGTTTCGTCGAGATACACACAAGAGTGAGATTGCTCATC   46

PUTATIVE BSAO1    GGTTTAAAGATTTGGGAGAAGAACATTTTAAAGGCCTGGTACTGATTGCCTTTTC  110
BSA cDNA          GGTTTAAAGATTTGGGAGAAGAACATTTTAAAGGCCTGGTACTGATTGCCTTTTC  101

PUTATIVE BSAO1    TCAGTATCTCCAGCAGTGTCCATTTGATGAGCATGTAAAATTAGTGAACGAACTA  165
BSA cDNA          TCAGTATCTCCAGCAGTGTCCATTTGATGAGCATGTAAAATTAGTGAACGAACTA  156

PUTATIVE BSAO1    ACTGAGTTTGCAAAAACATGTGTTGCTGATGAGTCCCATGCCGGCTGTGAAAAGT  220
BSA cDNA          ACTGAGTTTGCAAAAACATGTGTTGCTGATGAGTCCCATGCCGGCTGTGAAAAGT  211

PUTATIVE BSAO1    CACTTCACACTCTCTTTGGAGATGAATTGTGTAAAGTTGCATCCCTTCGTGAAAC  275
BSA cDNA          CACTTCACACTCTCTTTGGAGATGAATTGTGTAAAGTTGCATCCCTTCGTGAAAC  266

PUTATIVE BSAO1    CTATGGTGACATGGCTGACTGCTGTGAGAAACAAGAGCCTGAAAGAAATGAATGC  330
BSA cDNA          CTATGGTGACATGGCTGACTGCTGTGAGAAACAAGAGCCTGAAAGAAATGAATGC  321

PUTATIVE BSAO1    TTCCTGAGCCACAAAGATGATAGCCCAGACCTCCCTAAATTGAAACCAGACCCCA  385
BSA cDNA          TTCCTGAGCCACAAAGATGATAGCCCAGACCTCCCTAAATTGAAACCAGACCCCA  376

PUTATIVE BSAO1    ATACTTTGTGTGATGAGTTTAAGGCAGATGAAAAGAAGTTTTGGGGAAAATACCT  440
BSA cDNA          ATACTTTGTGTGATGAGTTTAAGGCAGATGAAAAGAAGTTTTGGGGAAAATACCT  431

PUTATIVE BSAO1    ATACGAAATTGCTAGAAGACATCCCTACTTTTATGCACCAGAACTCCTTTACTAT  495
BSA cDNA          ATACGAAATTGCTAGAAGACATCCCTACTTTTATGCACCAGAACTCCTTTACTAT  486

PUTATIVE BSAO1    GCTAATAAATATAATGGAGTTTTTCAAGAATGCTGCCAAGCTGAAGATAAAGGTG  550
BSA cDNA          GCTAATAAATATAATGGAGTTTTTCAAGAATGCTGCCAAGCTGAAGATAAAGGTG  541

PUTATIVE BSAO1    CCTGCCTGCTACCAAAGATTGAAACTATGAGAGAAAAAGTACTGACTTCATCTGC  605
BSA cDNA          CCTGCCTGCTACCAAAGATTGAAACTATGAGAGAAAAAGTACTGACTTCATCTGC  596

PUTATIVE BSAO1    CAGACAGAGACTCAGGTGTGCCAGTATTCAAAAATTTGGAGAAAGAGCTTTAAAA  660
BSA cDNA          CAGACAGAGACTCAGGTGTGCCAGTATTCAAAAATTTGGAGAAAGAGCTTTAAAA  651

PUTATIVE BSAO1    GCATGGTCAGTAGCTCGCCTGAGCCAGAAATTTCCCAAGGCTGAGTTTGTAGAAG  715
BSA cDNA          GCATGGTCAGTAGCTCGCCTGAGCCAGAAATTTCCCAAGGCTGAGTTTGTAGAAG  706

PUTATIVE BSAO1    TTACCAAGCTAGTGACAGATCTCACAAAAGTCCACAAGGAATGCTGCCATGGTGA  770
BSA cDNA          TTACCAAGCTAGTGACAGATCTCACAAAAGTCCACAAGGAATGCTGCCATGGTGA  761

PUTATIVE BSAO1    CCTACTTGAATGCGCAGATGACAGGGCAGATCTTGCCAAGTACATATGTGATAAT  825
BSA cDNA          CCTACTTGAATGCGCAGATGACAGGGCAGATCTTGCCAAGTACATATGTGATAAT  816

PUTATIVE BSAO1    CAAGATACAATCTCCAGTAAACTGAAGGAATGCTGTGATAAGCCTTTGTTGGAAA  880
BSA cDNA          CAAGATACAATCTCCAGTAAACTGAAGGAATGCTGTGATAAGCCTTTGTTGGAAA  871

PUTATIVE BSAO1    AATCCCACTGCATTGCTGAGGTAGAAAAAGATGCCATACCTGAAAACCTGCCCCC  935
BSA cDNA          AATCCCACTGCATTGCTGAGGTAGAAAAAGATGCCATACCTGAAAACCTGCCCCC  926

PUTATIVE BSAO1    ATTAACTGCTGACTTTGCTGAAGATAAGGATGTTTGCAAAAACTATCAGGAAGCA  990
BSA cDNA          ATTAACTGCTGACTTTGCTGAAGATAAGGATGTTTGCAAAAACTATCAGGAAGCA  981

PUTATIVE BSAO1    AAAGATGCCTTCCTGGGCTCGTTTTTGTATGAATATTCAAGAAGGCATCCTGAAT  1045
BSA cDNA          AAAGATGCCTTCCTGGGCTCGTTTTTGTATGAATATTCAAGAAGGCATCCTGAAT  1036

PUTATIVE BSAO1    ATGCTGTCTCAGTGCTATTGAGACTTGCCAAGGAATATGAAGCCACACTGGAGGA  1100
BSA cDNA          ATGCTGTCTCAGTGCTATTGAGACTTGCCAAGGAATATGAAGCCACACTGGAGGA  1091

PUTATIVE BSAO1    ATGCTGTGCCAAAGATGATCCACATGCATGCTATTCCACAGTGTTTGACAAACTT  1155
BSA cDNA          ATGCTGTGCCAAAGATGATCCACATGCATGCTATTCCACAGTGTTTGACAAACTT  1146

PUTATIVE BSAO1    AAGCATCTTGTGGATGAGCCTCAGAATTTAATCAAACAAAACTGTGACCAATTCG  1210
BSA cDNA          AAGCATCTTGTGGATGAGCCTCAGAATTTAATCAAACAAAACTGTGACCAATTCG  1201
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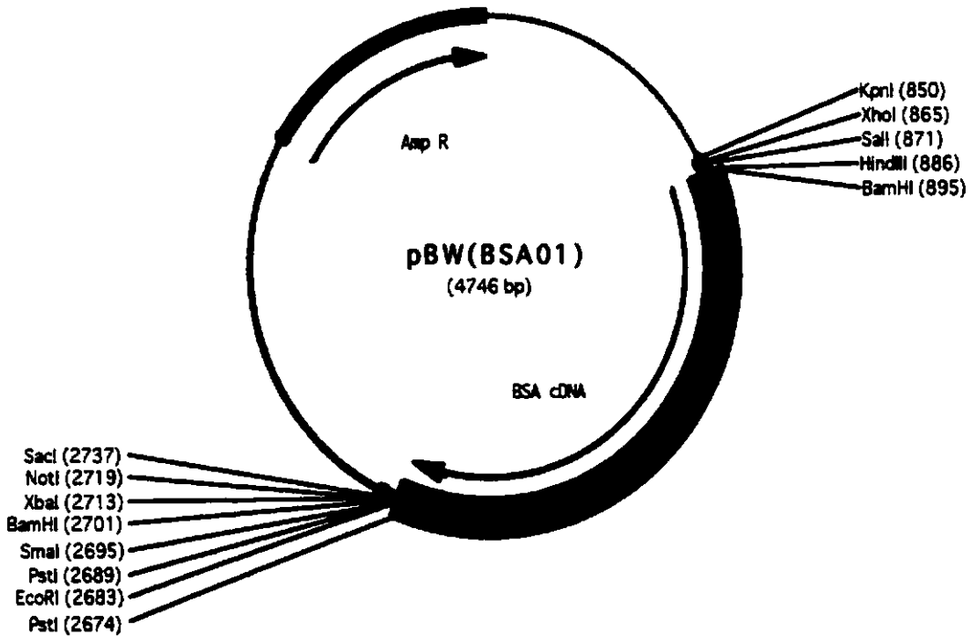
PUTATIVE *BSAO1*	AAAAACTTGGAGAGTATGGATTCCAAAATGCGCTCATAGTTCGTTACACCAGGAA	1265
BSA cDNA	AAAAACTTGGAGAGTATGGATTCCAAAATGCGCTCATAGTTCGTTACACCAGGAA	1256
PUTATIVE *BSAO1*	AGTACCCCAAGTGTCAACTCCAACTCTCGTGGAGGTTTCAAGAAGCCTAGGAAAA	1320
BSA cDNA	AGTACCCCAAGTGTCAACTCCAACTCTCGTGGAGGTTTCAAGAAGCCTAGGAAAA	1311
PUTATIVE *BSAO1*	GTGGGTACTAGGTGTTGTACAAAGCCGGAATCAGAAAGAATGCCCTGTACTGAAG	1375
BSA cDNA	GTGGGTACTAGGTGTTGTACAAAGCCGGAATCAGAAAGAATGCCCTGTACTGAAG	1366
PUTATIVE *BSAO1*	ACTATCTGAGCTTGATCCTGAACCGGTTGTGCGTGCTGCATGAGAAGACACCAGT	1430
BSA cDNA	ACTATCTGAGCTTGATCCTGAACCGGTTGTGCGTGCTGCATGAGAAGACACCAGT	1421
PUTATIVE *BSAO1*	GAGTGAAAAAGTCACCAAGTGCTGCACAGAGTCATTGGTGAACAGACGGCCATGT	1485
BSA cDNA	GAGTGAAAAAGTCACCAAGTGCTGCACAGAGTCATTGGTGAACAGACGGCCATGT	1476
PUTATIVE *BSAO1*	TTCTCTGCTCTGACACCTGATGAAACATATGTACCCAAAGCCTTTGATGAGAAAT	1540
BSA cDNA	TTCTCTGCTCTGACACCTGATGAAACATATGTACCCAAAGCCTTTGATGAGAAAT	1531
PUTATIVE *BSAO1*	TGTTCACCTTCCATGCAGATATATGCACACTTCCCGATACTGAGAAACAAATCAA	1595
BSA cDNA	TGTTCACCTTCCATGCAGATATATGCACACTTCCCGATACTGAGAAACAAATCAA	1586
PUTATIVE *BSAO1*	GAAACAAACTGCACTTGTTGAGCTGTTGAAACACAAGCCCAAGGCAACAGAGGAA	1650
BSA cDNA	GAAACAAACTGCACTTGTTGAGCTGTTGAAACACAAGCCCAAGGCAACAGAGGAA	1641
PUTATIVE *BSAO1*	CAACTGAAAACCGTCATGGAGAATTTTGTGGCTTTTGTAGACAAGTGCTGTGCAG	1705
BSA cDNA	CAACTGAAAACCGTCATGGAGAATTTTGTGGCTTTTGTAGACAAGTGCTGTGCAG	1696
PUTATIVE *BSAO1*	CTGATGACAAAGAGGCCTGCTTTGCTGTGGAGGGTCCAAAACTTGTTGTTTCAAC	1760
BSA cDNA	CTGATGACAAAGAGGCCTGCTTTGCTGTGGAGGGTCCAAAACTTGTTGTTTCAAC	1751
PUTATIVE *BSAO1*	TCAAACAGCCTTAGCCTAACTGCAG	1785
BSA cDNA	TCAAACAGCCTTAGCCTAA	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 *Eco*RV restriction site. The detailed sequence of the insert is shown along with some flanking sequences on the page following the plasmid map.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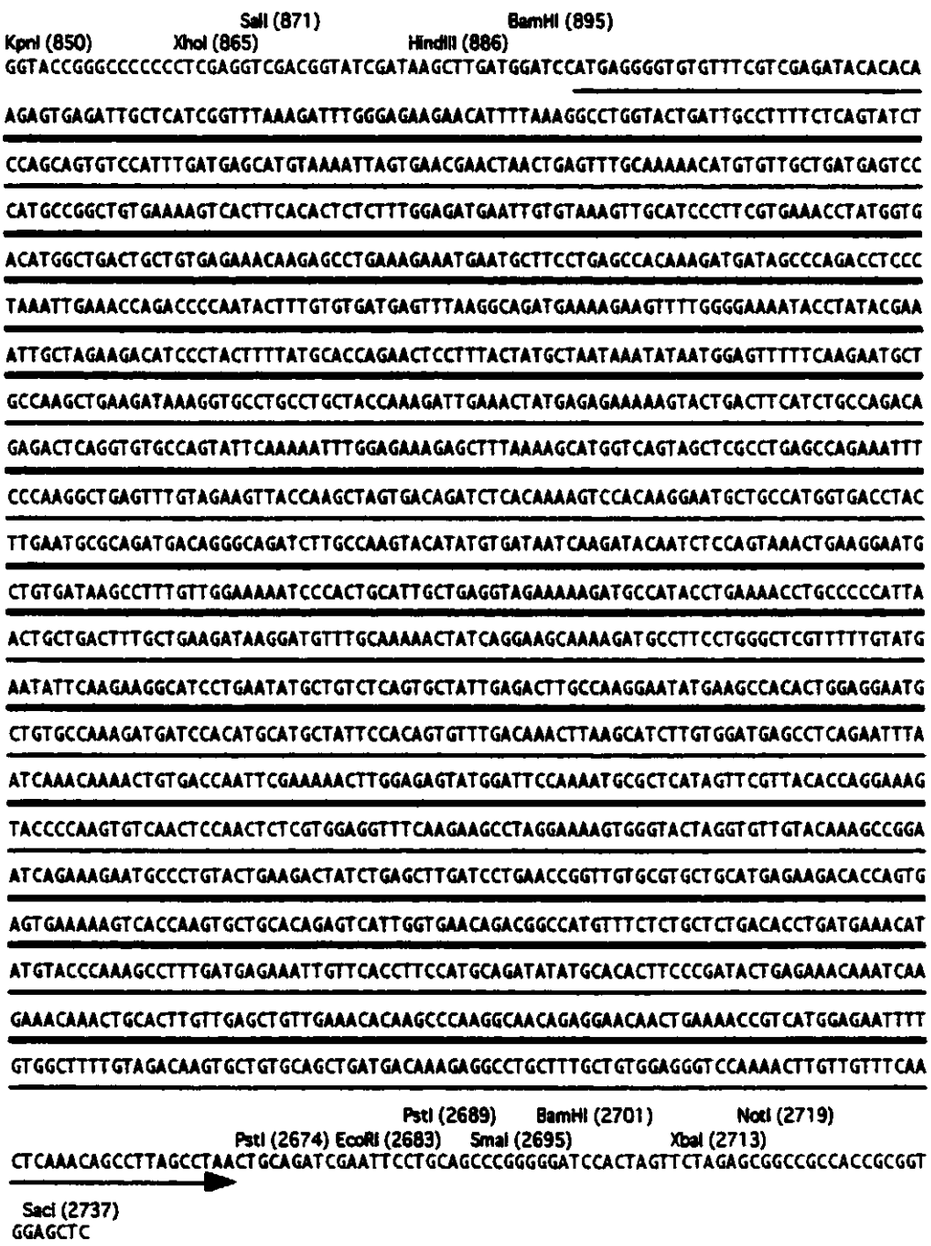
pBW(BSA01)
(4746 bp)
BSA cDNA
KpnI (850)
XhoI (865)
SalI (871)
HindIII (886)
BamHI (895)
SacI (2737)
NotI (2719)
XbaI (2713)
BamHI (2701)
SmaI (2695)
PstI (2689)
EcoRI (2683)
PstI (2674)


SalI (871)
BamHI (895)
KpnI (850)
XhoI (865)
HindIII (886)
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CCAGCAGTGTCCATTTGATGAGCATGTAAAATTAGTGAACGAACTAACTGAGTTTGCAAAAACATGTGTTGCTGATGAGTCC
CATGCCGGCTGTGAAAAGTCACTTCACACTCTCTTTGGAGATGAATTGTGTAAAGTTGCATCCCTTCGTGAAACCTATGGTG
ACATGGCTGACTGCTGTGAGAAACAAGAGCCTGAAAGAAATGAATGCTTCCTGAGCCACAAAGATGATAGCCCAGACCTCCC
TAAATTGAAACCAGACCCCAATACTTTGTGTGATGAGTTTAAGGCAGATGAAAAGAAGTTTTGGGGAAAATACCTATACGAA
ATTGCTAGAAGACATCCCTACTTTTATGCACCAGAACTCCTTTACTATGCTAATAAATATAATGGAGTTTTTCAAGAATGCT
GCCAAGCTGAAGATAAAGGTGCCTGCCTGCTACCAAAGATTGAAACTATGAGAGAAAAAGTACTGACTTCATCTGCCAGACA
GAGACTCAGGTGTGCCAGTATTCAAAAATTTGGAGAAAGAGCTTTAAAAGCATGGTCAGTAGCTCGCCTGAGCCAGAAATTT
CCCAAGGCTGAGTTTGTAGAAGTTACCAAGCTAGTGACAGATCTCACAAAAGTCCACAAGGAATGCTGCCATGGTGACCTAC
TTGAATGCGCAGATGACAGGGCAGATCTTGCCAAGTACATATGTGATAATCAAGATACAATCTCCAGTAAACTGAAGGAATG
CTGTGATAAGCCTTTGTTGGAAAAATCCCACTGCATTGCTGAGGTAGAAAAAGATGCCATACCTGAAAACCTGCCCCCATTA
ACTGCTGACTTTGCTGAAGATAAGGATGTTTGCAAAAACTATCAGGAAGCAAAAGATGCCTTCCTGGGCTCGTTTTTGTATG
AATATTCAAGAAGGCATCCTGAATATGCTGTCTCAGTGCTATTGAGACTTGCCAAGGAATATGAAGCCACACTGGAGGAATG
CTGTGCCAAAGATGATCCACATGCATGCTATTCCACAGTGTTTGACAAACTTAAGCATCTTGTGGATGAGCCTCAGAATTTA
ATCAAACAAAACTGTGACCAATTCGAAAAACTTGGAGAGTATGGATTCCAAAATGCGCTCATAGTTCGTTACACCAGGAAAG
TACCCCAAGTGTCAACTCCAACTCTCGTGGAGGTTTCAAGAAGCCTAGGAAAAGTGGGTACTAGGTGTTGTACAAAGCCGGA
ATCAGAAAGAATGCCCTGTACTGAAGACTATCTGAGCTTGATCCTGAACCGGTTGTGCGTGCTGCATGAGAAGACACCAGTG
AGTGAAAAAGTCACCAAGTGCTGCACAGAGTCATTGGTGAACAGACGGCCATGTTTCTCTGCTCTGACACCTGATGAAACAT
ATGTACCCAAAGCCTTTGATGAGAAATTGTTCACCTTCCATGCAGATATATGCACACTTCCCGATACTGAGAAACAAATCAA
GAAACAAACTGCACTTGTTGAGCTGTTGAAACACAAGCCCAAGGCAACAGAGGAACAACTGAAAACCGTCATGGAGAATTTT
GTGGCTTTTGTAGACAAGTGCTGTGCAGCTGATGACAAAGAGGCCTGCTTTGCTGTGGAGGGTCCAAAACTTGTTGTTTCAA
PstI (2689)
BamHI (2701)
NotI (2719)
PstI (2674)
EcoRI (2683)
SmaI (2695)
XbaI (2713)
CTCAAACAGCCTTAGCCTAACTGCAGATCGAATTCCTGCAGCCCGGGGGATCCACTAGTTCTAGAGCGGCCGCCACCGCGGT
SacI (2737)
GGAGCTC

Figure 23. Restriction analysis using *Bam*HI and *Pst*I on plasmid DNA from putative pBW(BSA02) 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/*Hin*dIII 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.

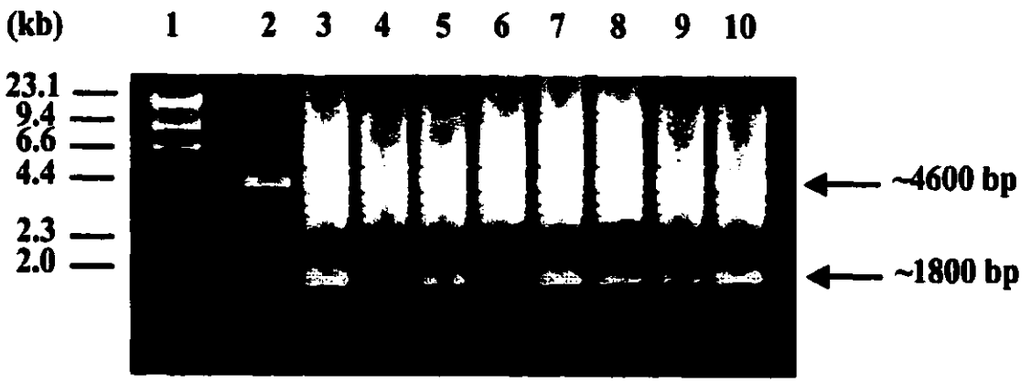
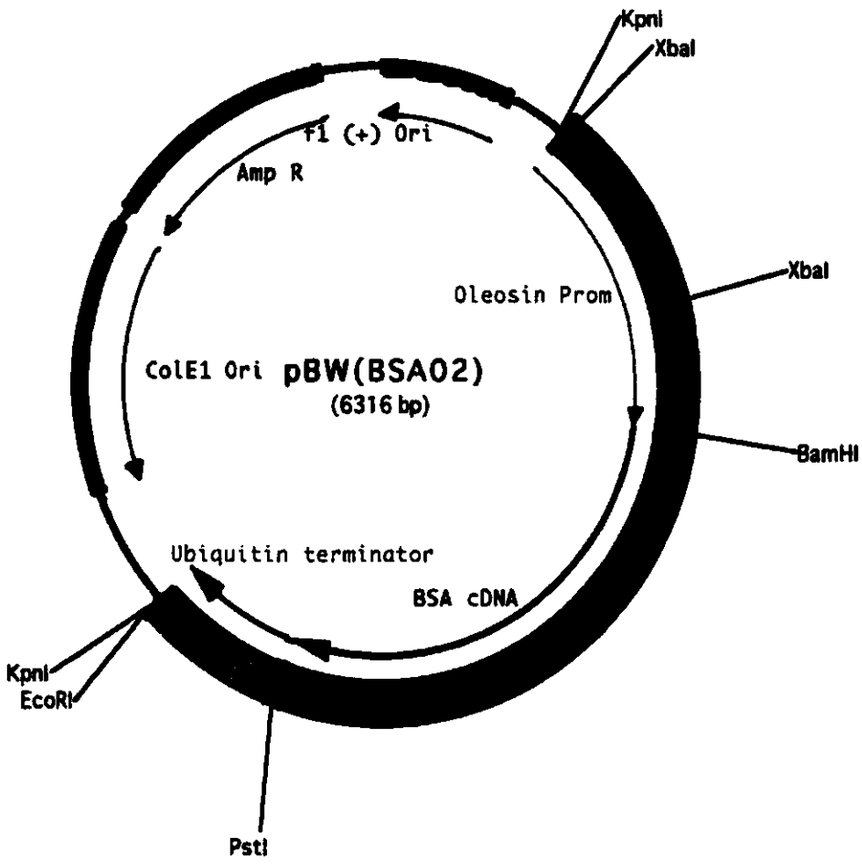
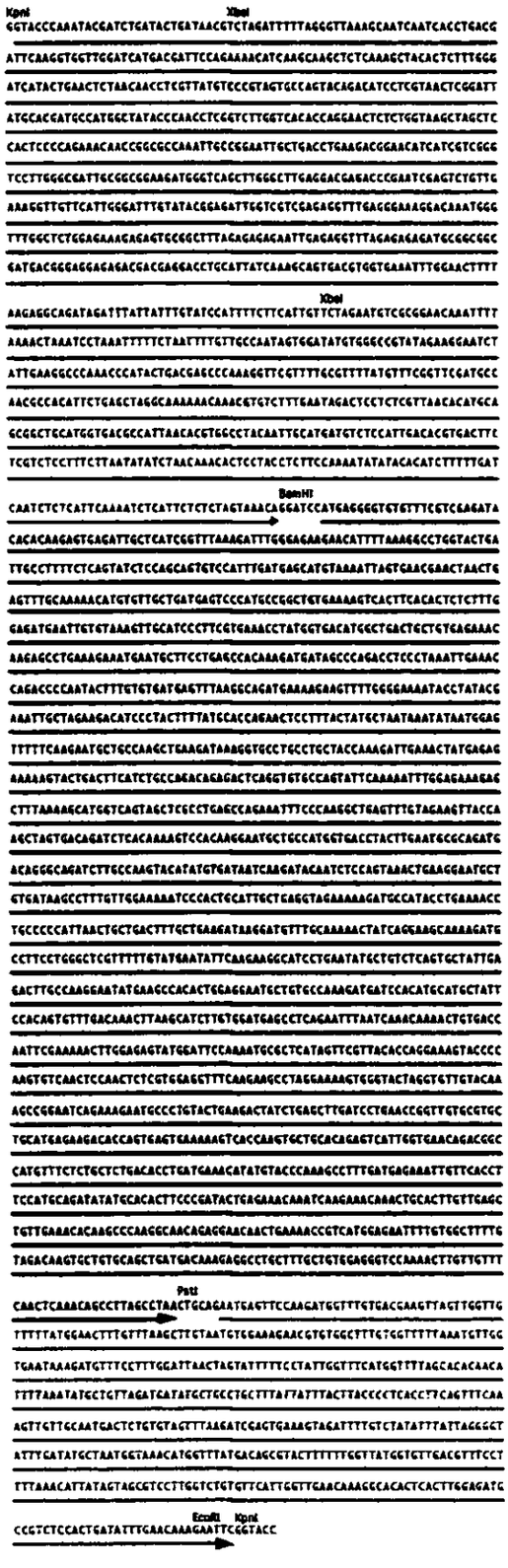

(kb)
1
2
3
4
5
6
7
8
9
10
23.1
9.4
6.6
4.4
2.3
2.0
~4600 bp
~1800 bp

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
f1 (+) Ori
Amp R
XbaI
Oleosin Prom
ColE1 Ori
pBW(BSA02)
(6316 bp)
BamHI
Ubiquitin terminator
BSA cDNA
KpnI
EcoRI
PstI


KpnI
XbaI
XbaI
BamHI
PstI
EcoRI
KpnI

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 *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 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 *Kpn*I (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 *Bam*HI (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 *Kpn*I 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/*Hin*dIII Ladder, and lanes 2 and 3 contain restricted pCGN1559 and pBW(ASP02), respectively.

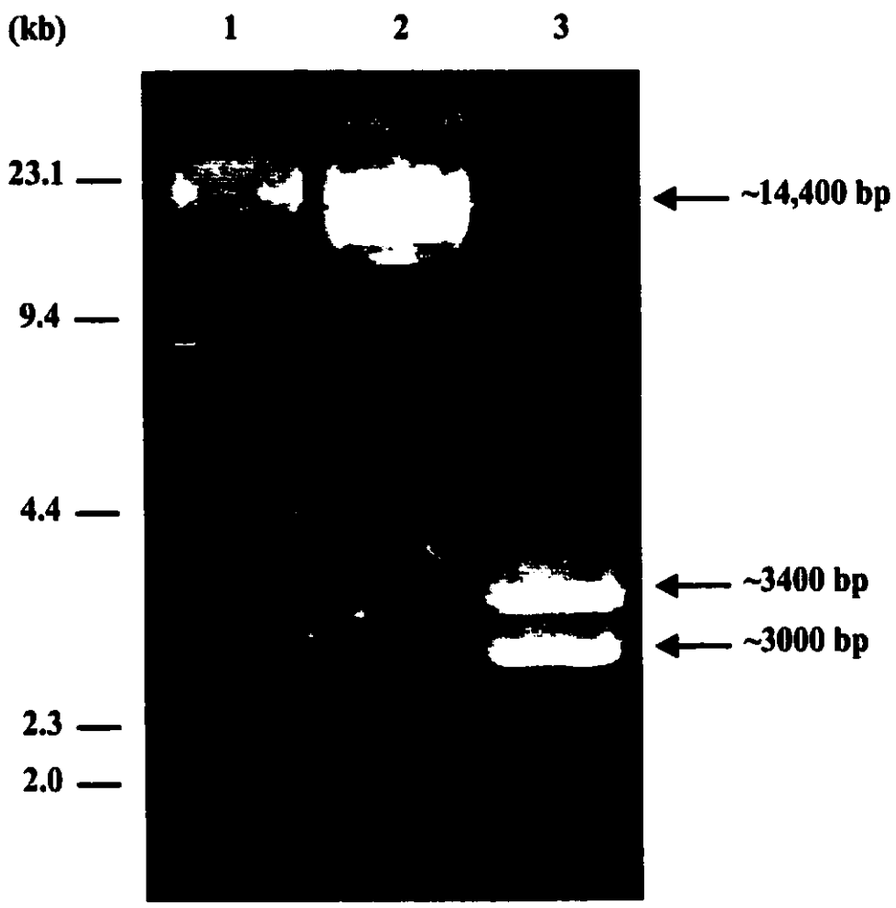

(kb)
1
2
3
23.1
9.4
4.4
2.3
2.0
~14,400 bp
~3400 bp
~3000 bp

Figure 26. Restriction analysis using *Kpn*I 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/*Hin*dIII Ladder, and lane 2 contains restricted plasmid DNA from the putative colony.

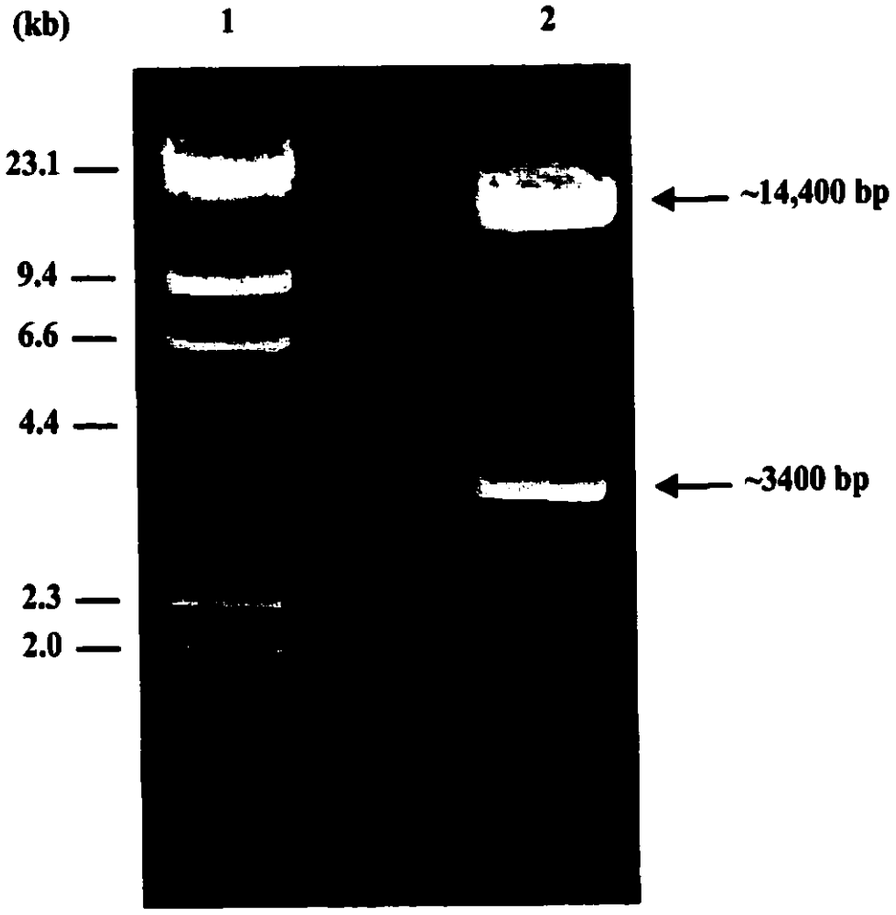

(kb)
1
2
23.1
9.4
6.6
4.4
2.3
2.0
~14,400 bp
~3400 bp

Figure 27. Orientation analysis using *Bam*HI on plasmid DNA from putative pBW(Bin-BSA03) clones. 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/*Hin*dIII Ladder, and lanes 2 through 4 contain restricted plasmid DNA from colonies 1 through 3, respectively.

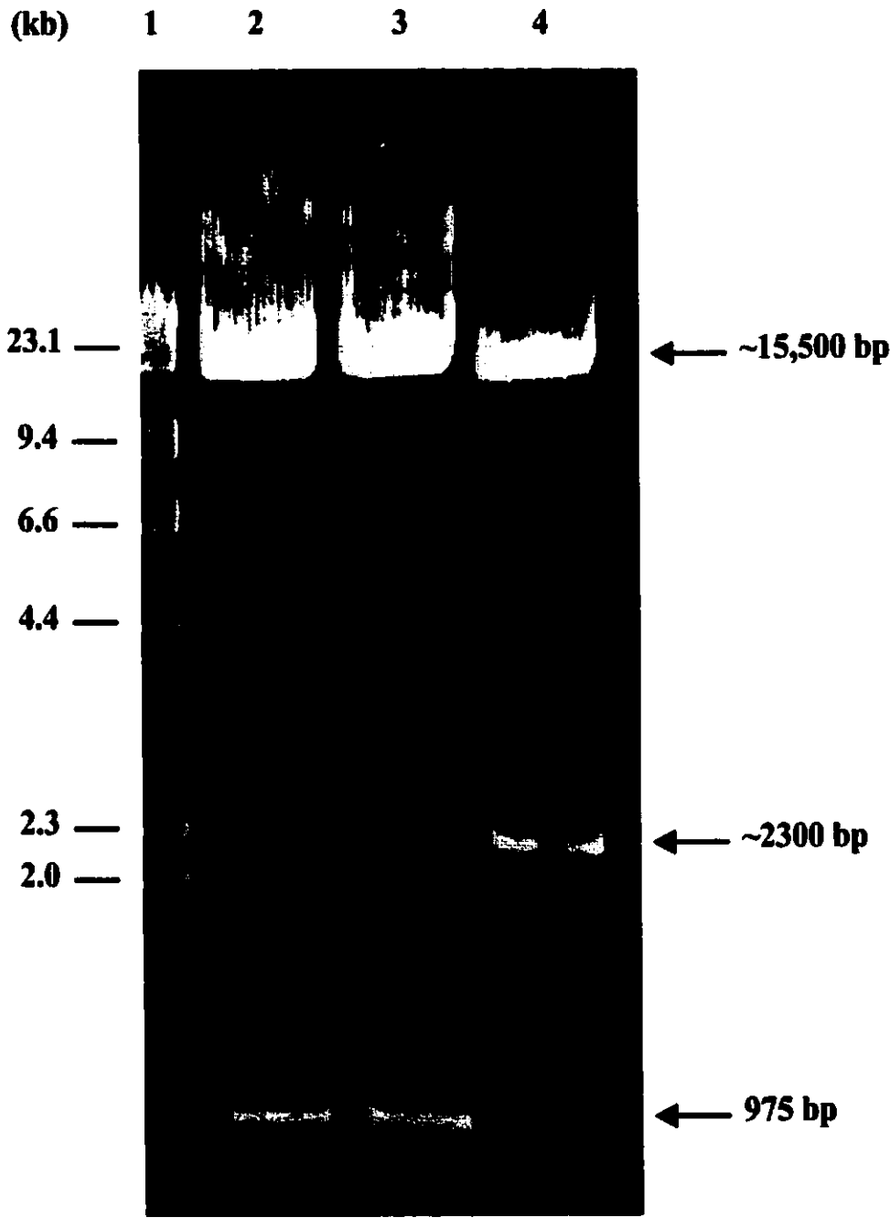

(kb)
1
2
3
4
23.1
9.4
6.6
4.4
2.3
2.0
~15,500 bp
~2300 bp
975 bp

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 *BSAO3*	TGGCTATACCCAACCTCGGTCTTGGTCACACCAGGAACTCTCTGGTAAGCTAGCT	55
OLEOSIN PROMOTER	TGGCTATACCCAACCTCGGTCTTGGTCACACCAGGAACTCTCTGGTAAGCTAGCT	55
PUTATIVE *BSAO3*	CCACTCCCCAGAAACAACCGGCGCCAAATTGCCGGAATTGCTGACCTGAAGACGG	110
OLEOSIN PROMOTER	CCACTCCCCAGAAACAACCGGCGCCAAATTGCCGGAATTGCTGACCTGAAGACGG	110
PUTATIVE *BSAO3*	AACATCATCGTCGGGTCCTTGGGCGATTGCGGCGGAAGATGGGTCAGCTTGGGCT	165
OLEOSIN PROMOTER	AACATCATCGTCGGGTCCTTGGGCGATTGCGGCGGAAGATGGGTCAGCTTGGGCT	165
PUTATIVE *BSAO3*	TGAGGACGAGACCCGAATCGAGTCTGTTGAAAGGTTGTTCATTGGGATTTGTATA	220
OLEOSIN PROMOTER	TGAGGACGAGACCCGAATCGAGTCTGTTGAAAGGTTGTTCATTGGGATTTGTATA	220
PUTATIVE *BSAO3*	CGGAGATTGGTCGTCGAGAGGTTTGAGGGAAAGGACAAATGGGTTTGGCTCTGGA	275
OLEOSIN PROMOTER	CGGAGATTGGTCGTCGAGAGGTTTGAGGGAAAGGACAAATGGGTTTGGCTCTGGA	275
PUTATIVE *BSAO3*	GAAAGAGAGTGCGGCTTTAGAGAGAGAATTGAGAGGTTTAGAGAGAGATGCGGCG	330
OLEOSIN PROMOTER	GAAAGAGAGTGCGGCTTTAGAGAGAGAATTGAGAGGTTTAGAGAGAGATGCGGCG	330
PUTATIVE *BSAO3*	GCGATGACGGGAGGAGAGACGACGAGGACCTGCATTATCAAAGCAGTGACGTGGT	385
OLEOSIN PROMOTER	GCGATGACGGGAGGAGAGACGACGAGGACCTGCATTATCAAAGCAGTGACGTGGT	385
PUTATIVE *BSAO3*	GAAATTTGGAACTTTTAAGAGGCAGATAGATTTATTATTTGTATCCATTTTCTTC	440
OLEOSIN PROMOTER	GAAATTTGGAACTTTTAAGAGGCAGATAGATTTATTATTTGTATCCATTTTCTTC	440
PUTATIVE *BSAO3*	ATTGTTCTAGAATGTCGCGGAACAAATTTTAAAACTAAATCCTAAATTTTTCTAA	495
OLEOSIN PROMOTER	ATTGTTCTAGAATGTCGCGGAACAAATTTTAAAACTAAATCCTAAATTTTTCTAA	495
PUTATIVE *BSAO3*	TTTTGTTGCCAATAGTGGATATGTGGGCCGTATAGAAGGAATCTATTGAAGGCCC	550
OLEOSIN PROMOTER	TTTTGTTGCCAATAGTGGATATGTGGGCCGTATAGAAGGAATCTATTGAAGGCCC	550
PUTATIVE *BSAO3*	AAACCCATACTGACGAGCCCAAAGGTTCGTTTTGCGTTTTATGTTTCGGTTCGAT	605
OLEOSIN PROMOTER	AAACCCATACTGACGAGCCCAAAGGTTCGTTTTGCGTTTTATGTTTCGGTTCGAT	605
PUTATIVE *BSAO3*	GCCAACGCCACATTCTGAGCTAGGCAAAAAACAAACGTGTCTTTGAATAGACTCC	660
OLEOSIN PROMOTER	GCCAACGCCACATTCTGAGCTAGGCAAAAAACAAACGTGTCTTTGAATAGACTCC	660
PUTATIVE *BSAO3*	TCTCGTTAACACATGCAGCGGCTGCATGGTGACGCCATTAACACGTGGCCTACAA	715
OLEOSIN PROMOTER	TCTCGTTAACACATGCAGCGGCTGCATGGTGACGCCATTAACACGTGGCCTACAA	715
PUTATIVE *BSAO3*	TTGCATGATGTCTCCATTGACACGTGACTTCTCGTCTCCTTTCTTAATATATCTA	770
OLEOSIN PROMOTER	TTGCATGATGTCTCCATTGACACGTGACTTCTCGTCTCCTTTCTTAATATATCTA	770
PUTATIVE *BSAO3*	ACAAACACTCCTACCTCTTCCAAAATATATACACATCTTTTTGATCAATCTCTCA	825
OLEOSIN PROMOTER	ACAAACACTCCTACCTCTTCCAAAATATATACACATCTTTTTGATCAATCTCTCA	825
PUTATIVE *BSAO3*	TTCAAAATCTCATTCTCTCTAGTAAACAGGATCCATGAGGGGTGTGTTTCGTCGA	880
OLEOSIN PROMOTER/BSA	TTCAAAATCTCATTCTCTCTAGTAAACA AGGGGTGTGTTTCGTCGA	847/18
PUTATIVE *BSAO3*	GATACACACAAGAGTGAGATTGCTCATCGGTTTAAAGATTTGGGAGAAGAACATT	935
BSA	GATACACACAAGAGTGAGATTGCTCATCGGTTTAAAGATTTGGGAGAAGAACATT	73
PUTATIVE *BSAO3*	TTAAAGGCCTGGTACTGATTGCCTTTTCTCAGTATCTCCAGCAGTGTCCATTTGA	990
BSA	TTAAAGGCCTGGTACTGATTGCCTTTTCTCAGTATCTCCAGCAGTGTCCATTTGA	128
PUTATIVE *BSAO3*	TGAGCATGTAAAATTAGTGAACGAACTAACTGAGTTTGCAAAAACATGTGTTGCT	1045
BSA	TGAGCATGTAAAATTAGTGAACGAACTAACTGAGTTTGCAAAAACATGTGTTGCT	183
PUTATIVE *BSAO3*	GATGAGTCCCATGCCGGCTGTGAAAAGTC	1074
BSA	GATGAGTCCCATGCCGGCTGTGAAAAGTC	212

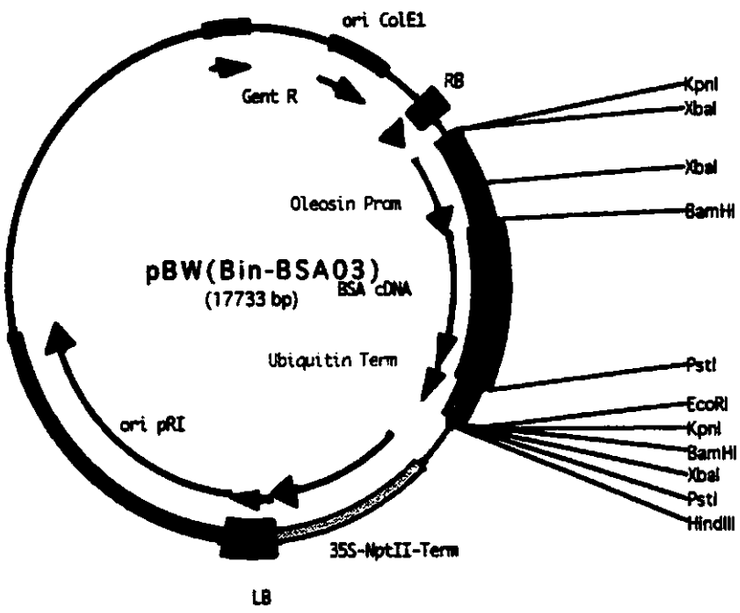
4.1.9. Identification of Transgenic Plants: NptII Assay

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 *Kpn*I 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.


ori ColE1
RB
Gent R
KpnI
XbaI
XbaI
BamHI
Oleosin Prom
pBW(Bin-BSA03)
(17733 bp)
BSA cDNA
Ubiquitin Term
PstI
EcoRI
KpnI
BamHI
XbaI
PstI
HindIII
ori pRI
35S-NptII-Term
LB

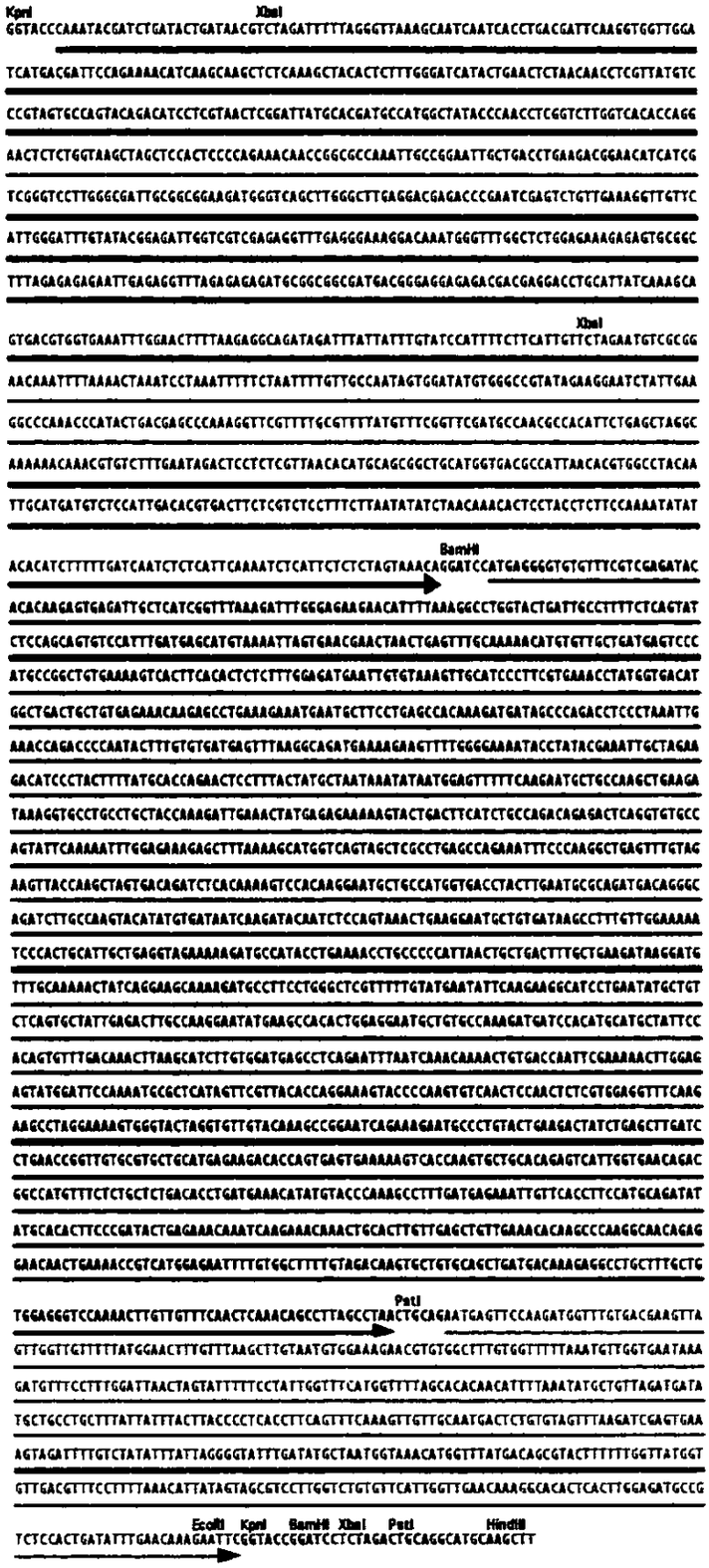

KpnI
XbaI
GGTACCCAAATACGATCTGATACTGATAACGTCTAGATTTTTAGGGTTAAAGCAATCAATCACCTGACGATTCAAGGTGGTTGGA
TCATGACGATTCCAGAAAACATCAAGCAAGCTCTCAAAGCTACACTCTTTGGGATCATACTGAACTCTAACAACCTCGTTATGTC
CCGTAGTGCCAGTACAGACATCCTCGTAACTCGGATTATGCACGATGCCATGGCTATACCCAACCTCGGTCTTGGTCACACCAGG
AACTCTCTGGTAAGCTAGCTCCACTCCCCAGAAACAACCGGCGCCAAATTGCCGGAATTGCTGACCTGAAGACGGAACATCATCG
TCGGGTCCTTGGGCGATTGCGGCGGAAGATGGGTCAGCTTGGGCTTGAGGACGAGACCCGAATCGAGTCTGTTGAAAGGTTGTTC
ATTGGGATTTGTATACGGAGATTGGTCGTCGAGAGGTTTGAGGGAAAGGACAAATGGGTTTGGCTCTGGAGAAAGAGAGTGCGGC
TTTAGAGAGAGAATTGAGAGGTTTAGAGAGAGATGCGGCGGCGATGACGGGAGGAGAGACGACGAGGACCTGCATTATCAAAGCA
XbaI
GTGACGTGGTGAAATTTGGAACTTTTAAGAGGCAGATAGATTTATTATTTGTATCCATTTTCTTCATTGTTCTAGAATGTCGCGG
AACAAATTTTAAAACTAAATCCTAAATTTTTCTAATTTTGTTGCCAATAGTGGATATGTGGGCCGTATAGAAGGAATCTATTGAA
GGCCCAAACCCATACTGACGAGCCCAAAGGTTCGTTTTGCGTTTTATGTTTCGGTTCGATGCCAACGCCACATTCTGAGCTAGGC
AAAAAACAAACGTGTCTTTGAATAGACTCCTCTCGTTAACACATGCAGCGGCTGCATGGTGACGCCATTAACACGTGGCCTACAA
TTGCATGATGTCTCCATTGACACGTGACTTCTCGTCTCCTTTCTTAATATATCTAACAAACACTCCTACCTCTTCCAAAATATAT
BamHI
ACACATCTTTTTGATCAATCTCTCATTCAAAATCTCATTCTCTCTAGTAAACAGGATCCATGAGGGGTGTGTTTCGTCGAGATAC
ACACAAGAGTGAGATTGCTCATCGGTTTAAAGATTTCGGAGAAGAACATTTTAAAGGCCTGGTACTGATTGCCTTTTCTCAGTAT
CTCCAGCAGTGTCCATTTGATGAGCATGTAAAATTAGTGAACGAACTAACTGAGTTTGCAAAAACATGTGTTGCTGATGAGTCCC
ATGCCGGCTGTGAAAAGTCACTTCACACTCTCTTTGGAGATGAATTGTGTAAAGTTGCATCCCTTCGTGAAACCTATGGTGACAT
GGCTGACTGCTGTGAGAAACAAGAGCCTGAAAGAAATGAATGCTTCCTGAGCCACAAAGATGATAGCCCAGACCTCCCTAAATTG
AAACCAGACCCCAATACTTTGTGTGATGAGTTTAAGGCAGATGAAAAGAAGTTTTGGGGAAAATACCTATACGAAATTGCTAGAA
GACATCCCTACTTTTATGCACCAGAACTCCTTTACTATGCTAATAAATATAATGGAGTTTTTCAAGAATGCTGCCAAGCTGAAGA
TAAAGGTGCCTGCCTGCTACCAAAGATTGAAACTATGAGAGAAAAAGTACTGACTTCATCTGCCAGACAGAGACTCAGGTGTGCC
AGTATTCAAAAATTTGGAGAAAGAGCTTTAAAAGCATGGTCAGTAGCTCGCCTGAGCCAGAAATTTCCCAAGGCTGAGTTTGTAG
AAGTTACCAAGCTAGTGACAGATCTCACAAAAGTCCACAAGGAATGCTGCCATGGTGACCTACTTGAATGCGCAGATGACAGGGC
AGATCTTGCCAAGTACATATGTGATAATCAAGATACAATCTCCAGTAAACTGAAGGAATGCTGTGATAAGCCTTTGTTGGAAAAA
TCCCACTGCATTGCTGAGGTAGAAAAAGATGCCATACCTGAAAACCTGCCCCCATTAACTGCTGACTTTGCTGAAGATAAGGATG
TTTGCAAAAACTATCAGGAAGCAAAAGATGCCTTCCTGGGCTCGTTTTTGTATGAATATTCAAGAAGGCATCCTGAATATGCTGT
CTCAGTGCTATTGAGACTTGCCAAGGAATATGAAGCCACACTGGAGGAATGCTGTGCCAAAGATGATCCACATGCATGCTATTCC
ACAGTGTTTGACAAACTTAAGCATCTTGTGGATGAGCCTCAGAATTTAATCAAACAAAACTGTGACCAATTCGAAAAACTTGGAG
AGTATGGATTCCAAAATGCGCTCATAGTTCGTTACACCAGGAAAGTACCCCAAGTGTCAACTCCAACTCTCGTGGAGGTTTCAAG
AAGCCTAGGAAAAGTGGGTACTAGGTGTTGTACAAAGCCGGAATCAGAAAGAATGCCCTGTACTGAAGACTATCTGAGCTTGATC
CTGAACCGGTTGTGCGTGCTGCATGAGAAGACACCAGTGAGTGAAAAAGTCACCAAGTGCTGCACAGAGTCATTGGTGAACAGAC
GGCCATGTTTCTCTGCTCTGACACCTGATGAAACATATGTACCCAAAGCCTTTGATGAGAAATTGTTCACCTTCCATGCAGATAT
ATGCACACTTCCCGATACTGAGAACAAATCAAGAAACAAACTGCACTTGTTGAGCTGTTGAAACACAAGCCCAAGGCAACAGAG
GAACAACTGAAAACCGTCATGGAGAATTTTGTGGCTTTTGTAGACAAGTGCTGTGCAGCTGATGACAAAGAGGCCTGCTTTGCTG
PstI
TGGAGGGTCCAAAACTTGTTGTTTCAACTCAAACAGCCTTAGCCTAACTGCAGAATGAGTTCCAAGATGGTTTGTGACGAAGTTA
GTTGGTTGTTTTTATGGAACTTTGTTTAAGCTTGTAATGTGGAAAGAACGTGTGGCTTTGTGGTTTTTAAATGTTGGTGAATAAA
GATGTTTCCTTTGGATTAACTAGTATTTTTCCTATTGGTTTCATGGTTTTAGCACACAACATTTTAAATATGCTGTTAGATGATA
TGCTGCCTGCTTTATTATTTACTTACCCCTCACCTTCAGTTTCAAAGTTGTTGCAATGACTCTGTGTAGTTTAAGATCGAGTGAA
AGTAGATTTTGTCTATATTTATTAGGGGTATTTGATATGCTAATGGTAAACATGGTTTATGACAGCGTACTTTTTTGGTTATGGT
GTTGACGTTTCCTTTTAAACATTATAGTAGCGTCCTTGGTCTGTGTTCATTGGTTGAACAAAGGCACACTCACTTGGAGATGCCG
EcoRI KpnI BamHI XbaI PstI HindIII
TCTCCACTGATATTTGAACAAAGAATTCGGTACCGGATCCTCTAGACTGCAGGCATGCAAGCTT

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

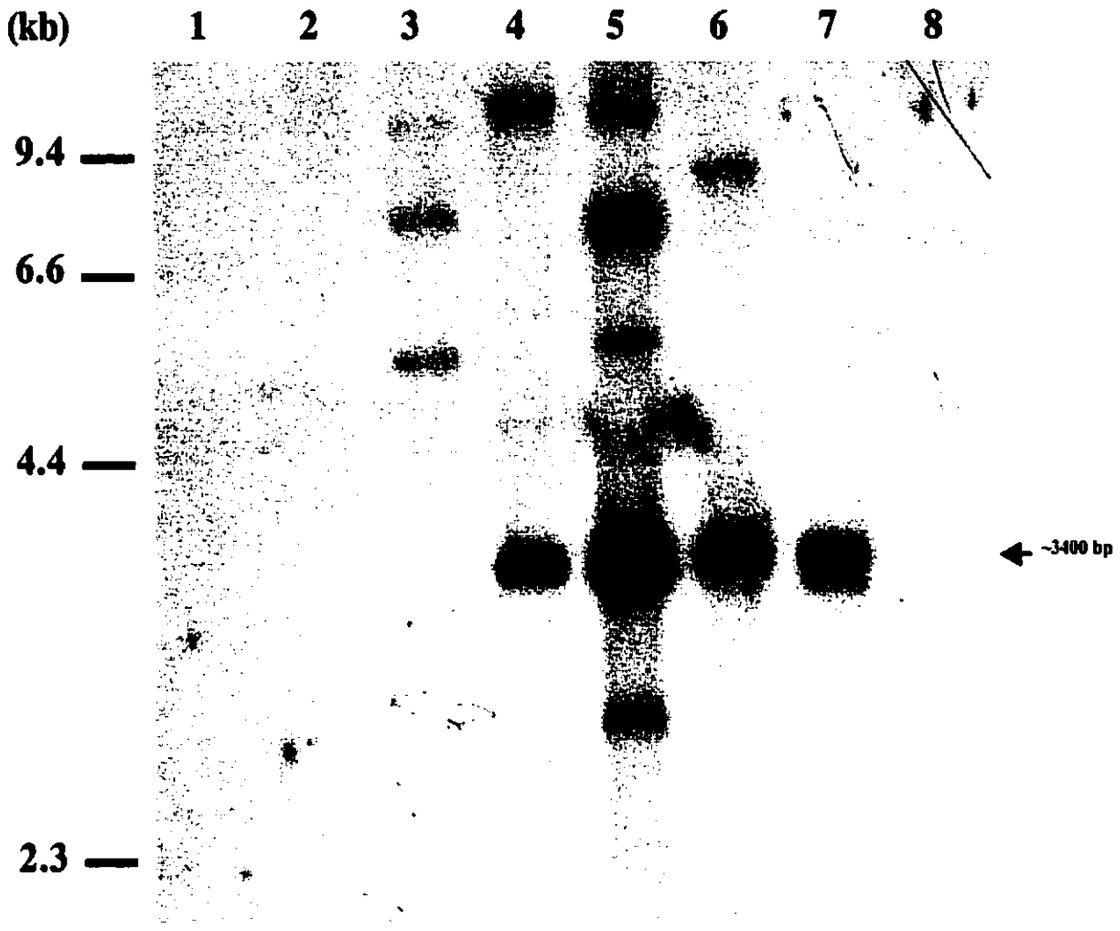
Plant	NptII Positive or Negative
BSA 1	Negative
BSA 2	Positive
BSA 3	Positive
BSA 4	Negative
BSA 5	Positive
BSA 6	Negative
BSA 7	Positive
BSA 8	Negative
BSA 9	Negative
BSA 10	Negative
BSA 11	Positive
BSA 12	Positive
BSA 13	Negative
BSA 14	Positive
BSA 15	Negative
BSA 16	Negative
BSA 17	Positive

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 [^{32}P]-*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 [^{32}P]-*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.


(kb)
1
2
3
4
5
6
7
8
9.4
6.6
4.4
2.3
~3400 bp

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).

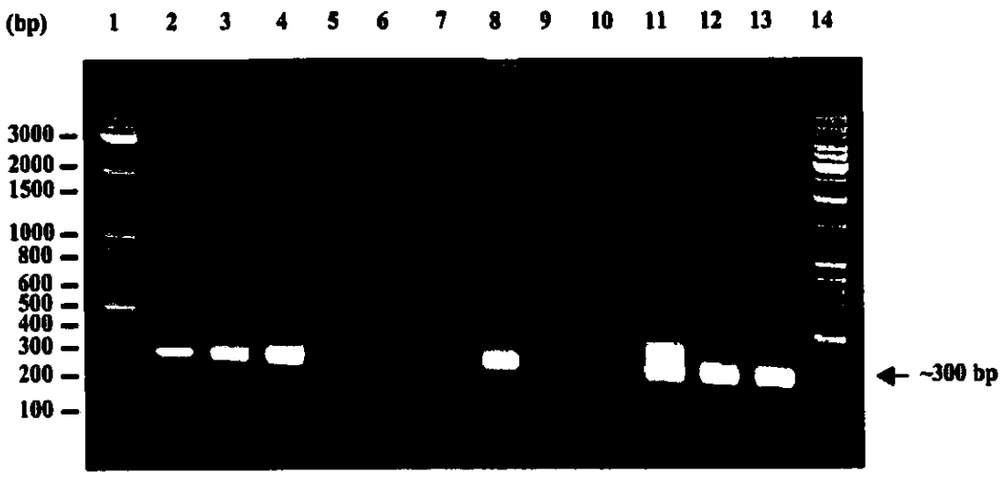
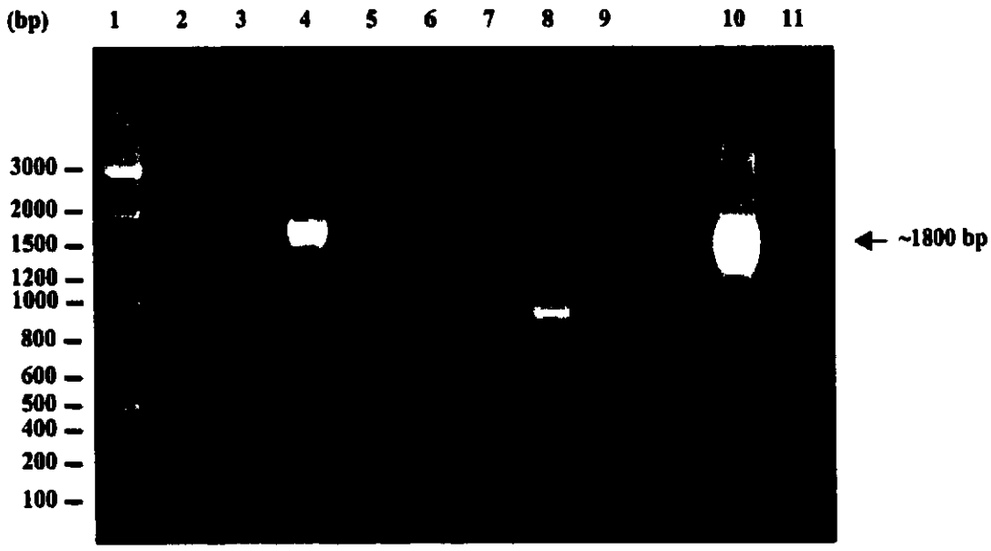

(bp)
1
2
3
4
5
6
7
8
9
10
11
12
13
14
3000
2000
1500
1000
800
600
500
400
300
200
100
~300 bp

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, 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, 5 μL of RT-PCR reaction from pBSA (positive control); 11, PCR mixture without template (negative control).


(bp)
1
2
3
4
5
6
7
8
9
10
11
3000
2000
1500
1200
1000
800
600
500
400
200
100
~1800 bp

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 ± one standard deviation was 34.2 ± 3.0% for gravimetric analysis and 37.0 ± 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

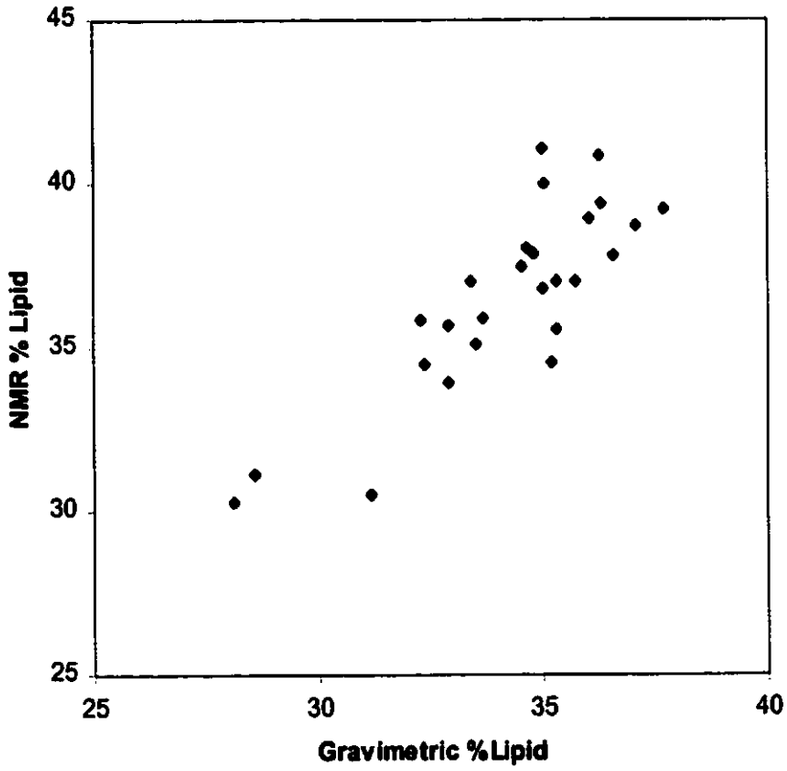
None of the transgenic plants were statistically different in total lipid component compared to negative control plants. Having said that, BSA 11 did have noticeably lower total lipid than the negative control plants, even though the numbers were not statistically significant. When measured gravimetrically, 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 ± 3.0% gravimetrically and 37.0 ± 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 ($r^2 = 0.70$, $P < 0.0001$).


45
40
35
30
25
NMR % Lipid
25
30
35
40
Gravimetric %Lipid

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	15.6	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

[A] $P = 0.05$
[B] $P = 0.04$

(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	18.6[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

[A] $P = 0.04$
[B] $P = 0.02$

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 ± 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)

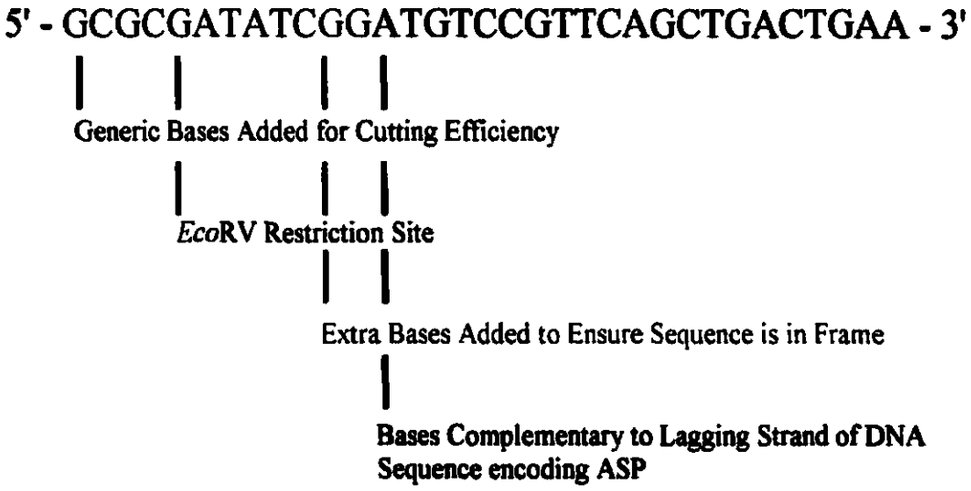
In developing seed tissues, FAs are formed in the plastids and are exported to the ER for assembly into TAGs (Murphy *et al.*, 1993). These TAGs are stored in oil bodies that bud from the ER. Oleosins are embedded within the oil bodies and are thought to stabilize them and prevent coalescence with other 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

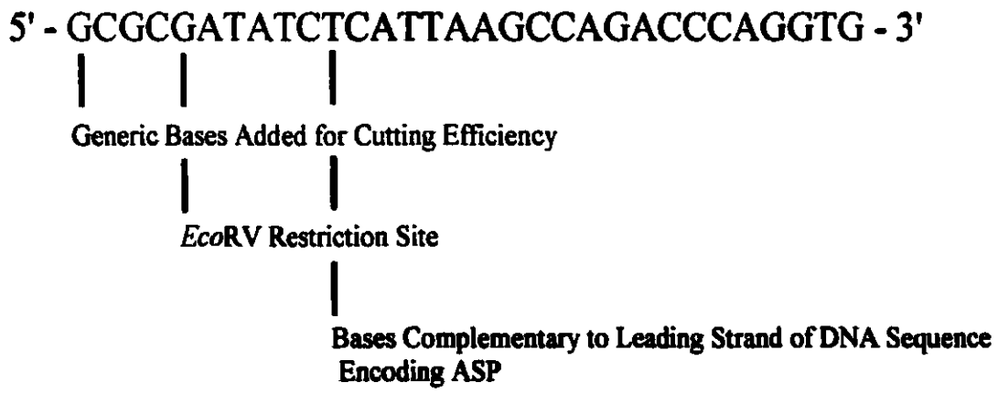
PCR was performed using *Pfu* polymerase on the original vector carrying the ASP cDNA. The primers ASP871 and ASP872 (Figure 34) were selected so that the DNA sequence encoding ASP would be amplified. *Eco*RV restriction sites were engineered on the 5' and 3' ends of the ASP DNA sequence, respectively,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

Figure 34. Primers designed to amplify the DNA sequence encoding ASP for insertion into pSBS2050. An *Eco*RV 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 *Swa*I 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



Reverse Primer: ASP872



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/*Eco*RI+*Hin*dIII Ladder (MBI Fermentas, Flamborough, ON) and lane 2 contains 5μL of the PCR reaction.

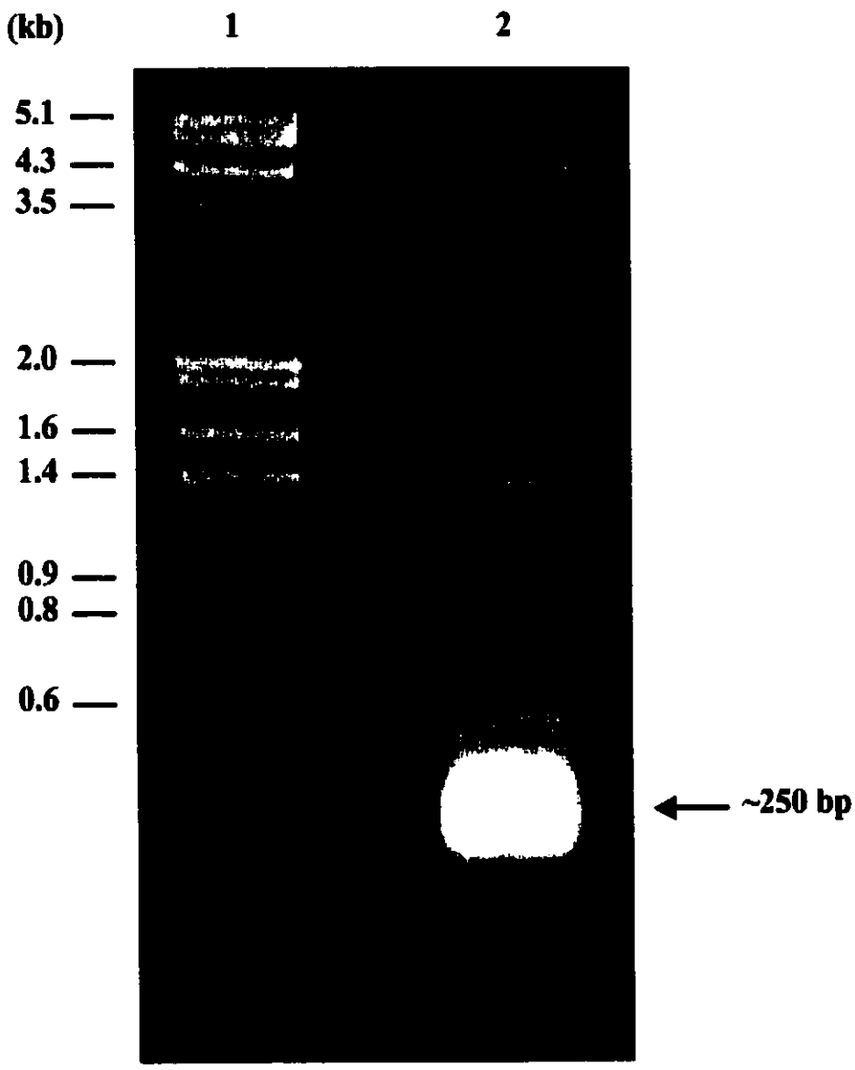

(kb)
1
2
5.1
4.3
3.5
2.0
1.6
1.4
0.9
0.8
0.6
~250 bp

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/*Eco*RI+*Hin*dIII Ladder (MBI Fermentas, Flamborough, ON); 2, 5 μL of the PCR reaction from colony 1; 3, 5 μL of the PCR reaction from colony 2; 4, 5 μL of the PCR reaction from colony 3.

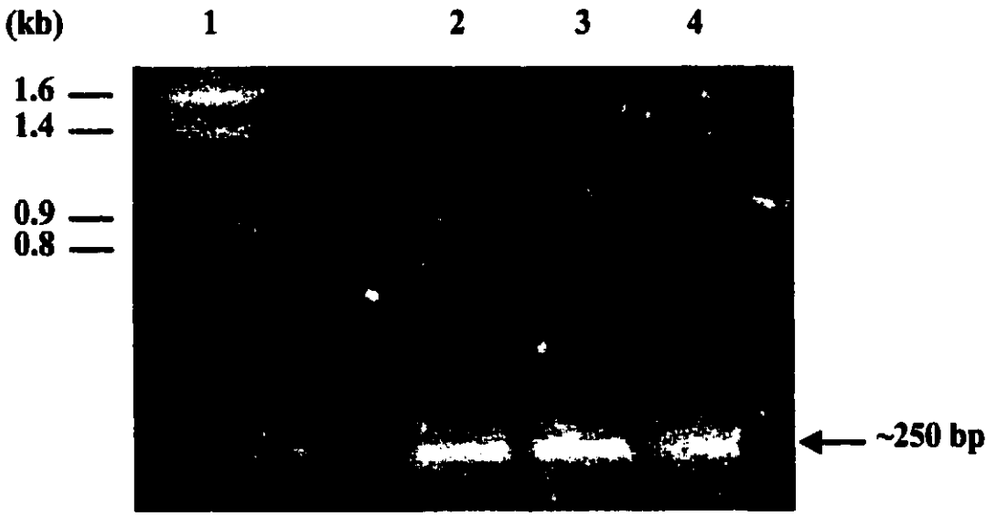

(kb)
1
2
3
4
1.6
1.4
0.9
0.8
~250 bp

Figure 38. Restriction analysis using *Eco*RV 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/*Eco*RI+*Hin*dIII 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.

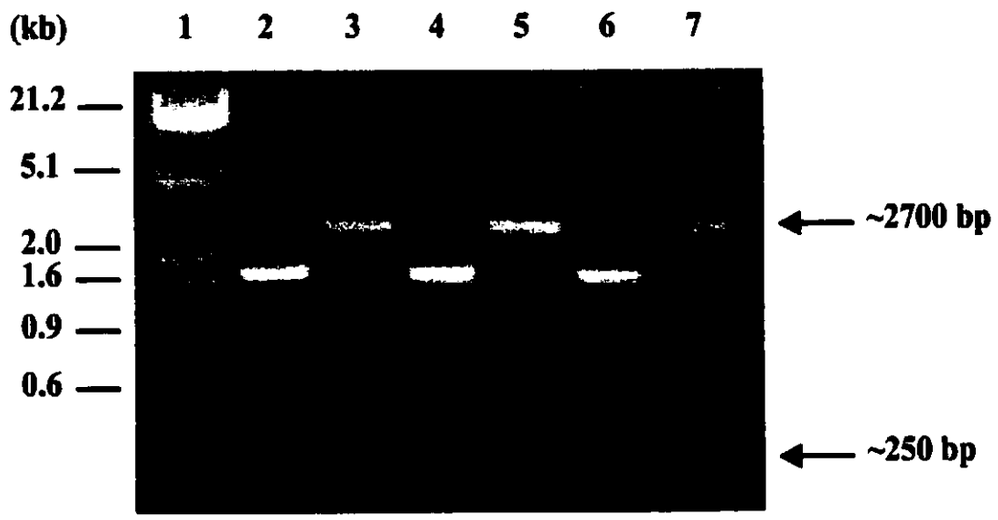
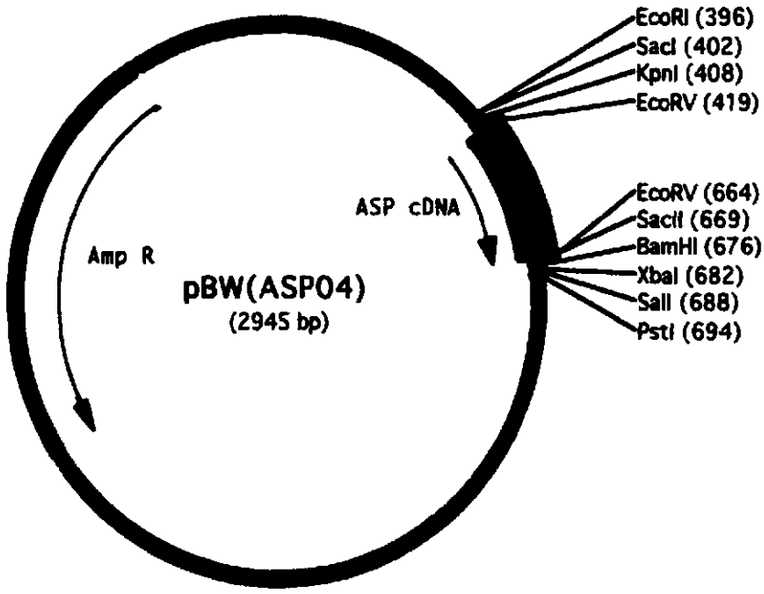

(kb)
1
2
3
4
5
6
7
21.2
5.1
2.0
1.6
0.9
0.6
~2700 bp
~250 bp

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, *Eco*RV sites are in blue, and additional Gs are in green.

PUTATIVE ASP04	GCGCGATATCGGATGTCCGTTCAGCTGACTGAAAAACGTATGGATAAAGTTGGTA	55
ASP	ATGTCCGTTCAGCTGACTGAAAAACGTATGGATAAAGTTGGTA	43
PUTATIVE ASP04	AGTACCCGAAGGAACTGCGTAAATGTTGCGAAGACGGGATGCGTGAAAACCCGAT	110
ASP	AGTACCCGAAGGAACTGCGTAAATGTTGCGAAGACGGGATGCGTGAAAACCCGAT	98
PUTATIVE ASP04	GCGTTTCTCTTGCCAACGTCGTACCCGCTTCATCTCCCTGGGCGAAGCTTGCAAA	165
ASP	GCGTTTCTCTTGCCAACGTCGTACCCGCTTCATCTCCCTGGGCGAAGCTTGCAAA	153
PUTATIVE ASP04	AAGGTTTTCCTGGACTGCTGCAACTACATCACTGAACTGCGTCGTCAGCACGCTC	220
ASP	AAGGTTTTCCTGGACTGCTGCAACTACATCACTGAACTGCGTCGTCAGCACGCTC	208
PUTATIVE ASP04	GTGCTTCTCACCTGGGTCTGGCTTAATGAGATATCGCGC	259
ASP	GTGCTTCTCACCTGGGTCTGGCTTAATGA	237

Figure 40. Plasmid map for pBW(ASP04). The vector contains an ampicllin 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)
GAATTCGAGCTCGGTACCCGCGCGATATCGGATGTCCGTTCAGCTGACTGAAAAACGTATGGATAAAGTT

GGTAAGTACCCGAAGGAACTGCGTAAATGTTGCGAAGACGGGATGCGTGAAAACCCGATGCGTTTCTCTT

CCAACGTCGTACCCGCTTCATCTCCCTGGGCGAAGCTTGCAAAAAGGTTTTCCTGGACTGCTGCAACTA

SacII (669)
EcoRV (664)
CATCACTGAACTGCGTCGTCAGCACGCTCGTGCTTCTCACCTGGGTCTGGCTTAATGAGATATCCGCGGG

XbaI (682) PstI (694)
BamHI (676) SalI (688)
GGATCCTCTAGAGTCGACCTGCAG

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

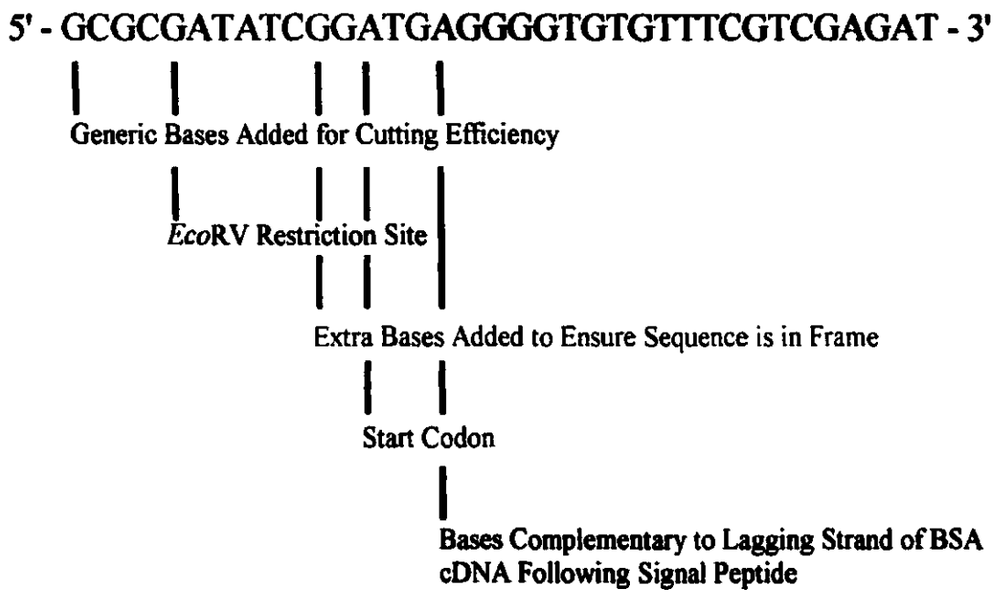
4.3.2. Genetic Constructs to Facilitate Expression of BSA in the ER

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. *Eco*RV 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 *Eco*RV 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 *Swa*I 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 *Sma*I. 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 *Eco*RV 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



Reverse Primer: BSA862

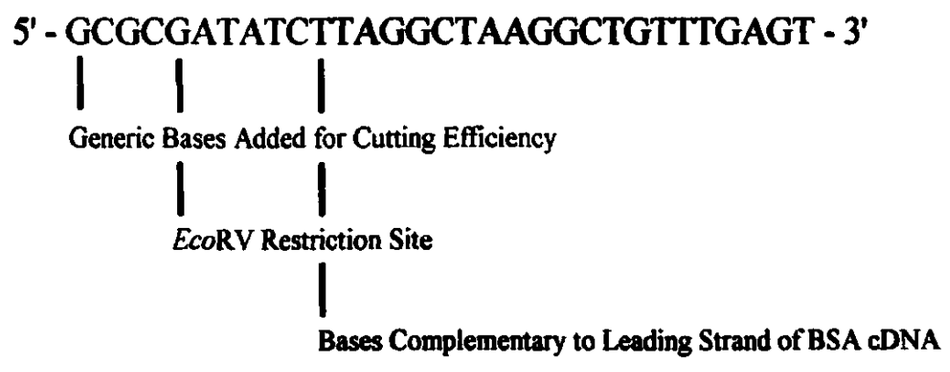
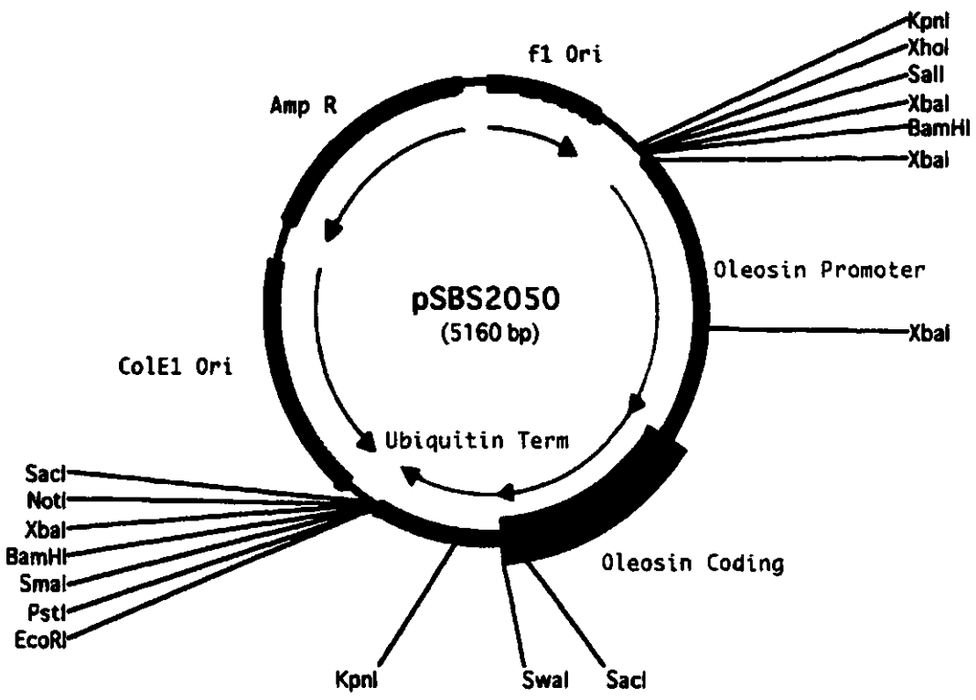


Figure 42. Plasmid map for pSBS2050. The vector contains an ampicillin resistance gene (solid black section and solid black arrow) as well as a 1.1 Kb *Arabidopsis* oleosin promoter (light blue arrow), an oleosin cDNA (solid green arrow) and a ubiquitin terminator (second light blue arrow). The detailed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


f1 Ori
Amp R
KpnI
XhoI
SalI
XbaI
BamHI
XbaI
Oleosin Promoter
XbaI
pSBS2050
(5160 bp)
ColE1 Ori
Ubiquitin Term
SacI
NotI
XbaI
BamHI
SmaI
PstI
EcoRI
Oleosin Coding
KpnI
SwaI
SacI

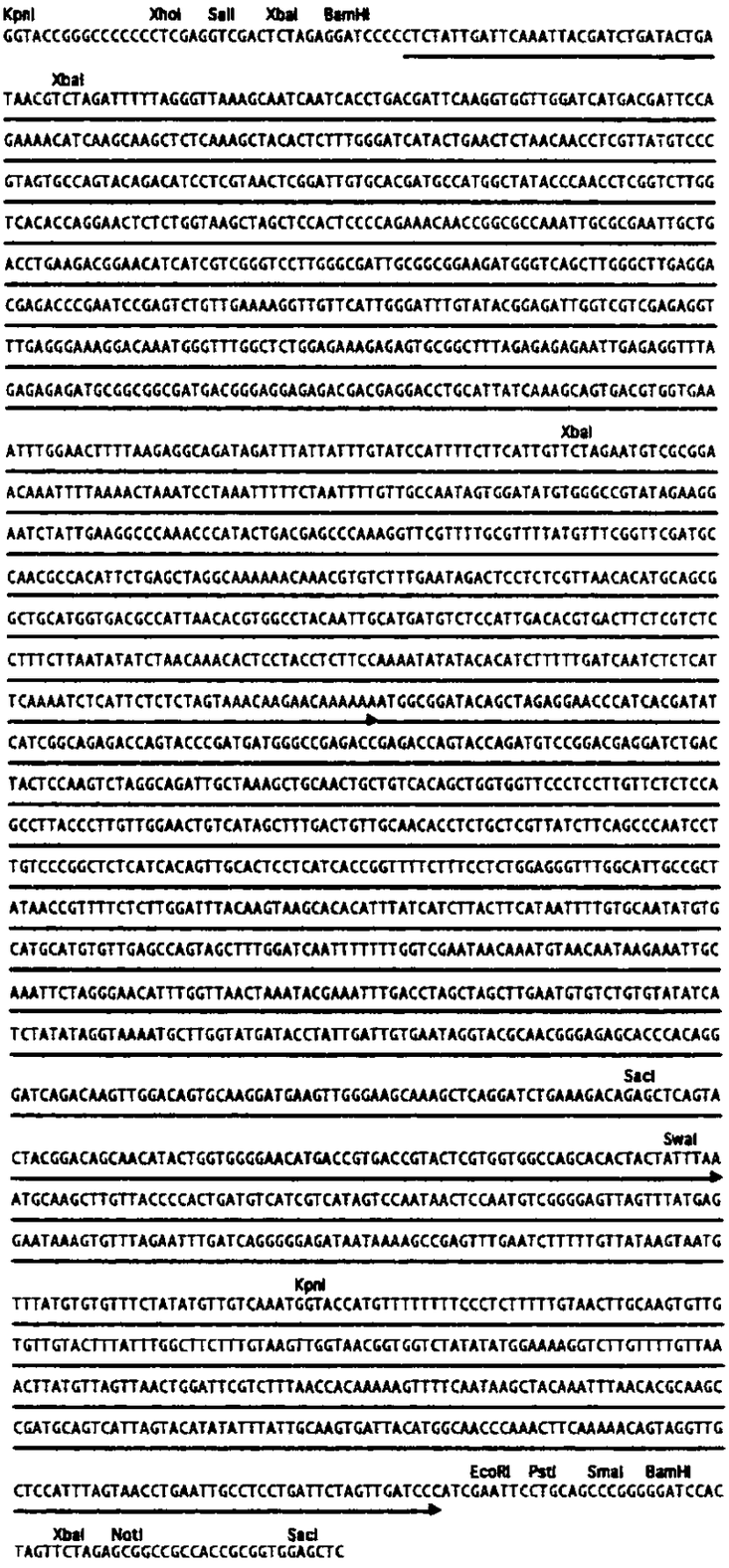

KpnI XhoI SalI XbaI BamHI
GGTACCGGGCCCCCCCTCGAGGTCGACTCTAGAGGATCCCCCTCTATTGATTCAAATTACGATCTGATACTGA
XbaI
TAACGTCTAGATTTTTAGGGTTAAAGCAATCAATCACCTGACGATTCAAGGTGGTTGGATCATGACGATTCCA
GAAAACATCAAGCAAGCTCTCAAAGCTACACTCTTTGGGATCATACTGAACTCTAACAACCTCGTTATGTCCC
GTAGTGCCAGTACAGACATCCTCGTAACTCGGATTGTGCACGATGCCATGGCTATACCCAACCTCGGTCTTGG
TCACACCAGGAACTCTCTGGTAAGCTAGCTCCACTCCCCAGAAACAACCGGCGCCAAATTGCGCGAATTGCTG
ACCTGAAGACGGAACATCATCGTCGGGTCCTTGGGCGATTGCGGCGGAAGATGGGTCAGCTTGGGCTTGAGGA
CGAGACCCGAATCCGAGTCTGTTGAAAAGGTTGTTCATTGGGATTTGTATACGGAGATTGGTCGTCGAGAGGT
TTGAGGGAAAGGACAAATGGGTTTGGCTCTGGAGAAAGAGAGTGCGGCTTTAGAGAGAGAATTGAGAGGTTTA
GAGAGAGATGCGGCGGCGATGACGGGAGGAGAGACGACGAGGACCTGCATTATCAAAGCAGTGACGTGGTGAA
XbaI
ATTTGGAACTTTTAAGAGGCAGATAGATTTATTATTTGTATCCATTTTCTTCATTGTTCTAGAATGTCGCGGA
ACAAATTTTAAAACTAAATCCTAAATTTTTCTAATTTTGTTGCCAATAGTGGATATGTGGGCCGTATAGAAGG
AATCTATTGAAGGCCCAAACCCATACTGACGAGCCCAAAGGTTCGTTTTGCGTTTTATGTTTCGGTTCGATGC
CAACGCCACATTCTGAGCTAGGCAAAAAACAAACGTGTCTTTGAATAGACTCCTCTCGTTAACACATGCAGCG
GCTGCATGGTGACGCCATTAACACGTGGCCTACAATTGCATGATGTCTCCATTGACACGTGACTTCTCGTCTC
CTTTCTTAATATATCTAACAAACACTCCTACCTCTTCCAAAATATATACACATCTTTTTGATCAATCTCTCAT
TCAAAATCTCATTCTCTCTAGTAAACAAGAACAAAAAAATGGCGGATACAGCTAGAGGAACCCATCACGATAT
CATCGGCAGAGACCAGTACCCGATGATGGGCCGAGACCGAGACCAGTACCAGATGTCCGGACGAGGATCTGAC
TACTCCAAGTCTAGGCAGATTGCTAAAGCTGCAACTGCTGTCACAGCTGGTGGTTCCCTCCTTGTTCTCTCCA
GCCTTACCCTTGTTGGAACTGTCATAGCTTTGACTGTTGCAACACCTCTGCTCGTTATCTTCAGCCCAATCCT
TGTCCCGGCTCTCATCACAGTTGCACTCCTCATCACCGGTTTTCTTTCCTCTGGAGGGTTTGGCATTGCCGCT
ATAACCGTTTTCTCTTGGATTTACAAGTAAGCACACATTTATCATCTTACTTCATAATTTTGTGCAATATGTG
CATGCATGTGTTGAGCCAGTAGCTTTGGATCAATTTTTTTGGTCGAATAACAAATGTAACAATAAGAAATTGC
AAATTCTAGGGAACATTTGGTTAACTAAATACGAAATTTGACCTAGCTAGCTTGAATGTGTCTGTGTATATCA
TCTATATAGGTAAAATGCTTGGTATGATACCTATTGATTGTGAATAGGTACGCAACGGGAGAGCACCCACAGG
SacI
GATCAGACAAGTTGGACAGTGCAAGGATGAAGTTGGGAAGCAAAGCTCAGGATCTGAAAGACAGAGCTCAGTA
SwaI
CTACGGACAGCAACATACTGGTGGGGAACATGACCGTGACCGTACTCGTGGTGGCCAGCACACTACTATTTAA
ATGCAAGCTTGTTACCCCACTGATGTCATCGTCATAGTCCAATAACTCCAATGTCGGGGAGTTAGTTTATGAG
GAATAAAGTGTTTAGAATTTGATCAGGGGGAGATAATAAAAGCCGAGTTTGAATCTTTTTGTTATAAGTAATG
KpnI
TTTATGTGTGTTTCTATATGTTGTCAAATGGTACCATGTTTTTTTTCCCTCTTTTTGTAACTTGCAAGTGTTG
TGTTGTACTTTATTTGGCTTCTTTGTAAGTTGGTAACGGTGGTCTATATATGGAAAAGGTCTTGTTTTGTTAA
ACTTATGTTAGTTAACTGGATTCGTCTTTAACCACAAAAAGTTTTCAATAAGCTACAAATTTAACACGCAAGC
CGATGCAGTCATTAGTACATATATTTATTGCAAGTGATTACATGGCAACCCAAACTTCAAAAACAGTAGGTTG
EcoRI PstI SmaI BamHI
CTCCATTTAGTAACCTGAATTGCCTCCTGATTCTAGTTGATCCCATCGAATTCCTGCAGCCCGGGGGATCCAC
XbaI NotI SacI
TAGTTCTAGAGCGGCCGCCACCGCGGTGGAGCTC

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 *Eco*RV 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/*Eco*RI+*Hin*dIII Ladder (MBI Fermentas, Flamborough, ON) and lane 2 contains 5μL of the PCR reaction.

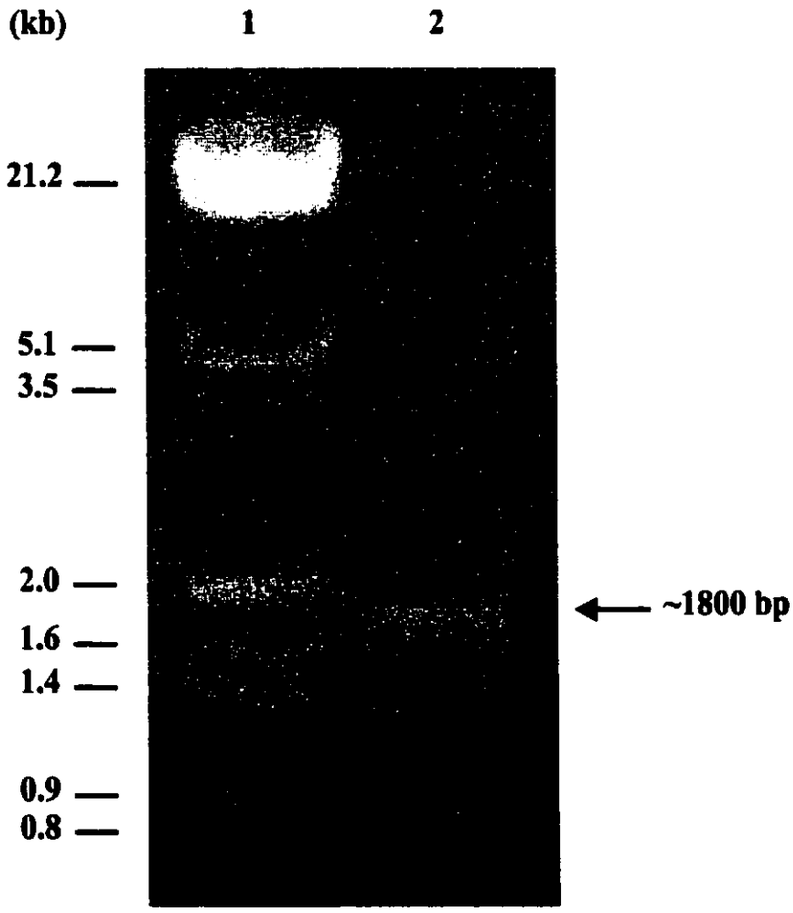
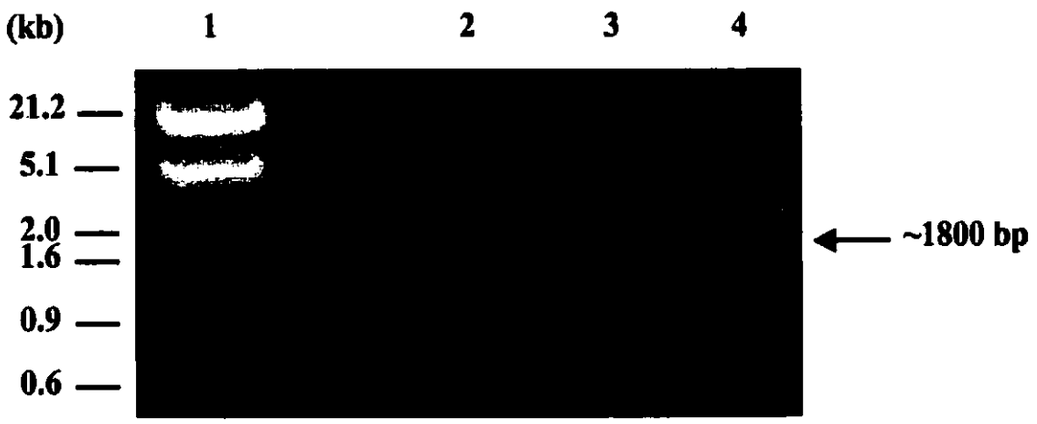

(kb)
1
2
21.2
5.1
3.5
2.0
1.6
1.4
0.9
0.8
~1800 bp

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/*Eco*RI+*Hin*dIII Ladder (MBI Fermentas, Flamborough, ON); 2, 5 μL of the PCR reaction from colony 1; 3, 5 μL of the PCR reaction from colony 2; 4, 5 μL of the PCR reaction from colony 3.


(kb)
1
2
3
4
21.2
5.1
2.0
1.6
0.9
0.6
~1800 bp

carrying plasmid DNA that produced a DNA fragment of ~1800 bp when amplified using Primers BSA861 and BSA862. Restriction analysis with *Eco*RV was performed on the plasmid DNA from colony 1 as compared to linearized pUC19 to ensure that the engineered *Eco*RV 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 *Eco*RV 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 *Eco*RV and the fragment was subsequently gel extracted and gene-cleaned. This fragment was ligated into gel purified pSBS2050 that had been previously restricted with *Sma*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 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 *Pst*I and *Sca*I and electrophoresed on an agarose gel (Figure 50). The distinguishing DNA fragment that was expected for a

Figure 46. Restriction analysis using *Eco*RV on plasmid DNA from colony 1, which was presumed to contain the *BSA05* gene. 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+*Hin*dIII Ladder (MBI Fermentas, Flamborough, ON); 2, pUC19 linearized *Sma*I; 3, restricted colony 1 plasmid DNA.

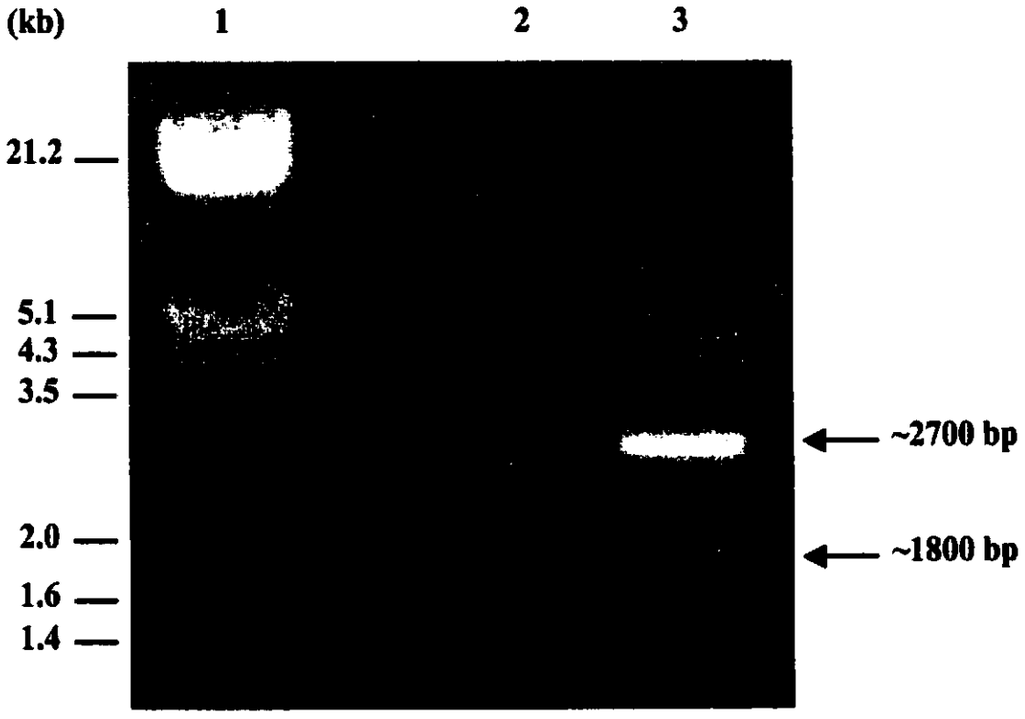

(kb)
1
2
3
21.2
5.1
4.3
3.5
2.0
1.6
1.4
~2700 bp
~1800 bp

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, *Eco*RV sites are in blue, additional Gs are in green and the engineered start codon is in red.

```
PUTATIVE BSA05   GCGCGATATCGGATGAGGGGTGTGTTTCGTCGAGATACACACAAGAGTGAGATTG   55
BSA                          AGGGGTGTGTTTCGTCGAGATACACACAAGAGTGAGATTG   40

PUTATIVE BSA05   CTCATCGGTTTAAAGATTTGGGAGAAGAACATTTTAAAGGCCTGGTACTGATTGC   110
BSA              CTCATCGGTTTAAAGATTTGGGAGAAGAACATTTTAAAGGCCTGGTACTGATTGC   95

PUTATIVE BSA05   CTTTTCTCAGTATCTCCAGCAGTGTCCATTTGATGAGCATGTAAAATTAGTGAAC   165
BSA              CTTTTCTCAGTATCTCCAGCAGTGTCCATTTGATGAGCATGTAAAATTAGTGAAC   150

PUTATIVE BSA05   GAACTAACTGAGTTTGCAAAAACATGTGTTGCTGATGAGTCCCATGCCGGCTGTG   220
BSA              GAACTAACTGAGTTTGCAAAAACATGTGTTGCTGATGAGTCCCATGCCGGCTGTG   205

PUTATIVE BSA05   AAAAGTCACTTCACACTCTCTTTGGAGATGAATTGTGTAAAGTTGCATCCCTTCG   275
BSA              AAAAGTCACTTCACACTCTCTTTGGAGATGAATTGTGTAAAGTTGCATCCCTTCG   260

PUTATIVE BSA05   TGAAACCTATGGTGACATGGCTGACTGCTGTGAGAAACAAGAGCCTGAAAGAAAT   330
BSA              TGAAACCTATGGTGACATGGCTGACTGCTGTGAGAAACAAGAGCCTGAAAGAAAT   315

PUTATIVE BSA05   GAATGCTTCCTGAGCCACAAAGATGATAGCCCAGACCTCCCTAAATTGAAACCAG   385
BSA              GAATGCTTCCTGAGCCACAAAGATGATAGCCCAGACCTCCCTAAATTGAAACCAG   370

PUTATIVE BSA05   ACCCCAATACTTTGTGTGATGAGTTTAAGGCAGATGAAAAGAAGTTTTGGGGAAA   440
BSA              ACCCCAATACTTTGTGTGATGAGTTTAAGGCAGATGAAAAGAAGTTTTGGGGAAA   425

PUTATIVE BSA05   ATACCTATACGAAATTGCTAGAAGACATCCCTACTTTTATGCACCAGAACTCCTT   495
BSA              ATACCTATACGAAATTGCTAGAAGACATCCCTACTTTTATGCACCAGAACTCCTT   480

PUTATIVE BSA05   TACTATGCTAATAAATATAATGGAGTTTTTCAAGAATGCTGCCAAGCTGAAGATA   550
BSA              TACTATGCTAATAAATATAATGGAGTTTTTCAAGAATGCTGCCAAGCTGAAGATA   535

PUTATIVE BSA05   AAGGTGCCTGCCTGCTACCAAAGATTGAAACTATGAGAGAAAAAGTACTGACTTC   605
BSA              AAGGTGCCTGCCTGCTACCAAAGATTGAAACTATGAGAGAAAAAGTACTGACTTC   590

PUTATIVE BSA05   ATCTGCCAGACAGAGACTCAGGTGTGCCAGTATTCAAAAATTTGGAGAAAGAGCT   660
BSA              ATCTGCCAGACAGAGACTCAGGTGTGCCAGTATTCAAAAATTTGGAGAAAGAGCT   645

PUTATIVE BSA05   TTAAAAGCATGGTCAGTAGCTCGCCTGAGCCAGAAATTTCCCAAGGCTGAGTTTG   715
BSA              TTAAAAGCATGGTCAGTAGCTCGCCTGAGCCAGAAATTTCCCAAGGCTGAGTTTG   700

PUTATIVE BSA05   TAGAAGTTACCAAGCTAGTGACAGATCTCACAAAAGTCCACAAGGAATGCTGCCA   770
BSA              TAGAAGTTACCAAGCTAGTGACAGATCTCACAAAAGTCCACAAGGAATGCTGCCA   755

PUTATIVE BSA05   TGGTGACCTACTTGAATGCGCAGATGACAGGGCAGATCTTGCCAAGTACATATGT   825
BSA              TGGTGACCTACTTGAATGCGCAGATGACAGGGCAGATCTTGCCAAGTACATATGT   810

PUTATIVE BSA05   GATAATCAAGATACAATCTCCAGTAAACTGAAGGAATGCTGTGATAAGCCTTTGT   880
BSA              GATAATCAAGATACAATCTCCAGTAAACTGAAGGAATGCTGTGATAAGCCTTTGT   865

PUTATIVE BSA05   TGGAAAAATCCCACTGCATTGCTGAGGTAGAAAAAGATGCCATACCTGAAAACCT   935
BSA              TGGAAAAATCCCACTGCATTGCTGAGGTAGAAAAAGATGCCATACCTGAAAACCT   920

PUTATIVE BSA05   GCCCCCATTAACTGCTGACTTTGCTGAAGATAAGGATGTTTGCAAAAACTATCAG   990
BSA              GCCCCCATTAACTGCTGACTTTGCTGAAGATAAGGATGTTTGCAAAAACTATCAG   975

PUTATIVE BSA05   GAAGCAAAAGATGCCTTCCTGGGCTCGTTTTTGTATGAATATTCAAGAAGGCATC   1045
BSA              GAAGCAAAAGATGCCTTCCTGGGCTCGTTTTTGTATGAATATTCAAGAAGGCATC   1030

PUTATIVE BSA05   CTGAATATGCTGTCTCAGTGCTATTGAGACTTGCCAAGGAATATGAAGCCACACT   1100
BSA              CTGAATATGCTGTCTCAGTGCTATTGAGACTTGCCAAGGAATATGAAGCCACACT   1085

PUTATIVE BSA05   GGAGGAATGCTGTGCCAAAGATGATCCACATGCATGCTATTCCACAGTGTTTGAC   1155
BSA              GGAGGAATGCTGTGCCAAAGATGATCCACATGCATGCTATTCCACAGTGTTTGAC   1140

PUTATIVE BSA05   AAACTTAAGCATCTTGTGGATGAGCCTCAGAATTTAATCAAACAAAACTGTGACC   1210
BSA              AAACTTAAGCATCTTGTGGATGAGCCTCAGAATTTAATCAAACAAAACTGTGACC   1195
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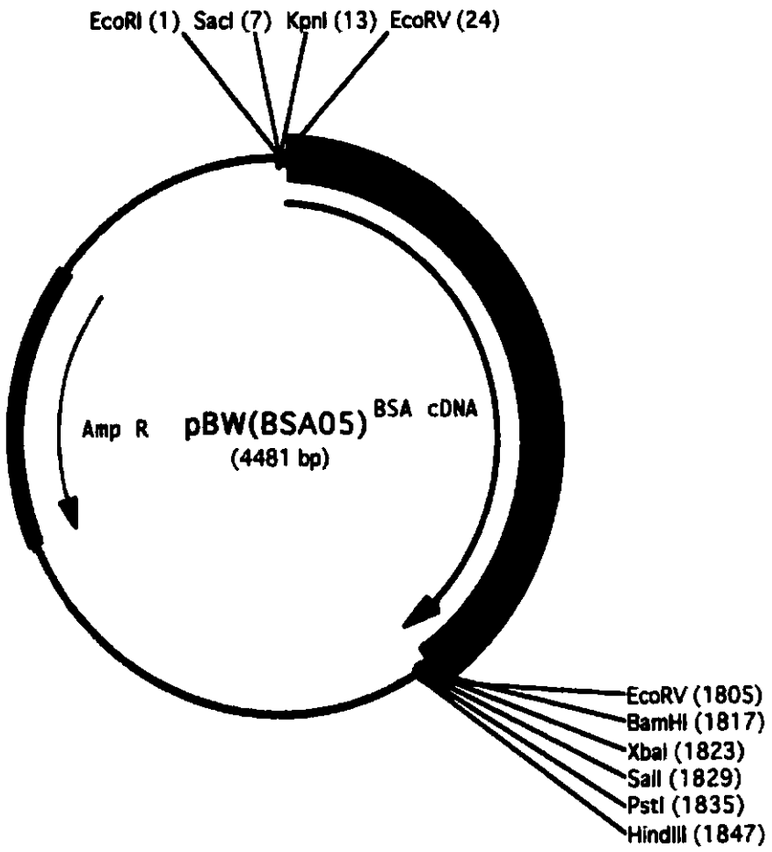
PUTATIVE *BSA05*	AATTCGAAAAACTTGGAGAGTATGGATTCCAAAATGCGCTCATAGTTCGTTACAC	1265
BSA	AATTCGAAAAACTTGGAGAGTATGGATTCCAAAATGCGCTCATAGTTCGTTACAC	1250
PUTATIVE *BSA05*	CAGGAAAGTACCCCAAGTGTCAACTCCAACTCTCGTGGAGGTTTCAAGAAGCCTA	1320
BSA	CAGGAAAGTACCCCAAGTGTCAACTCCAACTCTCGTGGAGGTTTCAAGAAGCCTA	1305
PUTATIVE *BSA05*	GGAAAAGTGGGTACTAGGTGTTGTACAAAGCCGGAATCAGAAAGAATGCCCTGTA	1375
BSA	GGAAAAGTGGGTACTAGGTGTTGTACAAAGCCGGAATCAGAAAGAATGCCCTGTA	1360
PUTATIVE *BSA05*	CTGAAGACTATCTGAGCTTGATCCTGAACCGGTTGTGCGTGCTGCATGAGAAGAC	1430
BSA	CTGAAGACTATCTGAGCTTGATCCTGAACCGGTTGTGCGTGCTGCATGAGAAGAC	1415
PUTATIVE *BSA05*	ACCAGTGAGTGAAAAAGTCACCAAGTGCTGCACAGAGTCATTGGTGAACAGACGG	1485
BSA	ACCAGTGAGTGAAAAAGTCACCAAGTGCTGCACAGAGTCATTGGTGAACAGACGG	1470
PUTATIVE *BSA05*	CCATGTTTCTCTGCTCTGACACCTGATGAAACATATGTACCCAAAGCCTTTGATG	1540
BSA	CCATGTTTCTCTGCTCTGACACCTGATGAAACATATGTACCCAAAGCCTTTGATG	1525
PUTATIVE *BSA05*	AGAAATTGTTCACCTTCCATGCAGATATATGCACACTTCCCGATACTGAGAAACA	1595
BSA	AGAAATTGTTCACCTTCCATGCAGATATATGCACACTTCCCGATACTGAGAAACA	1580
PUTATIVE *BSA05*	AATCAAGAAACAAACTGCACTTGTTGAGCTGTTGAAACACAAGCCCAAGGCAACA	1650
BSA	AATCAAGAAACAAACTGCACTTGTTGAGCTGTTGAAACACAAGCCCAAGGCAACA	1635
PUTATIVE *BSA05*	GAGGAACAACTGAAAACCGTCATGGAGAATTTTGTGGCTTTTGTAGACAAGTGCT	1705
BSA	GAGGAACAACTGAAAACCGTCATGGAGAATTTTGTGGCTTTTGTAGACAAGTGCT	1690
PUTATIVE *BSA05*	GTGCAGCTGATGACAAAGAGGCCTGCTTTGCTGTGGAGGGTCCAAAACTTGTTGT	1760
BSA	GTGCAGCTGATGACAAAGAGGCCTGCTTTGCTGTGGAGGGTCCAAAACTTGTTGT	1745
PUTATIVE *BSA05*	TTCAACTCAAACAGCCTTAGCCTAAGATATCGCGC	1795
BSA	TTCAACTCAAACAGCCTTAGCCTAA	1770

Figure 48. Plasmid map for pBW(BSA05). The vector contains an ampicllin resistance gene (solid black arrow). The BSA 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 on the page following the plasmid map.


EcoRI (1)
SacI (7)
KpnI (13)
EcoRV (24)
Amp R
pBW(BSA05)
(4481 bp)
BSA cDNA
EcoRV (1805)
BamHI (1817)
XbaI (1823)
SalI (1829)
PstI (1835)
HindIII (1847)

SacI (7)
EcoRI (1) KpnI (13) EcoRV (24)
GAATTCGAGCTCGGTACCCGCGCGATATCGGATGAGGGGTGTGTTTCGTCGAGATACACACAAGAGTGAG

ATTGCTCATCGGTTTAAAGATTTGGGAGAAGAACATTTTAAAGGCCTGGTACTGATTGCCTTTTCTCAGT

ATCTCCAGCAGTGTCCATTTGATGAGCATGTAAAATTAGTGAACGAACTAACTGAGTTTGCAAAAACATG

TGTTGCTGATGAGTCCCATGCCGGCTGTGAAAAGTCACTTCACACTCTCTTTGGAGATGAATTGTGTAAA

GTTGCATCCCTTCGTGAAACCTATGGTGACATGGCTGACTGCTGTGAGAAACAAGAGCCTGAAAGAAATG

AATGCTTCCTGAGCCACAAAGATGATAGCCCAGACCTCCCTAAATTGAAACCAGACCCCAATACTTTGTG

TGATGAGTTTAAGGCAGATGAAAAGAAGTTTTGGGGAAAATACCTATACGAAATTGCTAGAAGACATCCC

TACTTTTATGCACCAGAACTCCTTTACTATGCTAATAAATATAATGGAGTTTTTCAAGAATGCTGCCAAG

CTGAAGATAAAGGTGCCTGCCTGCTACCAAAGATTGAAACTATGAGAGAAAAAGTACTGACTTCATCTGC

CAGACAGAGACTCAGGTGTGCCAGTATTCAAAAATTTGGAGAAAGAGCTTTAAAAGCATGGTCAGTAGCT

CGCCTGAGCCAGAAATTTCCCAAGGCTGAGTTTGTAGAAGTTACCAAGCTAGTGACAGATCTCACAAAAG

TCCACAAGGAATGCTGCCATGGTGACCTACTTGAATGCGCAGATGACAGGGCAGATCTTGCCAAGTACAT

ATGTGATAATCAAGATACAATCTCCAGTAAACTGAAGGAATGCTGTGATAAGCCTTTGTTGGAAAAATCC

CACTGCATTGCTGAGGTAGAAAAAGATGCCATACCTGAAAACCTGCCCCCATTAACTGCTGACTTTGCTG

AAGATAAGGATGTTTGCAAAAACTATCAGGAAGCAAAAGATGCCTTCCTGGGCTCGTTTTTGTATGAATA

TTCAAGAAGGCATCCTGAATATGCTGTCTCAGTGCTATTGAGACTTGCCAAGGAATATGAAGCCACACTG

GAGGAATGCTGTGCCAAAGATGATCCACATGCATGCTATTCCACAGTGTTTGACAAACTTAAGCATCTTG

TGGATGAGCCTCAGAATTTAATCAAACAAAACTGTGACCAATTCGAAAAACTTGGAGAGTATGGATTCCA

AAATGCGCTCATAGTTCGTTACACCAGGAAAGTACCCCAAGTGTCAACTCCAACTCTCGTGGAGGTTTCA

AGAAGCCTAGGAAAAGTGGGTACTAGGTGTTGTACAAAGCCGGAATCAGAAAGAATGCCCTGTACTGAAG

ACTATCTGAGCTTGATCCTGAACCGGTTGTGCGTGCTGCATGAGAAGACACCAGTGAGTGAAAAAGTCAC

CAAGTGCTGCACAGAGTCATTGGTGAACAGACGGCCATGTTTCTCTGCTCTGACACCTGATGAAACATAT

GTACCCAAAGCCTTTGATGAGAAATTGTTCACCTTCCATGCAGATATATGCACACTTCCCGATACTGAGA

AACAAATCAAGAAACAAACTGCACTTGTTGAGCTGTTGAAACACAAGCCCAAGGCAACAGAGGAACAACT

GAAAACCGTCATGGAGAATTTTGTGGCTTTTGTAGACAAGTGCTGTGCAGCTGATGACAAAGAGGCCTGC

EcoRV (1805) BamHI (
TTTGCTGTGGAGGGTCCAAAACTTGTTGTTTCAACTCAAACAGCCTTAGCCTAAGATATCCGCGGGGGAT

SalI (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+*Hin*dIII 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.

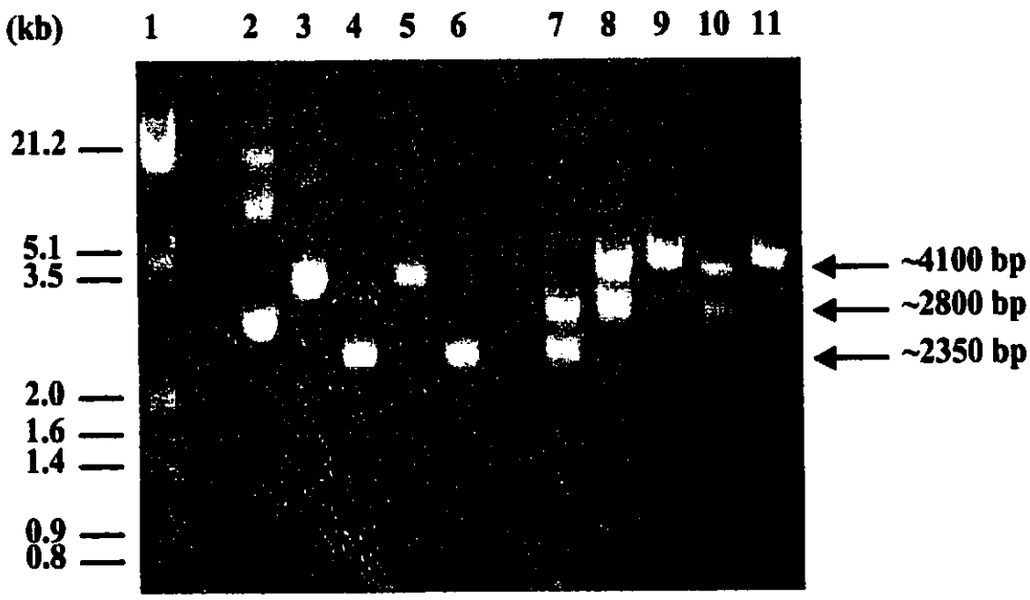
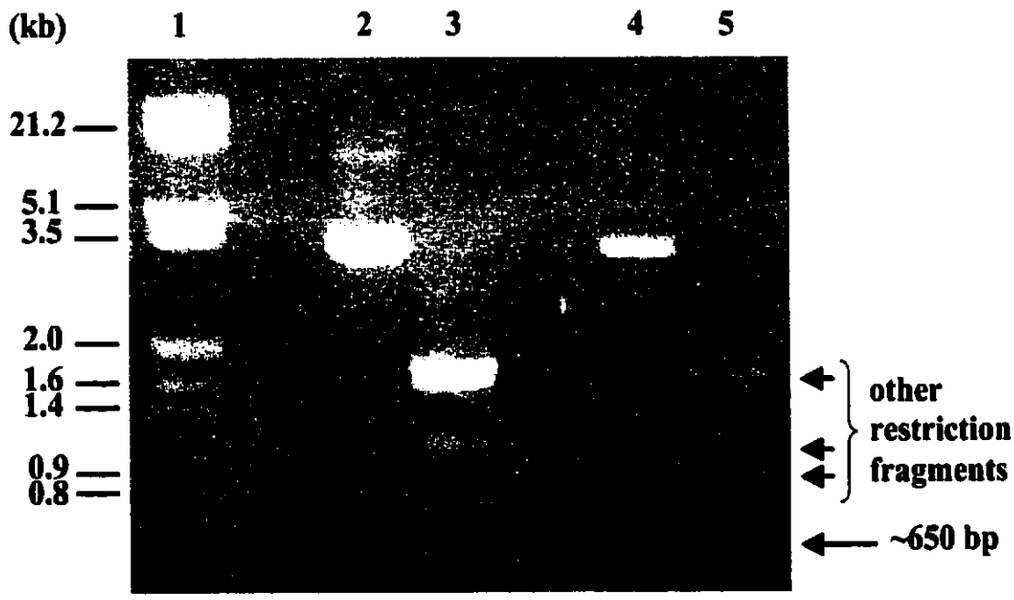

(kb)
1
2
3
4
5
6
7
8
9
10
11
21.2
5.1
3.5
2.0
1.6
1.4
0.9
0.8
~4100 bp
~2800 bp
~2350 bp

Figure 50. Orientation analysis using *Pst*I and *Sca*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+*Hin*dIII Ladder (MBI Fermentas, Flamborough, ON); 2, uncut colony 1 plasmid DNA; 3, restricted colony 1 plamid DNA; 4, uncut colony 5 plasmid DNA; 5, restricted colony 5 plasmid DNA.


(kb)
1
2
3
4
5
21.2
5.1
3.5
2.0
1.6
1.4
0.9
0.8
other
restriction
fragments
~650 bp

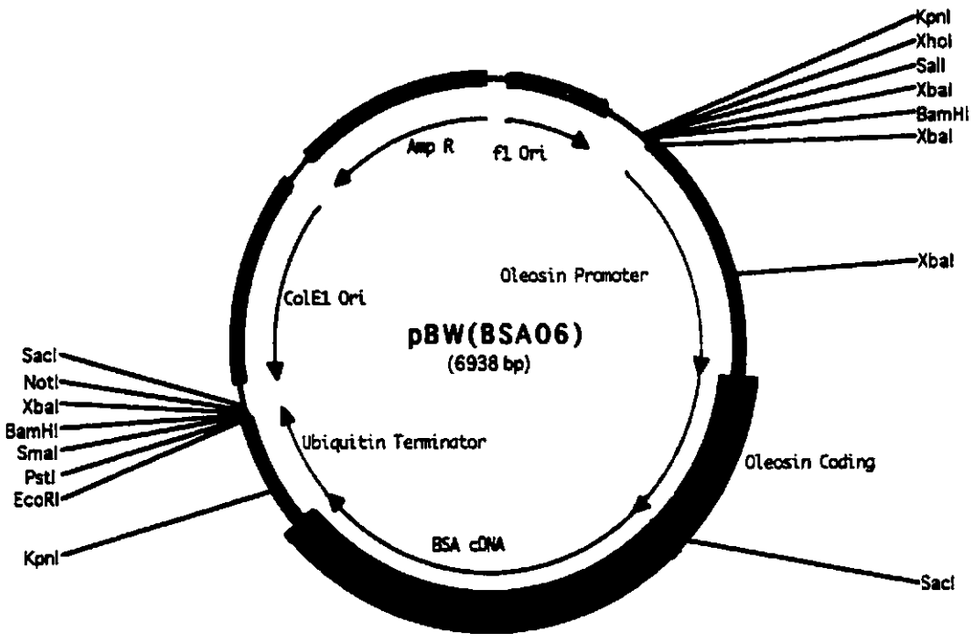
positive orientation was ~650 bp. Therefore, colony 1 was not only positive for the insert, but also had it in the correct orientation. The new plasmid containing the *Arabidopsis* oleosin promoter, DNA encoding oleosin protein, BSA cDNA and ubiquitin terminator was designated pBW(BSA06) (Figure 51). 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.

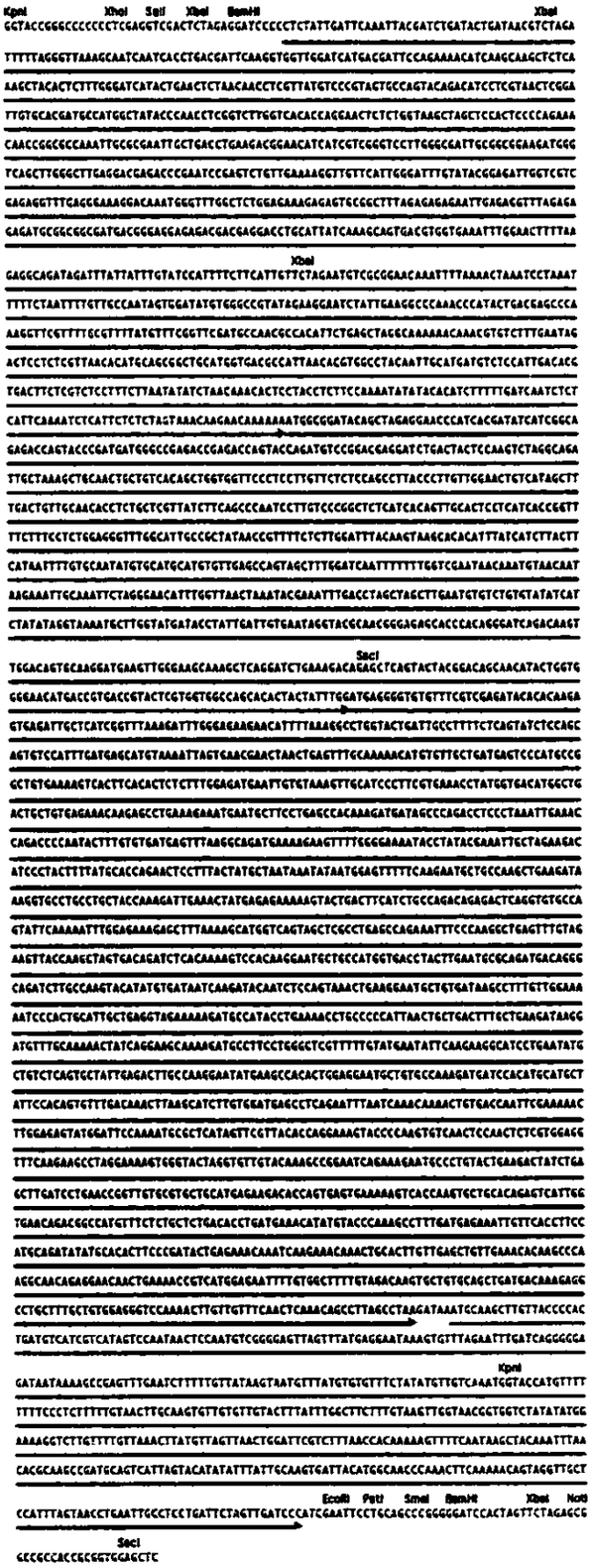
4.4. Bacterial Expression of BSA and a Truncation of the Protein

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 *Swa*I restriction site of pSBS2050 via restricting the *BSA05* gene with the blunt-ending restriction enzyme *Eco*RV. The detailed sequence of the insert is shown along with some flanking sequences on the page following the plasmid map.


KpnI
XhoI
SalI
XbaI
BamHI
XbaI
XbaI
Amp R
f1 Ori
Oleosin Promoter
ColE1 Ori
pBW(BSA06)
(6938 bp)
SacI
NotI
XbaI
BamHI
SmaI
PstI
EcoRI
KpnI
Ubiquitin Terminator
Oleosin Coding
BSA cDNA
SacI


KpnI XhoI SalI XbaI BamHI XbaI
XbaI
SacI
EcoRI PstI SmaI BamHI XbaI NotI
KpnI
SacI

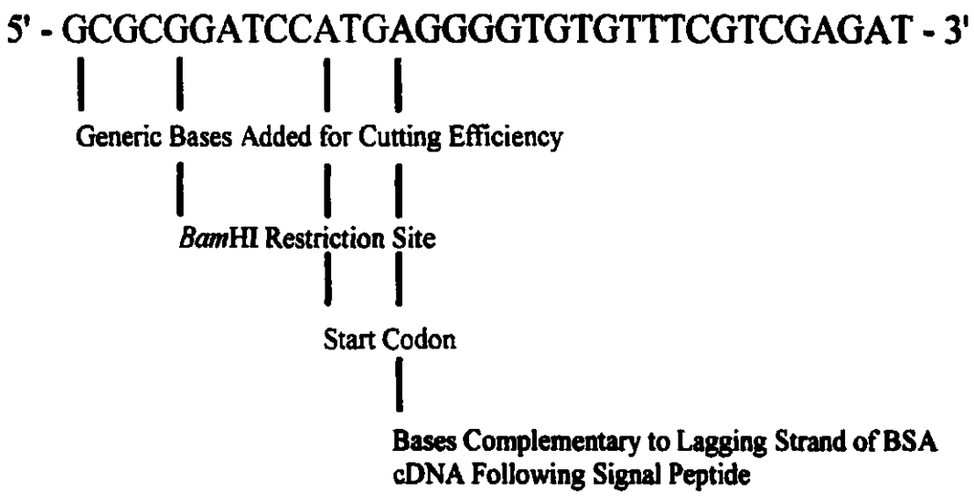
suggesting that the regions surrounding these residues may be responsible for FA binding (Reed, 1986). The sequence of amino acid residues at the presumed opening of the regions where FA bind to BSA were identified (Cistola *et al.*, 1987). The three binding sites consisted of two clusters of His-Arg-Arg (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



Reverse Primer: BSA976

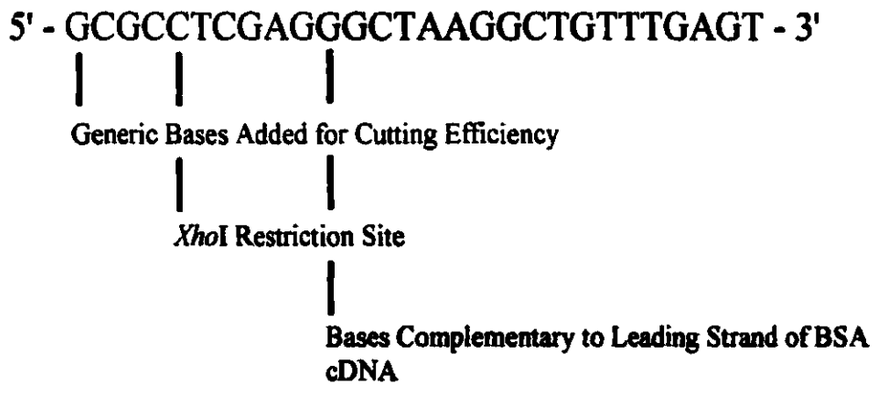


Figure 53. Agarose gel electrophoresis analysis of PCR amplification of BSA cDNA using *Pfu* polymerase and primers BSA975 and BSA976. The reaction conditions were 94°C for 1 minute (hot start) and 35 subsequent PCR cycles at 94°C for 45 seconds, 55°C for 1 minute and 72°C for 2 minutes. Following the 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/ *Eco*RI+*Hin*dIII Ladder (MBI Fermentas, Flamborough, ON) and lane 2 contains 5μL of the PCR reaction.

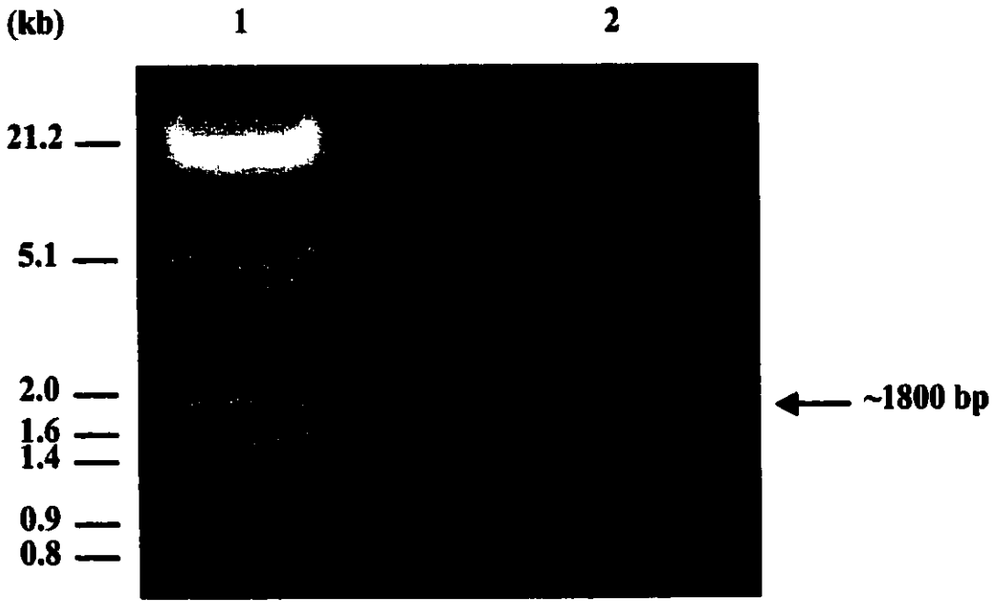
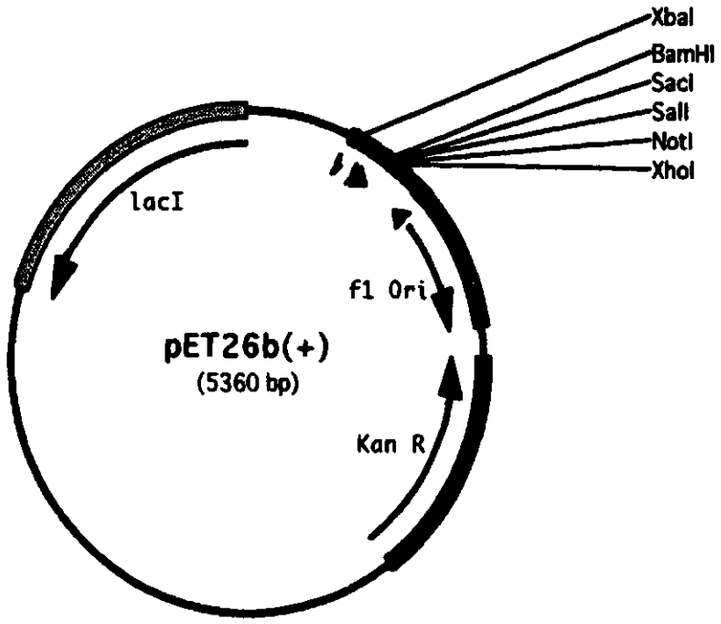

(kb)
1
2
21.2
5.1
2.0
1.6
1.4
0.9
0.8
~1800 bp

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

TTAACTTTAAGAAGGAGATATACATATGAAATACCTGCTGCCGACCGCTGCTGCTGGTCTGCT

BamHI SacI
GCTCCTCGCTGCCCAGCCGGCGATGGCCATGGATATCGGAATTAATTCGGATCCGAATTCGAG

SalI NotI XhoI
CTCCGTCGACAAGCTTGCGGCCGCACTCGAGCACCACCACCACCACCACTGAGATCCGGCTGC
TAACAAAGCCCGAAAGGAAGCTGAGTTGGCTGCTGCCACCGCTGAGCAATAACTAGCATAACC

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/ *Eco*RI+*Hin*dIII Ladder (MBI Fermentas, Flamborough, ON) and lanes 2 through 6 contain 5 μL of the PCR reaction from colonies 1 through 5, respectively.

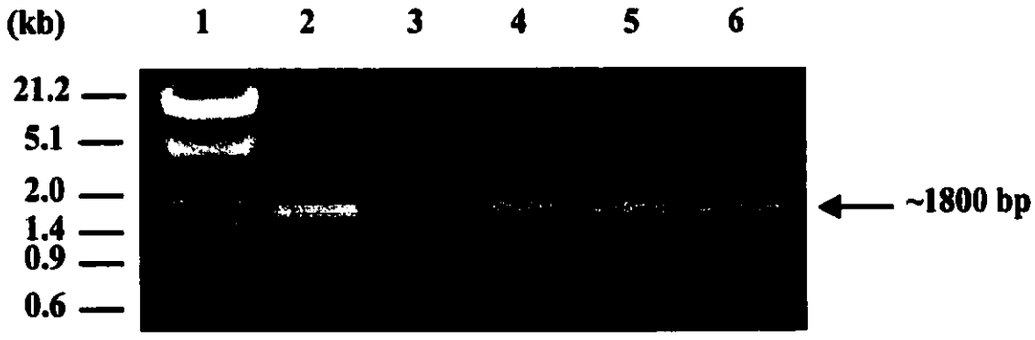

(kb)
1
2
3
4
5
6
21.2
5.1
2.0
1.4
0.9
0.6
~1800 bp

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+*Hin*dIII 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(+).

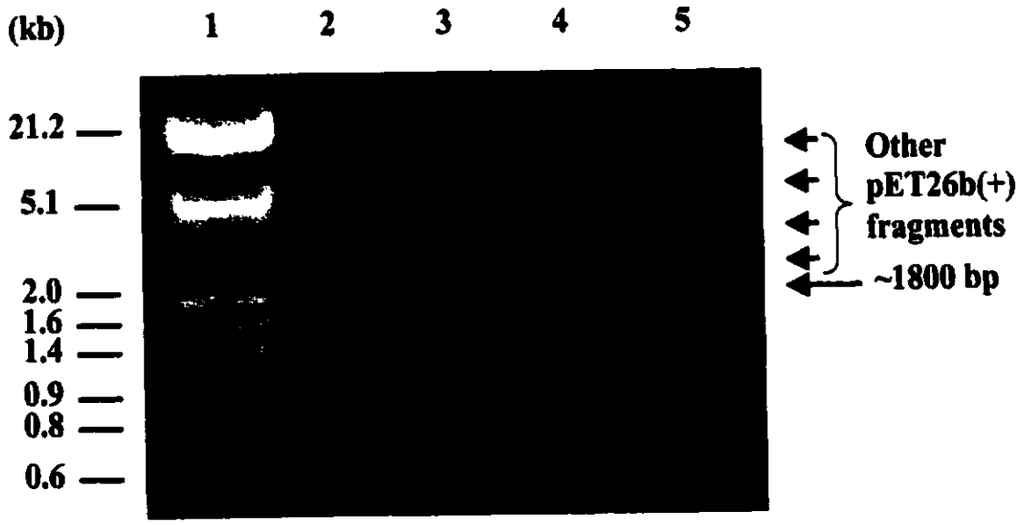

(kb)
1
2
3
4
5
21.2
5.1
2.0
1.6
1.4
0.9
0.8
0.6
Other
pET26b(+)
fragments
~1800 bp

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     TGGTCTGCTGCTCCTCGCTGCCCAGCCGGCGATGGCCATGGATATCGGAATTAAT   55
pET26B(+)          TGGTCTGCTGCTCCTCGCTGCCCAGCCGGCGATGGCCATGGATATCGGAATTAAT   55

PUTATIVE BSA07     TCGGATCCATGAGGGGTGTGTTTCGTCGAGATACACACAAGAGTGAGATTGCTCA  110
pET26B(+)/BSA      TC          AGGGGTGTGTTTCGTCGAGATACACACAAGAGTGAGATTGCTCA  57/44

PUTATIVE BSA07     TCGGTTTAAAGATTTGGGAGAAGAACATTTTAAAGGCCTGGTACTGATTGCCTTT  165
BSA                TCGGTTTAAAGATTTGGGAGAAGAACATTTTAAAGGCCTGGTACTGATTGCCTTT   99

PUTATIVE BSA07     TCTCAGTATCTCCAGCAGTGTCCATTTGATGAGCATGTAAAATTAGTGAACGAAC  220
BSA                TCTCAGTATCTCCAGCAGTGTCCATTTGATGAGCATGTAAAATTAGTGAACGAAC  154

PUTATIVE BSA07     TAACTGAGTTTGCAAAAACATGTGTTGCTGATGAGTCCCATGCCGGCTGTGAAAA  275
BSA                TAACTGAGTTTGCAAAAACATGTGTTGCTGATGAGTCCCATGCCGGCTGTGAAAA  209

PUTATIVE BSA07     GTCACTTCACACTCTCTTTGGAGATGAATTGTGTAAAGTTGCATCCCTTCGTGAA  330
BSA                GTCACTTCACACTCTCTTTGGAGATGAATTGTGTAAAGTTGCATCCCTTCGTGAA  264

PUTATIVE BSA07     ACCTATGGTGACATGGCTGACTGCTGTGAGAAACAAGAGCCTGAAAGAAATGAAT  385
BSA                ACCTATGGTGACATGGCTGACTGCTGTGAGAAACAAGAGCCTGAAAGAAATGAAT  319

PUTATIVE BSA07     GCTTCCTGAGCCACAAAGATGATAGCCCAGACCTCCCTAAATTGAAACCAGACCC  440
BSA                GCTTCCTGAGCCACAAAGATGATAGCCCAGACCTCCCTAAATTGAAACCAGACCC  374

PUTATIVE BSA07     CAATACTTTGTGTGATGAGTTTAAGGCAGATGAAAAGAAGTTTTGGGGAAAATAC  495
BSA                CAATACTTTGTGTGATGAGTTTAAGGCAGATGAAAAGAAGTTTTGGGGAAAATAC  429

PUTATIVE BSA07     CTATACGAAATTGCTAGAAGACATCCCTACTTTTATGCACCAGAACTCCTTTACT  550
BSA                CTATACGAAATTGCTAGAAGACATCCCTACTTTTATGCACCAGAACTCCTTTACT  484

PUTATIVE BSA07     ATGCTAATAAATATAATGGAGTTTTTCAAGAATGCTGCCAAGCTGAAGATAAAGG  605
BSA                ATGCTAATAAATATAATGGAGTTTTTCAAGAATGCTGCCAAGCTGAAGATAAAGG  539

PUTATIVE BSA07     TGCCTGCCTGCTACCAAAGATTGAAACTATGAGAGAAAAAGTACTGACTTCATCT  660
BSA                TGCCTGCCTGCTACCAAAGATTGAAACTATGAGAGAAAAAGTACTGACTTCATCT  594

PUTATIVE BSA07     GCCAGACAGAGACTCAGGTGTGCCAGTATTCAAAAATTTGGAGAAAGAGCTTTAA  715
BSA                GCCAGACAGAGACTCAGGTGTGCCAGTATTCAAAAATTTGGAGAAAGAGCTTTAA  649

PUTATIVE BSA07     AAGCATGGTCAGTAGCTCGCCTGAGCCAGAAATTTCCCAAGGCTGAGTTTGTAGA  770
BSA                AAGCATGGTCAGTAGCTCGCCTGAGCCAGAAATTTCCCAAGGCTGAGTTTGTAGA  704

PUTATIVE BSA07     AGTTACCAAGCTAGTGACAGATCTCACAAAAGTCCACAAGGAATGCTGCCATGGT  825
BSA                AGTTACCAAGCTAGTGACAGATCTCACAAAAGTCCACAAGGAATGCTGCCATGGT  759

PUTATIVE BSA07     GACCTACTTGAATGCGCAGATGACAGGGCAGATCTTGCCAAGTACATATGTGATA  880
BSA                GACCTACTTGAATGCGCAGATGACAGGGCAGATCTTGCCAAGTACATATGTGATA  814

PUTATIVE BSA07     ATCAAGATACAATCTCCAGTAAACTGAAGGAATGCTGTGATAAGCCTTTGTTGGA  935
BSA                ATCAAGATACAATCTCCAGTAAACTGAAGGAATGCTGTGATAAGCCTTTGTTGGA  869

PUTATIVE BSA07     AAAATCCCACTGCATTGCTGAGGTAGAAAAAGATGCCATACCTGAAAACCTGCCC  990
BSA                AAAATCCCACTGCATTGCTGAGGTAGAAAAAGATGCCATACCTGAAAACCTGCCC  924

PUTATIVE BSA07     CCATTAACTGCTGACTTTGCTGAAGATAAGGATGTTTGCAAAAACTATCAGGAAG 1045
BSA                CCATTAACTGCTGACTTTGCTGAAGATAAGGATGTTTGCAAAAACTATCAGGAAG  979

PUTATIVE BSA07     CAAAAGATGCCTTCCTGGGCTCGTTTTTGTATGAATATTCAAGAAGGCATCCTGA 1100
BSA                CAAAAGATGCCTTCCTGGGCTCGTTTTTGTATGAATATTCAAGAAGGCATCCTGA 1034

PUTATIVE BSA07     ATATGCTGTCTCAGTGCTATTGAGACTTGCCAAGGAATATGAAGCCACACTGGAG 1155
BSA                ATATGCTGTCTCAGTGCTATTGAGACTTGCCAAGGAATATGAAGCCACACTGGAG 1089


PUTATIVE BSA07     GAATGCTGTGCCAAAGATGATCCACATGCATGCTATTCCACAGTGTTTGACAAAC 1210
BSA                GAATGCTGTGCCAAAGATGATCCACATGCATGCTATTCCACAGTGTTTGACAAAC 1144
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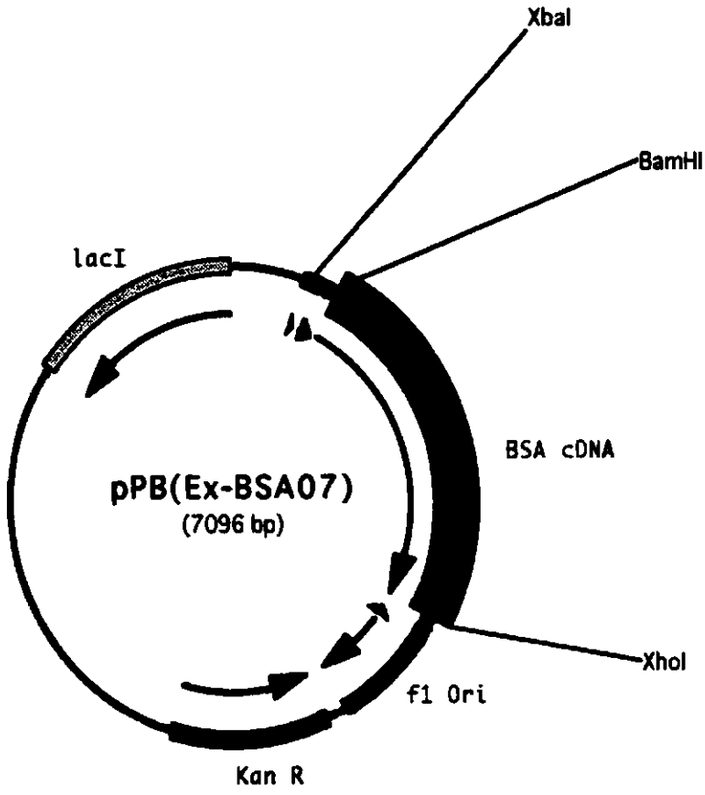
PUTATIVE *BSA07*	TTAAGCATCTTGTGGATGAGCCTCAGAATTTAATCAAACAAAACTGTGACCAATT	1265
BSA	TTAAGCATCTTGTGGATGAGCCTCAGAATTTAATCAAACAAAACTGTGACCAATT	1199
PUTATIVE *BSA07*	CGAAAAACTTGGAGAGTATGGATTCCAAAATGCGCTCATAGTTCGTTACACCAGG	1320
BSA	CGAAAAACTTGGAGAGTATGGATTCCAAAATGCGCTCATAGTTCGTTACACCAGG	1254
PUTATIVE *BSA07*	AAAGTACCCCAAGTGTCAACTCCAACTCTCGTGGAGGTTTCAAGAAGCCTAGGAA	1375
BSA	AAAGTACCCCAAGTGTCAACTCCAACTCTCGTGGAGGTTTCAAGAAGCCTAGGAA	1309
PUTATIVE *BSA07*	AAGTGGGTACTAGGTGTTGTACAAAGCCGGAATCAGAAAGAATGCCCTGTACTGA	1430
BSA	AAGTGGGTACTAGGTGTTGTACAAAGCCGGAATCAGAAAGAATGCCCTGTACTGA	1364
PUTATIVE *BSA07*	AGACTATCTGAGCTTGATCCTGAACCGGTTGTGCGTGCTGCATGAGAAGACACCA	1485
BSA	AGACTATCTGAGCTTGATCCTGAACCGGTTGTGCGTGCTGCATGAGAAGACACCA	1419
PUTATIVE *BSA07*	GTGAGTGAAAAAGTCACCAAGTGCTGCACAGAGTCATTGGTGAACAGACGGCCAT	1540
BSA	GTGAGTGAAAAAGTCACCAAGTGCTGCACAGAGTCATTGGTGAACAGACGGCCAT	1474
PUTATIVE *BSA07*	GTTTCTCTGCTCTGACACCTGATGAAACATATGTACCCAAAGCCTTTGATGAGAA	1595
BSA	GTTTCTCTGCTCTGACACCTGATGAAACATATGTACCCAAAGCCTTTGATGAGAA	1529
PUTATIVE *BSA07*	ATTGTTCACCTTCCATGCAGATATATGCACACTTCCCGATACTGAGAAACAAATC	1650
BSA	ATTGTTCACCTTCCATGCAGATATATGCACACTTCCCGATACTGAGAAACAAATC	1584
PUTATIVE *BSA07*	AAGAAACAAACTGCACTTGTTGAGCTGTTGAAACACAAGCCCAAGGCAACAGAGG	1705
BSA	AAGAAACAAACTGCACTTGTTGAGCTGTTGAAACACAAGCCCAAGGCAACAGAGG	1639
PUTATIVE *BSA07*	AACAACTGAAAACCGTCATGGAGAATTTTGTGGCTTTTGTAGACAAGTGCTGTGC	1760
BSA	AACAACTGAAAACCGTCATGGAGAATTTTGTGGCTTTTGTAGACAAGTGCTGTGC	1694
PUTATIVE *BSA07*	AGCTGATGACAAAGAGGCCTGCTTTGCTGTGGAGGGTCCAAAACTTGTTGTTTCA	1815
BSA	AGCTGATGACAAAGAGGCCTGCTTTGCTGTGGAGGGTCCAAAACTTGTTGTTTCA	1749
PUTATIVE *BSA07*	ACTCAAACAGCCTTAGCCCTCGAGCACCACCACCACCACCACTGA	1860
BSA/pET26B(+)	ACTCAAACAGCCTTAGCC CACCACCACCACCACCACTGA	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.


XbaI
BamHI
lacI
BSA cDNA
pPB(Ex-BSA07)
(7096 bp)
XhoI
f1 Ori
Kan R

BamHI
GGATCCATGAGGGGTGTGTTTCGTCGAGATACACACAAGAGTGAGATTGCTCATCGGTTTAAAG

ATTTGGGAGAAGAACATTTTAAAGGCCTGGTACTGATTGCCTTTTCTCAGTATCTCCAGCAGTG

TCCATTTGATGAGCATGTAAAATTAGTGAACGAACTAACTGAGTTTGCAAAAACATGTGTTGCT

GATGAGTCCCATGCCGGCTGTGAAAAGTCACTTCACACTCTCTTTGGAGATGAATTGTGTAAAG

TTGCATCCCTTCGTGAAACCTATGGTGACATGGCTGACTGCTGTGAGAAACAAGAGCCTGAAAG

AAATGAATGCTTCCTGAGCCACAAAGATGATAGCCCAGACCTCCCTAAATTGAAACCAGACCCC

AATACTTTGTGTGATGAGTTTAAGGCAGATGAAAAGAAGTTTTGGGGAAAATACCTATACGAAA

TTGCTAGAAGACATCCCTACTTTTATGCACCAGAACTCCTTTACTATGCTAATAAATATAATGG

AGTTTTTCAAGAATGCTGCCAAGCTGAAGATAAAGGTGCCTGCCTGCTACCAAAGATTGAAACT

ATGAGAGAAAAAGTACTGACTTCATCTGCCAGACAGAGACTCAGGTGTGCCAGTATTCAAAAAT

TTGGAGAAAGAGCTTTAAAAGCATGGTCAGTAGCTCGCCTGAGCCAGAAATTTCCCAAGGCTGA

GTTTGTAGAAGTTACCAAGCTAGTGACAGATCTCACAAAAGTCCACAAGGAATGCTGCCATGGT

GACCTACTTGAATGCGCAGATGACAGGGCAGATCTTGCCAAGTACATATGTGATAATCAAGATA

CAATCTCCAGTAAACTGAAGGAATGCTGTGATAAGCCTTTGTTGGAAAAATCCCACTGCATTGC

TGAGGTAGAAAAAGATGCCATACCTGAAAACCTGCCCCCATTAACTGCTGACTTTGCTGAAGAT

AAGGATGTTTGCAAAAACTATCAGGAAGCAAAAGATGCCTTCCTGGGCTCGTTTTTGTATGAAT

ATTCAAGAAGGCATCCTGAATATGCTGTCTCAGTGCTATTGAGACTTGCCAAGGAATATGAAGC

CACACTGGAGGAATGCTGTGCCAAAGATGATCCACATGCATGCTATTCCACAGTGTTTGACAAA

CTTAAGCATCTTGTGGATGAGCCTCAGAATTTAATCAAACAAAACTGTGACCAATTCGAAAAAC

TTGGAGAGTATGGATTCCAAAATGCGCTCATAGTTCGTTACACCAGGAAAGTACCCCAAGTGTC

AACTCCAACTCTCGTGGAGGTTTCAAGAAGCCTAGGAAAAGTGGGTACTAGGTGTTGTACAAAG

CCGGAATCAGAAAGAATGCCCTGTACTGAAGACTATCTGAGCTTGATCCTGAACCGGTTGTGCG

TGCTGCATGAGAAGACACCAGTGAGTGAAAAAGTCACCAAGTGCTGCACAGAGTCATTGGTGAA

CAGACGGCCATGTTTCTCTGCTCTGACACCTGATGAAACATATGTACCCAAAGCCTTTGATGAG

AAATTGTTCACCTTCCATGCAGATATATGCACACTTCCCGATACTGAGAAACAAATCAAGAAAC

AAACTGCACTTGTTGAGCTGTTGAAACACAAGCCCAAGGCAACAGAGGAACAACTGAAAACCGT

CATGGAGAATTTTGTGGCTTTTGTAGACAAGTGCTGTGCAGCTGATGACAAAGAGGCCTGCTTT

XhoI
GCTGTGGAGGGTCCAAAACTTGTTGTTTCAACTCAAACAGCCTTAGCCCTCGAGCACCACCACC

ACCACCACTGAGATCCGGCTGCTAACAAAGCCCGAAAGGAAGCTGAGTTGGCTGCTGCCACCGC
TGAGCAATAACTAGCATAACCCCTTGGGGCCTCTAAACGGGTCTTGAGGGGTTTTTTG

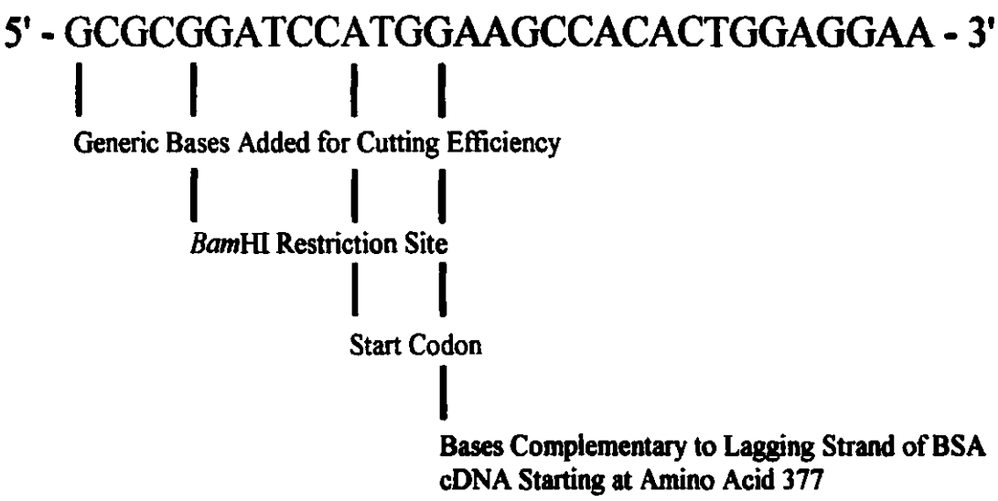
4.4.2. Genetic Construct to Facilitate Bacterial Expression of a Truncation of BSA

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



Reverse Primer: BSA843

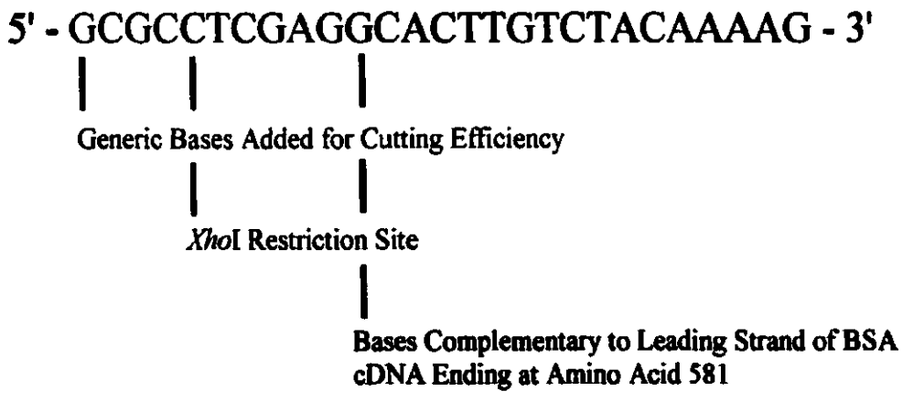


Figure 60. Agarose gel electrophoresis analysis of PCR amplification of BSA cDNA using *Pfu* polymerase and primers BSA842 and BSA843. The reaction conditions were 94°C for 1 minute (hot start) and 35 subsequent PCR cycles at 94°C for 45 seconds, 55°C for 1 minute and 72°C for 1 minute. Following the 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/ *Eco*RI+*Hin*dIII Ladder (MBI Fermentas, Flamborough, ON) and lane 2 contains 5μL of the PCR reaction.

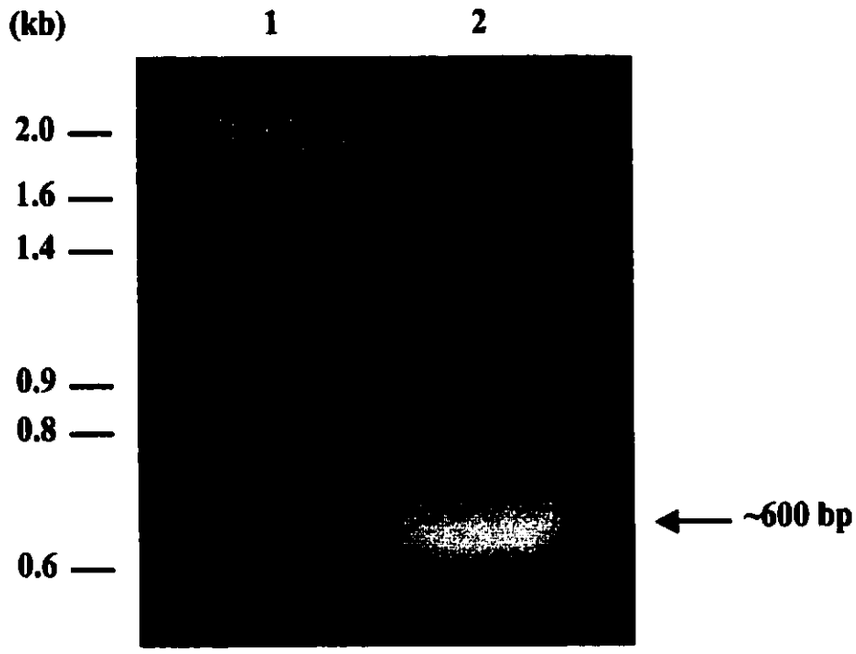
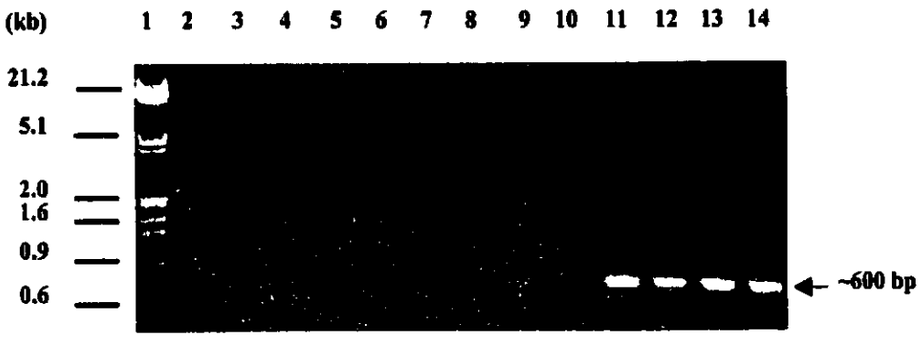

(kb)
1
2
2.0
1.6
1.4
0.9
0.8
0.6
~600 bp

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/ *Eco*RI+*Hin*dIII Ladder (MBI Fermentas, Flamborough, ON) and lanes 2 through 14 contain 5 μL of the PCR reaction from colonies 1 through 13, respectively.


(kb)
1 2 3 4 5 6 7 8 9 10 11 12 13 14
21.2
5.1
2.0
1.6
0.9
0.6
~600 bp

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.

```
PUTATIVE BSA04    TGGTCTGCTGCTCCTCGCTGCCCAGCCGGCGATGGCCATGGATATCGGAATTAAT   55
pET26b(+)         TGGTCTGCTGCTCCTCGCTGCCCAGCCGGCGATGGCCATGGATATCGGAATTAAT   55

PUTATIVE BSA04    TCGGATCCATGGAAGCCACACTGGAGGAATGCTGTGCCAAAGATGATCCACATGC   110
pET26b(+)/BSA     TC          GAAGCCACACTGGAGGAATGCTGTGCCAAAGATGATCCACATGC   57/44

PUTATIVE BSA04    ATGCTATTCCACAGTGTTTGACAAACTTAAGCATCTTGTGGATGAGCCTCAGAAT   165
BSA               ATGCTATTCCACAGTGTTTGACAAACTTAAGCATCTTGTGGATGAGCCTCAGAAT   99

PUTATIVE BSA04    TTAATCAAACAAAACTGTGACCAATTCGAAAAACTTGGAGAGTATGGATTCCAAA   220
BSA               TTAATCAAACAAAACTGTGACCAATTCGAAAAACTTGGAGAGTATGGATTCCAAA   154

PUTATIVE BSA04    ATGCGCTCATAGTTCGTTACACCAGGAAAGTACCCCAAGTGTCAACTCCAACTCT   275
BSA               ATGCGCTCATAGTTCGTTACACCAGGAAAGTACCCCAAGTGTCAACTCCAACTCT   209

PUTATIVE BSA04    CGTGGAGGTTTCAAGAAGCCTAGGAAAAGTGGGTACTAGGTGTTGTACAAAGCCG   330
BSA               CGTGGAGGTTTCAAGAAGCCTAGGAAAAGTGGGTACTAGGTGTTGTACAAAGCCG   264

PUTATIVE BSA04    GAATCAGAAAGAATGCCCTGTACTGAAGACTATCTGAGCTTGATCCTGAACCGGT   385
BSA               GAATCAGAAAGAATGCCCTGTACTGAAGACTATCTGAGCTTGATCCTGAACCGGT   319

PUTATIVE BSA04    TGTGCGTGCTGCATGAGAAGACACCAGTGAGTGAAAAAGTCACCAAGTGCTGCAC   440
BSA               TGTGCGTGCTGCATGAGAAGACACCAGTGAGTGAAAAAGTCACCAAGTGCTGCAC   374

PUTATIVE BSA04    AGAGTCATTGGTGAACAGACGGCCATGTTTCTCTGCTCTGACACCTGATGAAACA   495
BSA               AGAGTCATTGGTGAACAGACGGCCATGTTTCTCTGCTCTGACACCTGATGAAACA   429

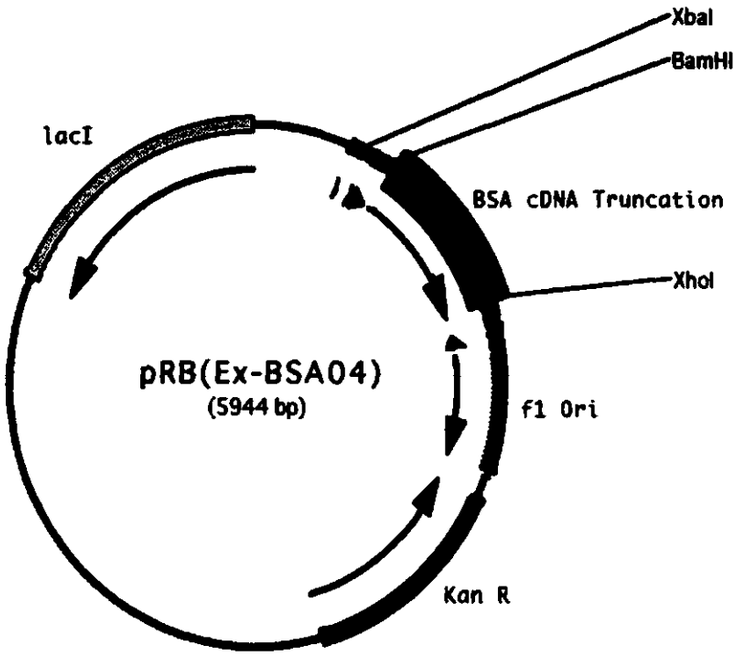
PUTATIVE BSA04    TATGTACCCAAAGCCTTTGATGAGAAATTGTTCACCTTCCATGCAGATATATGCA   550
BSA               TATGTACCCAAAGCCTTTGATGAGAAATTGTTCACCTTCCATGCAGATATATGCA   484

PUTATIVE BSA04    CACTTCCCGATACTGAGAAACAAATCAAGAAACAAACTGCACTTGTTGAGCTGTT   605
BSA               CACTTCCCGATACTGAGAAACAAATCAAGAAACAAACTGCACTTGTTGAGCTGTT   539

PUTATIVE BSA04    GAAACACAAGCCCAAGGCAACAGAGGAACAACTGAAAACCGTCATGGAGAATTTT   660
BSA               GAAACACAAGCCCAAGGCAACAGAGGAACAACTGAAAACCGTCATGGAGAATTTT   594

PUTATIVE BSA04    GTGGCTTTTGTAGACAAGCTCGAGCACCACCACCACCACCACTGA             705
BSA/pET26b(+)     GTGGCTTTTGTAGACAAG          CACCACCACCACCACCACTGA         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

GTTTGACAAACTTAAGCATCTTGTGGATGAGCCTCAGAATTTAATCAAACAAAACTGTGACCAATTCG

AAAAACTTGGAGAGTATGGATTCCAAAATGCGCTCATAGTTCGTTACACCAGGAAAGTACCCCAAGTG

TCAACTCCAACTCTCGTGGAGGTTTCAAGAAGCCTAGGAAAAGTGGGTACTAGGTGTTGTACAAAGCC

GGAATCAGAAAGAATGCCCTGTACTGAAGACTATCTGAGCTTGATCCTGAACCGGTTGTGCGTGCTGC

ATGAGAAGACACCAGTGAGTGAAAAAGTCACCAAGTGCTGCACAGAGTCATTGGTGAACAGACGGCCA

TGTTTCTCTGCTCTGACACCTGATGAAACATATGTACCCAAAGCCTTTGATGAGAAATTGTTCACCTT

CCATGCAGATATATGCACACTTCCCGATACTGAGAAACAAATCAAGAAACAAACTGCACTTGTTGAGC

TGTTGAAACACAAGCCCAAGGCAACAGAGGAACAACTGAAAACCGTCATGGAGAATTTTGTGGCTTTT

XhoI
GTAGACAAGTGCCTCGAGCACCACCACCACCACCACTGAGATCCGGCTGCTAACAAAGCCCGAAAGGA

AGCTGAGTTGGCTGCTGCCACCGCTGAGCAATAACTAGCATAACCCCTTGGGGCCTCTAAACGGGTCT

TGAGGGGTTTTTTG

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 or 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.

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