ABSTRACT

BYFIELD, Grace E. The effect of temperature on fatty acid desaturase gene expression and fatty acid composition in developing soybean seeds (Under the direction of Dr. Robert G. Upchurch).

Soybean [*Glycine max* (L.) Merr.] is the largest oilseed crop produced and consumed worldwide. As an oilseed, soybean provides high quality protein for livestock and poultry feed, and the oil is used extensively in the cooking and food manufacturing industries across the world. Soybean producers have targeted improved oil traits as a priority area of research to enhance the market share of U.S. soybean. Soybean seed is approximately 18% oil and standard commodity soybean oil is a mixture of five fatty acids; palmitic (11%), stearic (4%), oleic (22%), linoleic (53%), and linolenic (8%). Oils high in monounsaturated fatty acids possess increased oxidative stability which negates the need for hydrogenation and eliminates the production of trans-fats. Food products containing trans-fatty acids are currently a major health concern and as such, soybean breeders and molecular geneticists have been challenged to efficiently alter the fatty acid composition to meet the specific needs of the various industries. Fatty acid desaturases are enzymes responsible for the insertion of double bonds (normally in the *Z* or *cis* conformation) into alkyl chains, following the abstraction of two hydrogen atoms. The physical properties and nutritional value of many animal and plant storage lipids are determined by desaturases. The quality of soybean oil is of paramount importance both economically and from a nutritional stand point and depends to a large extent on the ratio of polyunsaturated to monounsaturated fatty acid.

Temperature also plays an important role in the final lipid content of oil seed plants. In this study, we designed and used gene-specific primers to the following fatty acid desaturases; stearoyl-ACP desaturase (SACPD), omega-6 fatty acid desaturase (FAD2-1), and omega-3 fatty acid desaturase (FAD3), to characterize soybean varieties. We further determined the effect of temperature on the expression of these desaturase genes by quantifying transcript accumulation at various stages of seed development.

We surveyed 51 soybean lines and found each contained two SACPD genes (A and B) with distinguishing amino acid variations in exon 3. The varieties also had two FAD2-1 (A and B) and three FAD3 (A, B, C) genes. Soluble ∆9 stearoyl-ACP desaturases introduce the first double bond into stearoyl-ACP (18:0-ACP) between carbons 9 and 10 to produce oleoyl-ACP ($18:1^{\Delta 9}$-ACP). Microsomal ω-6 desaturase catalyzes the first extra-plastidial desaturation and converts oleic acid to linoleic acid. Microsomal ω-3 fatty acid desaturases (FAD3s) catalyze the insertion of a third double bond into the linoleic (18:2) acid precursor to produce linolenic (18:3) acid.

An analysis of the effect of cold (22/18^{o}C), normal (26/22^{o}C), and warm (30/26^{o}C) temperatures on the transcript accumulation of these desaturase genes revealed some differences. Transcript accumulation of *SACPD-A* and *-B* decreased by up to 69% with increasing temperature in cultivars Dare, A6 (a high stearate line), and N01-3544 (a mid-oleic line). The oleic acid content of these three lines was inversely related to the levels of *SACPD* expression at the warm and cold temperatures. This suggests that transcription control of *SACPD* may not be a

crucial factor for regulating oleic acid content in soybean. FAD2-1A and FAD2-1B gene expression in stage 4 seeds was comparable at the normal temperature, but a change in growth temperature to either side of the norm resulted in increased expression of FAD2-1B over FAD2-1A, slight at the warm temperature, but more pronounced at the cold temperature. The three omega-3 fatty acid desaturase genes exhibited the highest levels of transcript accumulation in stage 4 seeds at the cold temperature, with *FAD3A* levels 1.3 to 1.8 fold higher than *3B* and *3C*.

The fatty acid composition of the seeds at different stages of development was determined in conjunction with steady state transcript levels. Results show that the stearic acid content of A6 had the most dramatic response of the three soybean lines to temperature manipulation. Stearic acid content increased at the warmer temperature for all stages of A6, but for Dare and N01-3544 the percentage change in 18:0 was slight and net negative. Conversely, growth at the cold temperature resulted in the most dramatic reduction (48%) of stearic acid content in A6. The oleate (18:1) concentration increased at the higher growth temperature compared to normal across all varieties and stages, with Dare showing the most dramatic (32 %) increase in oleic acid content at the warm temperature. The increase in *FAD2-1B* transcript accumulation with decreasing temperature was associated with increasing 18:2 content in two of the three varieties. Increased Gm*FAD3A* transcript accumulation was accompanied by an increase in 18:3 in all three soybean varieties examined.

The differences in steady state mRNA levels we observed could be due to changes in transcription rate or mRNA stability. In light of this, further studies to

measure desaturase activity under specific temperature regimes are needed in order to clarify the linkages of transcript level to enzyme activity and the fatty acid composition in developing soybean seeds.

THE EFFECT OF TEMPERATURE ON FATTY ACID DESATURASE GENE EXPRESSION AND FATTY ACID COMPOSITION IN DEVELOPING SOYBEAN SEEDS

by

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BIOGRAPHY

I grew up in the small town of Falmouth in Trelawny, Jamaica West Indies with three sisters and attended Westwood High School for girls. After graduating from high school, I completed a three-year diploma in Teacher Education at Mico Teachers' College, specializing in Mathematics and General Science. In 1993, I received a Bachelor's of Science in Botany with a minor in Chemistry from the University of the West Indies, Mona and went on to head the Chemistry Department at Wolmer's Boys' School where I met my husband, Brian. I subsequently worked with an environmental consulting company before setting up and managing Rototech International (Jamaica) Limited, the local branch of a manufacturing company established in Trinidad West Indies. Brian and I then decided to pursue graduate studies and in the summer of 1998, we migrated to the United States after he was accepted at UNC-Chapel Hill. I attended North Carolina Central University where I earned a Master's of Science in Biology with a minor in Education in 2002 under the direction of Dr. Goldie S. Byrd. During this time, I also managed to earn the title of Mom and thoroughly enjoy the company of my son Bakari, who is now four and a half years old. My graduate studies continued at North Carolina State University where I enrolled in the Doctoral program in the Department of Microbiology and was given the opportunity to combine my love for plants with molecular biology. The research project presented here was under the direction of Dr. Robert G. Upchurch and takes a look at soybean seeds at the molecular level.

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LITERATURE REVIEW

Introduction

Soybean [*Glycine max* (L.) Merr.] is the largest oilseed crop produced and consumed worldwide, accounting for 56 percent of the world oilseed production in 2003 (Soy Stats 2004). As an oilseed soybean provides high quality protein for livestock and poultry feed. The oil is used extensively in the cooking and food manufacturing industries across the world. The major nonfood market for vegetable oils is the production of soaps, detergents, and other surfactants. The United States has been for some time, and continues to be the leading producer of soybean. Soybean producers have targeted improved oil traits as a priority area of research to enhance the market share of U.S. soybean. The expanded use of soybean oil for additional industrial applications and renewable chemical feedstock is hampered by the physical and chemical properties of conventional soybean oil. In Nature, the vast majority of important food crops have a limited range of fatty acid. Palmate, oleate and linoleate represent approximately 80 percent of the total fatty acid with laurate, myristate, stearate, and linolenate, a further 15 percent (Harwood, 1998). Soybean seed is approximately 18% oil and standard commodity soybean oil is a mixture of five fatty acids; palmitic (11%), stearic (4%), oleic (22%), linoleic (53%), and linolenic (8%). These fatty acids differ greatly in melting points, oxidative stabilities, and chemical functionalities (Cahoon, 2003). Oils high in monounsaturated fatty acids possess increased oxidative stability which negates the need for hydrogenation and eliminates the production of trans-fats. Food products

containing trans-fatty acids are currently a major health concern and as such, soybean breeders and molecular geneticists have been challenged to increase monounsaturates to satisfy specific health concerns.

Fatty Acid Biosynthesis

In plants, lipids play important roles in energy storage, membrane structure, biological activity, and surface coverings, in addition to some specialized functions such as light harvesting during photosynthesis. Plants are fundamentally different from other eukaryotes in the molecular organization of the enzymes of fatty acid synthesis. The predominant plant fatty acids found in nature consist of just six or seven structures that have chain lengths of 16 or 18 carbons and one to three double bonds. These fatty acids are synthesized from acetyl-CoA by a series of reactions that are localized in the plastids. Acetyl-CoA carboxylases (ACCase), along with fatty acid synthase (FAS) facilitate the de novo synthesis of long chain fatty acid from acetyl CoA. The central carbon donor for fatty acid synthesis is the malonyl-CoA produced by ACCase. In the initial reactions, desaturases act on the preformed long-chain acyl esters, with O_2 and a reduced compound such as ferredoxin or cytochrome b_5 as cofactors. The assembly of fatty acids and the introduction of the first double bond occurs while these structures are attached to protein cofactor, acyl carrier protein (ACP). ACP is a small (9 kD) acidic protein that contains a prosthetic group to which the growing acyl chain is attached as a thioester. From this point on, all the reactions of the pathway involve ACP until the

16- or 18-carbon product is ready for transfer to glycerolipids or export from the plastid (Ohlrogge, 1995).

At least three separate condensing enzymes (3-ketoacyl-ACP synthases - KAS) are required to produce an 18-carbon fatty acid (Fig. 1). KAS III catalyzes the first condensation of acetyl-CoA and malonyl-ACP to form a 4-carbon product. KAS I produces chain lengths from six to sixteen carbons. The elongation of the 16-carbon palmitoyl-ACP to stearoyl-ACP is catalysed by KAS II. The initial product of each condensation is a 3-ketoacyl-ACP. This is followed by three additional reactions (reduction, dehydration, reduction) to form a saturated fatty acid. After each round of four reactions, the precursor fatty acid is lengthened by two carbons. The elongation of fatty acids in the plastids is terminated when the acyl group is removed from ACP.

Substrate selectivity of acyl-ACP thioesterase dictates that palmitate and stearate are the two products formed. The major fate of 16:0 and 18:0 acyl chains produced in the plastid is to form the hydrophobic portion of glycerolipid molecules which are components of all cellular membranes. Subsequent desaturation of these lipids to the highly unsaturated forms typical of plant cell membranes is carried out by membrane-bound desaturases of the chloroplast and the endoplasmic reticulum (ER) (Browse and Somerville, 1991; Heinz, 1993) (Fig. 2).

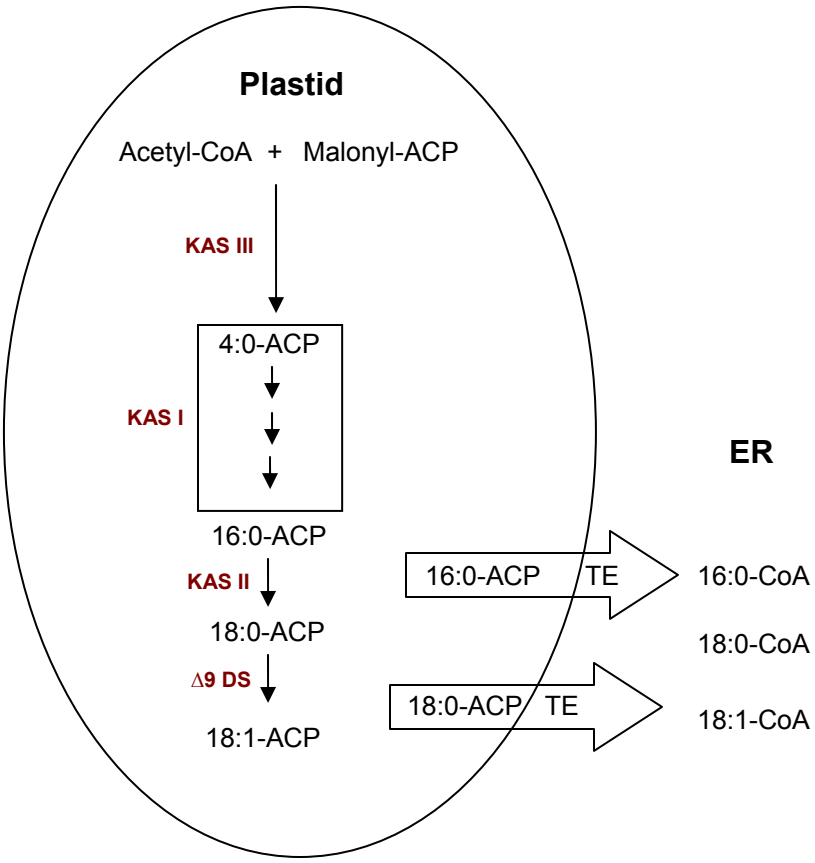

Figure 1. Representation of the fatty acid synthetic pathway in soybean. (Modified from Wilson *et al*., 2004).

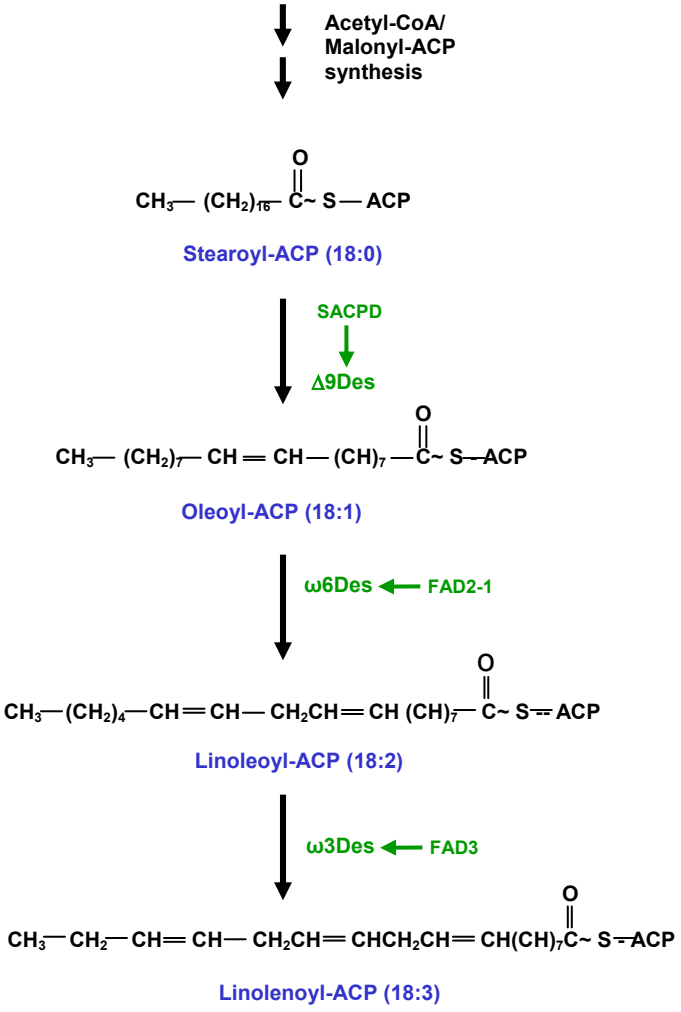

Figure 2. Flow diagram of lipid desaturation in plants.

Fatty Acid Desaturases (FADs)

Fatty acid desaturases are enzymes found in microbes, plants and animal cells and are essential in a number of physiological processes. They are responsible for the insertion of double bonds (normally in the *Z* or *cis* conformation) into alkyl chains, following the abstraction of two hydrogen atoms. Desaturases regulate the fluidity of membrane lipids and are also key enzymes in the biosynthesis of a variety of signaling molecules. The physical properties and nutritional value of many animal and plant storage lipids are determined by desaturases. Plant desaturases fall into two categories; soluble and membrane-bound. Membrane-bound desaturases are located in both ER and chloroplast membranes while soluble plant desaturases are localized in the chloroplast stroma. There is considerable sequence conservation among the membrane-bound desaturases (Shanklin, Whittle, and Fox, 1995). Each individual desaturase tends to be specific in the chain length and the double bond insertion position of its fatty acid substrate. All of the soluble desaturases use acyl-ACP substrates.

Stearoyl-ACP Desaturases

Soluble Δ_9 stearoyl-ACP desaturases (SACPD) are found in all plant cells and are essential for the biosynthesis of unsaturated membrane lipids. This enzyme introduces the first double bond into stearoyl-ACP (18:0-ACP) between carbons 9 and 10 to produce oleoyl-ACP ($18:1^{\Delta 9}$-ACP). SACPD is unique to the plant kingdom since all other known desaturases are integral membrane proteins (Ohlrogge and Browse, 1995). The first desaturase crystal structure published was a recombinant

form of the castor bean Δ_9 stearoyl-ACP desaturase (Lindqvist *et al*., 1996). This showed a homodimeric protein with two 37kDa subunits each having a diiron center which represents the active site of the desaturase. During the reaction, the reduced iron center binds oxygen and a high valent iron-oxygen complex likely abstracts hydrogen from the C-H bond (Whittle and Shanklin, 2001). SACPD occupies a key position in C18 fatty acid biosynthesis since perturbation of SACPD gene expression and/or enzyme activity may modulate the relative level of both stearic and oleic acid in soybean oil. Down-regulated SACPD expression or enzyme activity could produce oil with greater stearic acid, while increased expression of SACPD or enzyme activity could produce oil with greater oleic acid content.

Microsomal ω-6 Desaturases (FAD2)

Oil-producing crop plants such as soybean synthesize and store energy in the form of triacylglycerols (TAG), which are composed of a glycerol backbone molecule esterified by three saturated and/or unsaturated fatty acyl groups. In plants, the microsomal ω-6 desaturase-catalyzed pathway is the primary route of polyunsaturated lipid production. This membrane-bound enzyme catalyzes the first extra-plastidial desaturation in plants and converts oleic acid esterified to phosphatidylcholine to linoleic acid. FAD2 genes showing tissue-specific expression have also been identified in cotton (Liu *et al*., 1997), and sunflower (Martinez-Rivas *et al*., 2001). *Arabidopsis thaliana* has a single FAD2 gene (Okuley *et al*., 1994). Two different microsomal ω-6 desaturase genes, *FAD2-1* and *FAD2-2*, have been identified in soybean (Heppard *et al*., 1996). The FAD2-1 gene is strongly

expressed in developing seeds while the *FAD2-2* is constitutively expressed in both vegetative tissue and developing seeds. In a more recent study, Tang *et al*., (2005) identified two seed-specific isoforms of FAD2 designated FAD2-1A and FAD2-1B in the soybean genome. This seed-specific FAD2-1 gene is likely to play a major role in the conversion of oleic acid to linoleic acid within storage lipids during seed development.

Microsomal ω-3 Desaturases (FAD3)

Omega-3 fatty acid desaturases catalyze the insertion of a third double bond into linoleic acid precursors to produce linolenic acid. Seed linolenic acid levels have been shown to be determined by microsomal ω-3 desaturases (Yadav *et al*., 1993). *Arabidopsis thaliana* has one FAD3 gene (Yadav *et al*., 1993), while three independent microsomal ω-3 desaturase genes (Gm*FAD3A*, Gm*FAD3B* and Gm*FAD3C*) have been identified in soybean (Bilyeu *et al*., 2003). These three genes showed tissue-specific expression and the absence of FAD3A was linked to the low linolenic acid phenotype in the A5 soybean line. In another study (Vriten *et al*., 2005), identified two FAD3 desaturase genes (*FAD3A* and *FAD3B*) that control the level of linolenic acid in Flax. Analysis showed that in a low-linolenic-acid line, both genes contained point mutations leading to premature stop codons and consequently inactive truncated protein. These *FAD3* transcripts are seed specific with peak abundance approximately 20 DAF. In the low linolenic line, there was a dramatic reduction in the transcript levels of both genes.

Altering Soybean Fatty Acid Composition

Over 30 years ago, Howell *et al*. (1972) cited evidence that the enzymatic degradation or spontaneous oxidation of linoleic acid forms secondary products that give the oil a bad odor and flavor. Because linolenic acid results from the consecutive desaturation of oleic acid and linoleic acid, concentrations of oleic and linolenic acid are negatively correlated. So far, genetic approaches have been employed to reduce linoleic (18:2) and linolenic (18:3) acids while increasing oleic acid (18:1), to improve the stability of soybean oil. In addition, several natural gene mutations have been discovered that enable genetic flexibility in tailoring the fatty acid composition of soybean (Wilson, 2004). The process involves directed control of metabolic enzyme activity via gene manipulation as well as melding the functional combination of genes to produce novel fatty acid profiles.

A crude soybean oil having low- palmitic (16:0), mid to high- 18:1, and low- !8:3 concentration offers competitive market opportunities in numerous edible and industrial applications. Such oils would be widely used in salad/cooking oil, baking and frying fats. Soybean with a much higher 18:1 concentration could also be used in the manufacture of lubricants and hydraulic oil base stocks including soy diesel. In addition, a high 18:1 level should extend the utility of soybean oil in the manufacture of pharmaceuticals and cosmetic products including; bath oils, lipstick, make-up bases, aftershave lotions, detergents, shampoo and clear gels (USDA, 1996). Stearic acid concentration in soybean is genetically determined by alterations at the *Fas* locus. Induced mutations at this gene locus have resulted in

elevated 18:0 concentration. The FAM94-41 (9% 18:0) is the only known soybean germplasm that carries a natural mutation presumably at *Fas* (Pantalone, 2002).

Soybean Seed Development

Soybean seed ontogeny begins with a fertilized ovule and continues until seed maturity. The primary endosperm nucleus divides almost immediately following fertilization and these cycles of division continue for several days. By 8 days after fertilization, the heart-shaped embryo is completely embedded in cellular endosperm (Prakash and Chang, 1976). As the ovule continues to enlarge, both embryo and endosperm continue to grow at approximately the same rate until about day 14. The rapidly growing cotyledons rotate about 90^{o} and accumulate food reserves derived from the endosperm which is reduced to a thin aleurone layer and a few crushed cells by 20 days after fertilization (Pamplin, 1963).

The developing seed structure is attached to two large vascular bundles in the pod placenta. An embryonic sac separates the seed coat from the cotyledons of the developing embryo (Thorne, 1981). The total growth period for soybean may range from 108 to 144 days (Gay *et al*., 1980) and the duration of seed fill (seed growth prior to maturation) may range from 18 to 70 days (Reicosky *et al*., 1982). During this time, seed development progresses through at least five discernable stages shown in Table 1 below (Bils and Howell, 1963). The glycerolipid composition of soybean oil changes during seed development as does the concentration of individual fatty acids. In general, the concentration of stearic acid (18:0) remains

constant, the concentration of oleic (18:1) peaks between 30 and 45 days after flowering (DAF) then declines, linoleic acid (18:2) increases linearly throughout seed development, and linolenic acid (18:3) declines until about 60 DAF then increases slightly during the final stages of seed maturation (Simmons and Quackenbush, 1954; Hirayama and Hujii, 1965).

Table 1: Stages of soybean seed development

DAF	Activity
15	Cell division complete in cotyledons
R5 16-25	Increased metabolic activity, dry weight accumulation
R6 26-36	Proliferation of protein and lipid bodies
36-52	Protein and lipid accumulation, decline in starch content
53-60	Maximum dry weight achieved

Temperature and Fatty Acid Desaturases

The composition of saturated and unsaturated fatty acids of both membrane and storage lipids varies depending on environmental temperature (Heppard *et al*., 1996). There is a general inverse relationship between polyunsaturation of fatty acids and growth temperature; polyunsaturated fatty acids increase with decreasing temperature in membranes as well as seed storage lipids (Neidleman, 1987; Rennie and Tanner, 1989; Thompson, 1993). The overall reason for the increased enzyme activity in response to temperature change is probably related to the need to

maintain membrane fluidity, which is directly affected by the extent of unsaturation of the acyl lipid components of the bilayer (Murphy and Piffanelli, 1998). The mechanism of low-temperature adaptation in plants probably involves both transcriptional and/or post-translational regulation of the desaturases involved in polynsaturate formation. A direct effect of temperature on desaturase enzyme activity has been shown in developing soybean seeds (Cheesbrough, 1989). There are reports suggesting the existence of cold-inducible ω_3 and Δ_{12} desaturase genes in soybean (Kinney, 1994) and another identifying an isolated soybean Δ_{12} desaturase gene that is not regulated by low temperature (Heppard *et al*., 1996). There is a twenty-four amino acid difference between the FAD2-1A and FAD2-1B identified in soybean. Yeast expression studies revealed that the former is more unstable, particularly in cultures maintained at a higher growth temperature (Tang *et al*., 2005). Further, the results show that modulation of FAD2-1 enzyme activity may involve regulation at the post-translational level. Since many desaturases are encoded by multigene families, it is possible that some plant species may have both cold-inducible and non-cold-inducible forms of the same class of desaturase enzyme and/or gene (Murphy and Piffanelli, 1998). The leaves of transgenic tobacco plants with additional desaturase genes showed significant increase in chilling tolerance compared to their wild-type counterparts (Kodama *et al*., 1994, 1995; Ishizaki-Nishizawa *et al*., 1996).

Rationale and Objectives

The quality of soybean oil is of paramount importance both economically and from a nutritional stand point and depends to a large extent on the ratio of polyunsaturated to monounsaturated fatty acid. It is also a known fact that temperature plays a role in the final lipid content of oil seed plants. The hypotheses on which this research project is based are as follows:

- Desaturase gene expression partially governs seed fatty acid composition.
- Stearoyl ACP Desaturase (SACPD) genes may differ in enzyme activity.
- Temperature regulates desaturase gene expression and seed fatty acid composition.

Based on these, the following objectives were set out:

(i) Develop gene-specific primers for SACPD and the FAD genes involved in the C18 fatty acid biosynthetic pathway in soybean seed.

(ii) Use gene-specific primers to type soybean cultivars.

(iii) Determine the effect of temperature on the expression of different desaturase genes by quantifying transcript accumulation at various stages of seed development in five soybean cultivars.

(iv) Heterologously express SACPD genes to estimate enzyme activity.

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Two genes from soybean encoding soluble Δ9 stearoyl-ACP desaturases

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Abbreviations:	FAD	fatty acid desaturase
	RT-PCR	reverse transcription-polymerase chain reaction
	SACPD	stearoyl acyl carrier protein desaturase

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ABSTRACT

The Δ9 stearoyl acyl-carrier protein desaturase (*SACPD*) gene of soybean [*Glycine max* (L.) Merrill] encodes a soluble enzyme that converts stearic to oleic acid. Understanding the regulation of *SACPD* expression and enzyme activity are thus important steps towards developing soybean lines with altered stearic or oleic acid content. Using primers designed to a *G. max SACPD* cDNA sequence, a 3648 bp product was cloned and sequenced from the genome of cultivar Dare. Comparison of the third *SACPD* exon protein sequence with other available *Glycine SACPD* sequences revealed unique amino acid variability at positions 310 and 313. Sequence-specific primers were designed for real-time RT-PCR (reverse transcriptase-polymerase chain reaction) for this region of exon 3. Diagnostic and specific products were recovered with these primers using Dare cDNA template and Dare genomic DNA. Sequencing of a second genomic clone from Dare confirmed that there were two *SACPD* genes, designated *A* and *B*, in this cultivar. Survey of the genomes of 51 soybean lines and cultivars with PCR and the gene-specific primers indicated that all 51 had both *A* and *B*. Differences between *SACPD-A* and -*B* transcript abundance in soybean tissues while quantifiable were not dramatic. *SACPD-A* and -*B* transcript accumulation for three seed developmental stages between R5 and R6 was essentially equal. Biochemical analysis of the proteins encoded by these two *SACPD* genes may reveal whether the amino acid variability uncovered in this study has any relation to enzyme activity.

INTRODUCTION

Soybean oil is a mixture of five fatty acids (palmitic, stearic, oleic, linoleic, and linolenic) that differ greatly in melting points, oxidative stabilities, and chemical functionalities (Cahoon 2003). Although seed fatty acid composition is controlled by quantitative genetic traits, current research also includes a strong focus on the contribution and manipulation of fatty acid desaturase genes. The goal of efficiently altering fatty acid composition to meet the specific needs of industry therefore requires the efforts of soybean breeders and molecular geneticists.

Increasing the stearic acid content of soybean oil is desirable for certain food-processing applications since increased stearic acid content offers the potential for the production of solid fat products without hydrogenation (Spencer et al. 2003). This would reduce the content of trans-fat in food products and reduce a current health concern. High stearic acid oil could also be used as a replacement for tropical oils that are also high in palmitic acid which poses a heart disease risk.

Stearic acid concentration in soybean is genetically determined by alterations at the *Fas* locus. In addition to FAM94-41 (Pantalone 2002), a high stearic acid line carrying a natural mutation (*fasnc*), five other soybean germplasm lines have been reported to carry mutated stearic acid alleles that increase stearic acid: A6, fas^a (Hammond and Fehr 1983); FA41545, fas^b (Graef et al. 1985a); A81-606085, *fas* (Graef et al. 1985b); KK-2, st_1 (Rahman et al. 1997) and M25, st_2 (Rahman et al. 1997). Fas^a (30% stearic acid), fas^b (15% stearic acid), and *fas* (19% stearic acid) are allelic and represent different mutations in the same gene (Burton et al. 2004; Spencer, et al. 2003). One candidate gene, although unproven, for the *Fas* locus in

soybean, is the Δ9 stearoyl-acyl carrier protein (ACP) desaturase (Wilson 2004). The Δ9 stearoyl-ACP desaturase enzyme (SACPD), through the insertion of a double bond at C_9, converts stearic to oleic acid. Thus, *SACPD* occupies a key position in C18 fatty acid biosynthesis since perturbation of SACPD gene expression and/or enzyme activity may modulate the relative level of both stearic and oleic acid in soybean oil. Down-regulated *SACPD* expression or enzyme activity could produce oil with greater stearic acid, while increased expression of *SACPD* or enzyme activity could produce oil with greater oleic acid content.

In this study, we determined that there are two *SACPD* genes in soybean, a situation not previously recognized. The objective of this research was to characterize *SACPD* gene structure, determine the distribution of the *SACPD* genes in *Glycine*, and analyze *SACPD* expression levels in stages of seed development.

MATERIALS AND METHODS

Amplification, cloning, and sequencing

Genomic DNA was prepared form liquid nitrogen-frozen, powdered soybean leaves using the DNeasy Plant Mini kit from Qiagen (Valencia, CA) following manufacturer's protocol. Soybean leaves were harvested from five-week-old greenhouse grown plants. DNA samples were resuspended in sterile water. Soybean genomic *SACPD*s were amplified using oligonucleotide primers 5'GCGCCTTACATCACATAC 3'-forward and 5'GCTGCCCCTAACTGC 3'-reverse that were based on the GenBank mRNA sequence (accession number L34346) for a *Glycine max,* soluble stearoyl-acyl carrier protein desaturase (*SACPD*). In other experiments, *SACPD* exons 2 and exons 3 were amplified. Primers for exon 2 amplification were 5'CATCCTTACCCTATACCTG3'-forward and 5'CTACCATTGCGGAAGACC3'-reverse. Primers for exon 3 were 5'GATTCTTCATAGCTGCTGC3'-forward and 5'GCTGCCCCTAACTGC3'-reverse. Amplification reactions (25µl) contained 50 ng genomic DNA, 250 nM each primer, 2.5 µM each dNTP, 1 unit Taq DNA polymerase (Fisher, Atlanta, GA), and 1X PCR buffer (300 mM Tris-HCL, 75 mM $(NH_4)_2SO_4$, 7.5 mM $MgCl_2$, pH 8.5). Reaction conditions were 94^0C for 2 min (1 cycle), then 94^0C for 1 min, 55^0C for 2 min, 72^0C for 3 min (30 cycles), then 72^0C for 7 min.

Primers were also designed to a region of variability in exon 3 of *SACPD*. PCR with these primers produced products that discriminated between *SACPD* gene A (133 bp) and gene B (111 bp). These gene-specific primers were used to type soybean cultivars as well as to quantify steady state mRNA accumulation using real-

time RT-PCR. Gene-specific primers for *SACPD-A* (primer set A) were 5'CCTGTTTGATAACTACTCTGCC3'-forward and 5'TCTTCCCTCACCTGAAAGTCCG3'-reverse. Gene specific primers for *SACPD-B* (primer set B) were 5'CCTGTTTGATAGCTACTCTTCG3'-forward and 5'GTTAGCTGCTCCACCTCC3'-reverse. Primers for the soybean housekeeping gene actin were 5'GAGCTATGAATTGCCTGATGG3' forward and 5'CGTTTCATGAATTCCAGTAGC3' reverse derived from GenBank accession number U60500 (Moniz de Sa and Drouin 1996). Amplification of actin was done according to the protocol outlined above for *SACPD*. Amplification reactions (25µl) for PCR with *SACPD* gene-specific primers contained 50 ng of *Eco*RI restricted genomic DNA, 2 µM each primer, 250 µM dNTPs, 1 unit Taq DNA polymerase (Fisher, Atlanta, GA), and 1X PCR buffer (300 mM Tris-HCl, 75 mM $(NH_4)_2SO_4$, 7.5 mM $MgCl_2$, pH 8.5). Reaction conditions were 94^0C for 2 min (1 cycle), then 94^0C for 1 min, 66.8 ^{0}C for 2 min, 72^0C for 3 min (40 cycles), then 72^0C for 7 min.

PCR amplification reactions were performed in a MJ Research PTC-100 thermocycler (Watertown, MA). PCR products were analyzed on ethidium bromide stained, 1% agarose gels after electrophoresis with a 100 bp DNA ladder molecular wt. markers (Invitrogen, Carlsbad, CA). Amplicons were cloned into the sequencing vector pCR 2.1 using the TOPO TA cloning kit supplied by Invitrogen (Carlsbad, CA). Both strands of each PCR clone were sequenced at the Iowa State University Biotechnology Center, Ames, IA. Contigs were assembled using Vector NTI Advance 9.1 (Invitrogen). Assignment of open reading frames, translation to amino acid sequences, and sequence comparisons were done with the NCBI tools for data

mining (Gish and States, 1993; Altschul et al., 1990) and with Chromas (Technelysium.com.au). Sequences were aligned using Blossum62 Divide–and-Conquer Multiple Sequence Alignment v. 1.0 (BiBiServ, Bielefeld University Bioinformatics Server). Assignment of *SACPD* exons and introns was done using MacVector v. 7(IBI, New Haven, CT) and NetGene2 WWW Server (Center for Biological Sequence Analysis, The Technical University of Denmark).

Genomic DNA Blot Analysis

Genomic DNA (6 µg) was digested with restriction enzymes, electrophoresed through a 0.8% agarose gel, and transferred to Nytran Plus (Schleicher & Schuell, Keene, NH) membranes. The membranes were probed with [α-^{32}P] dCTP-labeled DNA fragments that correspond to an exon 3 region for either *SACPD-A* or *SACPD-B*, and exposed to x-ray film. Membrane hybridization and washing procedures were those described for Ultrahyb™ solution (Ambion, Austin, TX).

RNA Isolation and Processing

Glycine max Dare plants were grown under greenhouse conditions and plant tissues (pod, leaf, lateral root) harvested at 18 and 35 days after flowering (DAF). Dare plants were also grown under controlled day/night temperatures of 26/22^{0}C and seeds were harvested at four stages (18, 23, 28, and 35 DAF) of development between R5 and R6. Tissues and seeds were quickly frozen in liquid nitrogen and stored at -80^{0}C until RNA was extracted. Samples were pooled from three plants and RNA was isolated from 100 mg of frozen powdered tissue using the Qiagen

RNeasy Plant Mini kit (Valencia, CA) following the manufacturer's protocol. The RNA extraction was repeated on two other pooled samples. RNA samples were DNase treated with Ambion DNA-free (Austin, TX) according to the manufacturer's protocol. RNA concentrations were determined spectrophotometrically using absorbance at 260 nm. Samples were diluted to 50 ng/µl in sterile water and aliquots stored at -80^{0}C until use. To verify RNA integrity, 500 ng of total RNA of each sample was examined on a 1% agarose gel after electrophoresis stained with ethidium bromide.

Real-time reverse transcriptase PCR

Real-time reverse transcriptase PCR (Winer, J. W., et al. 1998; Bustin, S. A., 2002) was performed with the iCycler iQ™ (Bio-Rad, Hercules, CA) using the QuantiTech™ SYBR Green RT-PCR kit (Qiagen, Valencia, CA). Each reaction contained 12.5 µl of 2X SYBR Green PCR master mix, 250 nM forward and reverse gene-specific primers for *SACPD* gene A or gene B, 0.25 µl $MgCl_2$ (25 mM), 0.25 µl RT mix, 250 ng RNA, and nuclease-free water to 25 µl. The reactions were performed in a 96-well plates (0.2 ml tube volume) sealed with optical tape. Reverse transcription was carried out at 50^{0}C for 30 min followed by 95^{0}C for 15 min to inactivate the reverse transcriptases and to activate the HotStar Taq™ DNA polymerase. PCR amplification involved 45 cycles of 15 s at 94^{0}C, 30 s at 60^{0}C and 30 s at 72^{0}C followed by melt curve analysis over a 10^{0}C temperature gradient at 0.05^{0}Cs^{-1} from 78 to 88^{0}C. Duplicate reactions were done for each sample. Steady state transcript levels for SACPD genes were mathematically determined by

comparison of individual cycle threshold (Ct) values with a standard curve generated from serial dilutions of a PCR standard (Peirson, S. N., et al. 2003) from soybean genomic DNA. PCR efficiency ranged from 93 to 98% and negative control reactions did not produce any products. Targets (copy number), initially determined per microgram of total RNA isolated, were normalized as a percent of soybean *actin* gene expression level.

Determination of seed fatty acid composition

Fatty acid methyl esters (FAME) of mature soybean seed samples were prepared using acid methanolysis. Seed tissue, ground to a powder, was heated at 85°C for 90 min. in a 5% HCl-95% methanol solution. FAME was partitioned 2x into hexane and transferred to 2ml vials for analysis. The FAMEs were separated by gas chromatography using an HP 6890 GC (Agilent Technologies, Inc., Wilmington, DE) equipped with a DB-23 30 m x 0.53 mm column (same source). Operating conditions were 1 µl injection volume, a 20:1 split ratio, and He carrier gas flow of 6 ml min^{-1}. Temperatures were 250^0C, 200^0C, and 275^0C for the injector, oven and FID, respectively. Chromatograms were analyzed using HP ChemStation software.

RESULTS

Structural organization of the soybean soluble Δ9 stearoyl-ACP desaturase

Two 3648bp genomic fragments were identified from soybean cultivar Dare in a shotgun cloning experiment using PCR primers designed to terminal sequences of a soluble, Δ9 stearoyl-ACP desaturase cDNA (accession no.L34346) from soybean. Sequencing and analysis of these fragments revealed the *Glycine SACPD* gene structure (Fig.1A) to be the following: a 111 bp exon 1 sequence encoding a putative 37 amino acid transit sequence; a 1763 bp intron 1; a 504 bp exon 2 sequence encoding 168 amino acids; a 423 bp intron 2; and a 618 bp exon 3 sequence encoding 206 amino acids. The nucleotide and amino acid sequence data of the Dare *SACPDs* can be found at the GenBank database as accession numbers AY885234 (*SACPD-A*) and AY885233 (*SACPD-B*).

Translation of the 1233 bp transcript predicts a protein of 411 amino acids with a molecular mass of 47.2 kDa and a pI of 6.02. The amino acid sequence of the Dare *SACPDs* was scanned with the program TMpred (Hoffman and Stoffel 1993) and found not to contain transmembrane spanning sequences. Alignments of *Glycine max SACPD* deduced amino acid sequences (Fig. 1B) with *SACPDs* from *Arabidopsis* and *Ricinus* (Castor bean) indicates a high degree of sequence conservation. The Dare *SACPDs* have 67 and 71 percent sequence identity with *A. thaliana* (AAB64035) and *R. communis* (X56508) *SACPDs*, respectively. *G. max SACPD-A* and *-B* have 83 percent sequence identity with the database *G. max* sequence L34346. *SACPD-A* and *SACPD-B* are 98 percent identical in amino acid sequence. Amino acid variations were found in exon 3, but not in exon 1 and 2 of

the two Dare *SACPDs.* At position 210, *SACPD-A* has alanine; *SACPD-B* has threonine. At position 258, *SACPD-A* has glutamic acid; *SACPD-B* has glycine. At position 306, *SACPD-A* has asparagine; *SACPD-B* has aspartic acid. At position 310, *SACPD-A* has asparagine; *SACPD-B* has serine. At position 313, *SACPD-A* has alanine; *SACPD-B* has serine. At position 356, *SACPD-A* has valine; *SACPD-B* has isoleucine. At position 374, SACPD-A has glycine; SACPD-B has valine. These findings suggest that there are at least two *SACPD* genes, Gm*SACPD-A* and Gm*SACPD-B* in cultivar Dare. Interestingly, the primary sequence of *G. max* accession L34346 fits neither the amino acid substitution pattern *SACPD-A* nor *-B* exactly.

Alignment of other soybean SACPD exon 3 sequences

To assess the significance of the amino acid variability found in the *SACPD* exon 3s of Dare, other *Gm SACPD* exon 3s were PCR cloned and sequenced. We sequenced an exon 3 from *Gm* cultivar Bragg and an exon 3 from N01-3544, a mid-oleic acid *Gm* line. To these sequences were added 5 soybean cDNA exon 3-derived sequences from the GenBank database. Nucleic acid alignments for bp positions 3183 to 3211 in exon 3, depicted in Fig. 2A, show that the nine 9 *SACPD* exon 3 sequences can be differentiated into two groups: Dare-*A* , Bragg, N01-3544, AW 755581, and BG 882246 (Williams) designated group A, and Dare-*B*, AI 941223 (Williams), BI 471396 (also Bragg), and BG 363272 designated group B. Amino acid alignments of the corresponding region of the exon 3s (position 307 to 321) revealed that variation in amino acids at position 310 and 313 permit exactly the

same group A and B differentiation. Moreover, both *SACPD-A* and -*B* sequences were represented for cultivars Bragg (our sequence and BI 471396) and Williams (BG 882246 and AI 941223). Based on these results we hypothesized that soybean possesses at least two *SACPD* genes.

Detection of *SACPD-A* and *-B* in *Glycine*

PCR primers specific for *SACPD-A* and -*B* were designed based on the nucleic acid sequences encompassing the region of variability in exon 3. These primer sets were also designed to be used for real-time reverse transcriptase PCR to assess *A* and *B* transcript accumulation. The gel in Fig. 3 shows that amplification of Dare genomic DNA with primer set A produces a PCR product of 133 bp diagnostic for *SACPD-A*, and amplification of Dare genomic DNA with primer set B produces a product of 111 bp diagnostic for *SACPD-B*. Two distinct bands were observed when Dare genomic DNA, digested with either *BamH*I or *Eco*RI (Fig. 4), was hybridized with *SACPD-A* and *SACPD-B* exon-specific probes. These results suggest that there are at least two copies of each gene in the Dare soybean genome. Soybean is considered to be a stable allo-tetraploid with diploidized genomes [2n = 4x = 40] (Singh and Hymowitz 1988). As an allo-tetraploid, modern soybean was produced by the hybridization of two species resulting in the union of two separate chromosome sets and their subsequent doubling giving the appearance of a normal diploid. Thus, duplication (two copies) of each *SACPD* structural gene was not unexpected. Since our data suggested that the genome of *Glycine* possesses both *SACPD-A* and -*B,* a survey of the genomes of 51 *Glycine*

lines and cultivars was undertaken to determine gene *A* and *B* distribution. *Glycine* lines and cultivars in the survey (Table 1) represent different maturity groups, different stearate and oleate seed fatty acid compositions, include *G. soja* ancestral lines, and include the main contributors to the gene pool of modern *G. max* through 1988 (Gizlice et al. 1994). Table 1 shows that both *SACPD* gene *A* and *B* were present in all 51 *Glycine* lines and cultivars.

Relative expression of Gm*SACPD-A* and Gm*SACPD-B* in stages of seed development and tissues

For seed stage expression experiments, seeds and roots were harvested from plants grown under near optimal moisture, light and temperature conditions provided by a growth chamber in the Southeastern Environmental Research Center at N.C. State University, Raleigh. We measured steady-state transcript levels for *SACPD-A* and –*B*, a condition that reflects the maintenance of a constant transcript level, to determine whether the expression of one gene might differ from the other. The actin normalized pattern of *SACPD-A* and -*B* transcript accumulation at three seed developmental stages of cultivar Dare at 18, 28, and 35 DAF was essentially parallel and transcript accumulation equal (Fig. 5). At stage 2, 23 DAF however, *SACPD-A* transcript accumulation was approximately 30% greater than -*B*. By way of comparison, both *SACPD-A* and -*B* stage- specific accumulation were equal but of very low abundance (5-6%) in root tissue. Differences between *SACPD-A* and -*B* transcript accumulation in greenhouse-grown, non seed tissues while quantifiable, were not dramatic (data not shown). *SACPD-B* was higher (78% and 75%) in

abundance than *-A* (70% and 68%) in pod tissue at 18 and 35 DAF, respectively. *SACPD-A* and *-B* transcripts were similar in abundance (72%) in both young and mature leaves.

DISCUSSION

The soluble Δ9 stearoyl-ACP desaturase of soybean, like all the soluble desaturases using acyl-ACP substrates, is localized to the stroma fraction of plastids in developing seeds (Murphy and Piffanelli 1998). A short N-terminal transit peptide of 37 amino acids was identified for the soybean SACPDs that is presumably responsible for stroma targeting (Fig. 1A). Most of our knowledge about plant Δ9 stearoyl-ACP desaturases (SACPDs) comes from the study of the soluble enzyme from castor seed (Lindqvist et al. 1996). Structure analysis of the crystallized protein shows it to be a μ-oxo-bridged di-iron enzyme that belongs to the structural class I of large helix bundle proteins that catalyzes the NADPH and O_2-dependent insertion of a cis-double bond between C-9 and C-10 positions in stearoyl-ACP (Moche et al. 2003). The enzyme is a homodimer with each mature subunit of 41.6 kDa containing an independent binuclear iron cluster. At the core of the desaturase structure, two iron atoms are coordinated within a central four helix bundle in which the motif (D/E)-E-X-R-H is present in two of the four helices (Ohlrogge and Browse, 1995). During the desaturation reaction, the two-electron reduced, di-iron center binds oxygen and the high valent iron-oxygen complex formed abstracts hydrogen from the substrate C-H bond (Whittle and Shanklin 2001).

Inspection of *G. max* SACPD exon 3 protein sequences derived from cDNA data suggested that soybean contains two *SACPD* genes (Fig. 2A and B). Based on the amino acid variable region of exon 3, what we now call *SACPD-A* and *-B* were found in cultivar Williams. Further sequence analysis of exon 3 clones from cultivars Dare and Bragg indicated that they also contained *SACPD-A* and *-B*. Sequence analysis of Dare genomic clones confirmed that there are two different, soluble *SACPD* genes in this cultivar. Amino acid variability was found only in the Dare protein sequence of exon 3 and not in the transit peptide region (exon 1) or in exon 2. Previously, multiple plant genes were identified only for microsomal desaturases. For example, three different microsomal Δ12 oleate desaturase genes (*FAD2*s) were reported for sunflower (Martinez-Rivas et al. 2001), two microsomal *FAD2*s were reported each for soybean (Heppard et al., 1996) and for cotton (Liu et al., 1997), while three different microsomal ω-3 desaturase genes (*FAD3*s) were reported for soybean (Bilyeu et al., 2003).

Rather dramatic differences in microsomal desaturase gene expression levels and gene distribution have been found in soybean. Of the two soybean *FAD2* genes, *FAD2-1* was expressed specifically in seeds, and *FAD2-2* was expressed in all tissues (Heppard et al., 1996). Only one of the three soybean *FAD3* genes, *FAD3A*, was predominatly expressed in developing seeds (Bilyeu et al., 2003). In the same study, Bilyeu et al. found that the low linolenic acid breeding line A5 contained two of the *FAD3* genes, but lacked a third gene, *FAD3A*. In our study we found that the differences between the transcript abundance of the soluble *SACPD-A* and *-B* in soybean tissues while quantifiable, were not dramatic (Figs. 4 and 5).

However, both soybean *SACPD*s were much more highly expressed in seeds than in roots.

We thought that *Glycine* lines/cultivars might be identified that lacked one of the SACPD genes, however, both *A* and *B* genes were found in the genomes of all 51 *Glycine* lines/cultivars examined (Table 1). Group I consisted of eight *G. max* cultivars and lines of varying maturity, including the high stearic acid mutant A6 and the mid-oleic acid line NO1-3544, group II consisted of eight maturity group V *G. soja* lines varying in oleic acid content, and group III consisted of the 35 soybean cultivars of Gizlice, et al. (1994). Group III genotypes were chosen because they define the genetic base of North American soybean cultivars and represent 95% of the genes found in modern cultivars. Although GenBank accession L34346 may be indicative of a third soybean SACPD gene, efforts to find this sequence in cultivar Dare were unsuccessful. Since the primary sequence L34346 fits neither the amino acid substitution pattern *SACPD-A* nor *-B* exactly, L34346 may represent an allelic form of one of the two SACPD genes.

The manipulation of fatty acid desaturases to achieve a desired fatty acid composition in soybean oil has a strong rationale. Support for this rationale comes from research that has shown that down-regulation of the Δ-12 fatty acid desaturase gene *FAD2-1* elevates oleic acid content in the oil. Transgenic seeds with oleic acid content of approximately 75-80% of the total oil have been recovered after this gene was silenced in somatic embryos (Kinney, 1997) or the gene transcript was ribozyme-terminated (Buhr et al. 2002). As mentioned previously, the low linolenic acid breeding line A5 was found to lack the *FAD3A* gene at the *Fan* locus in

soybean (Bilyeu et al. 2003). In addition, FAM94-41 (Spencer et al. 2003), a high stearic acid line was found to carry a natural mutation, *fasnc*, at the *Fas* locus. Based on our findings, we will continue to characterize the enzymatic activity of the two soybean Δ9 soluble desaturases from both the wild type Dare cultivar and the high stearic acid mutant line A6. Results from these experiments may provide a means to achieve the stable production of high stearic acid soybean oil.

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Table 1. Detection of SACPD-A and –B genes in *Glycine* cultivars and lines that were typed by PCR with gene-specific primer pairs.

Cultivar/line	Maturity Group	% Stearate	% Oleate	*SACPD-A*	*SACPD-B*
Glycine max[a]					
Dare	V	3.1	20.5	+	+
Bragg	VI	4.1	18.6	+	+
Williams	III	3.2	22.7	+	+
A6	0	21.7	21.9	+	+
NO1-3544	IV	3.4	52.4	+	+
NC83-375	V	4.0	21.0	+	+
NC93-2007	VI	3.6	30.6	+	+
NC99-3170	VI	3.9	23.6	+	+
Glycine soja[b]	All V				
PI 407020		5.6	14.2	+	+
PI 424070B		3.7	28.5	+	+
PI 424073		3.7	11.7	+	+
PI 424083B		3.5	13.3	+	+
PI 424096		3.7	9.9	+	+
PI 424102B		4.1	19.6	+	+
PI 597461C		2.9	12.7	+	+
PI 562557		3.4	13.7	+	+
35 *G. max* cultivars of Gizlice et al. 1994[c]		ND[d]	ND	All+	All+

[a]*G. max* cultivars and lines from J.W. Burton, USDA-ARS, Raleigh, NC.

[b]*G. soja* lines from E. Peregrine, University of Illinois, Urbana-Champaign, IL.

[c]The 35 *G. max* cultivars of Gizlice et al.(1994) from T.A. Carter, USDA-ARS, Raleigh, NC.

[d]ND, not determined.

Legends

Figure 1. The soluble Δ9 stearoyl-ACP desaturase (*SACPD*) gene from soybean. **A**. Gene structural organization showing exon 1, encoding a transit peptide, exons 2 and 3, and introns 1 and 2. Exon 3 contains the region of amino acid sequence variability in *G. max* SACPDs. **B.** Alignment of *Glycine max* cultivar Dare SACPD-A and SACPD-B protein sequences with SACPD protein sequences from *Arabidopsis* Ab (AAB64035), *Ricinus communis* (Castor bean) Cb (X56508), and a *Glycine max* cDNA, Gm (L34346).

Figure 2. SACPD exon 3 sequence alignments. **A**. Nucleotide (Blastn) and **B.** Protein (Blastx) sequences of a section of SACPD exon 3 from 9 *Glycine* lines showing nucleotide and amino acid sequence variation. Sequences of Bragg-A, N01-3544, and Dare A and B were determined in this study. AW 755581, a cDNA of cultivar Jack, BG 882246 and AI 941223, cDNAs of cultivar Williams, BI 471396, a cDNA of cultivar Bragg-B, and BG 363272, a cDNA of cultivar Corolla are GenBank accessions.

Figure 3. Ethidium bromide stained gel showing amplicons produced by PCR using *Eco*R1 restricted Dare genomic DNA template with *SACPD* gene-specific primers (see Materials and Methods). Lane 1, 100bp DNA ladder; lane 2, 133 bp product produced with the *SACPD-A* primer pair; lane 3, 111 bp product produced with the *SACPD-B* primer pair.

Figure 4. Southern blots of Dare soybean cultivar genomic DNA hybridized with *SACPD-A* and *SACPD-B* exon 3 specific probes. Lane (1), genomic DNA digested with *BamH*I and lane (2), with *Eco*RI. Molecular size (kb) is indicated on the left.

Figure 5. Relative expression of cultivar Dare Gm*SACPD-A* and Gm*SACPD-B* at four stages of soybean seed development (between R5 and R6) at 18, 23, 28, and 35 DAF. Steady state mRNA levels were determined by real-time PCR after reverse transcription of total RNA. The values represent the percent of each gene normalized to the housekeeping gene *actin*. Bars indicate the standard error of the mean.

Figure 1A. Gene structural organization of the soybean SACPD gene

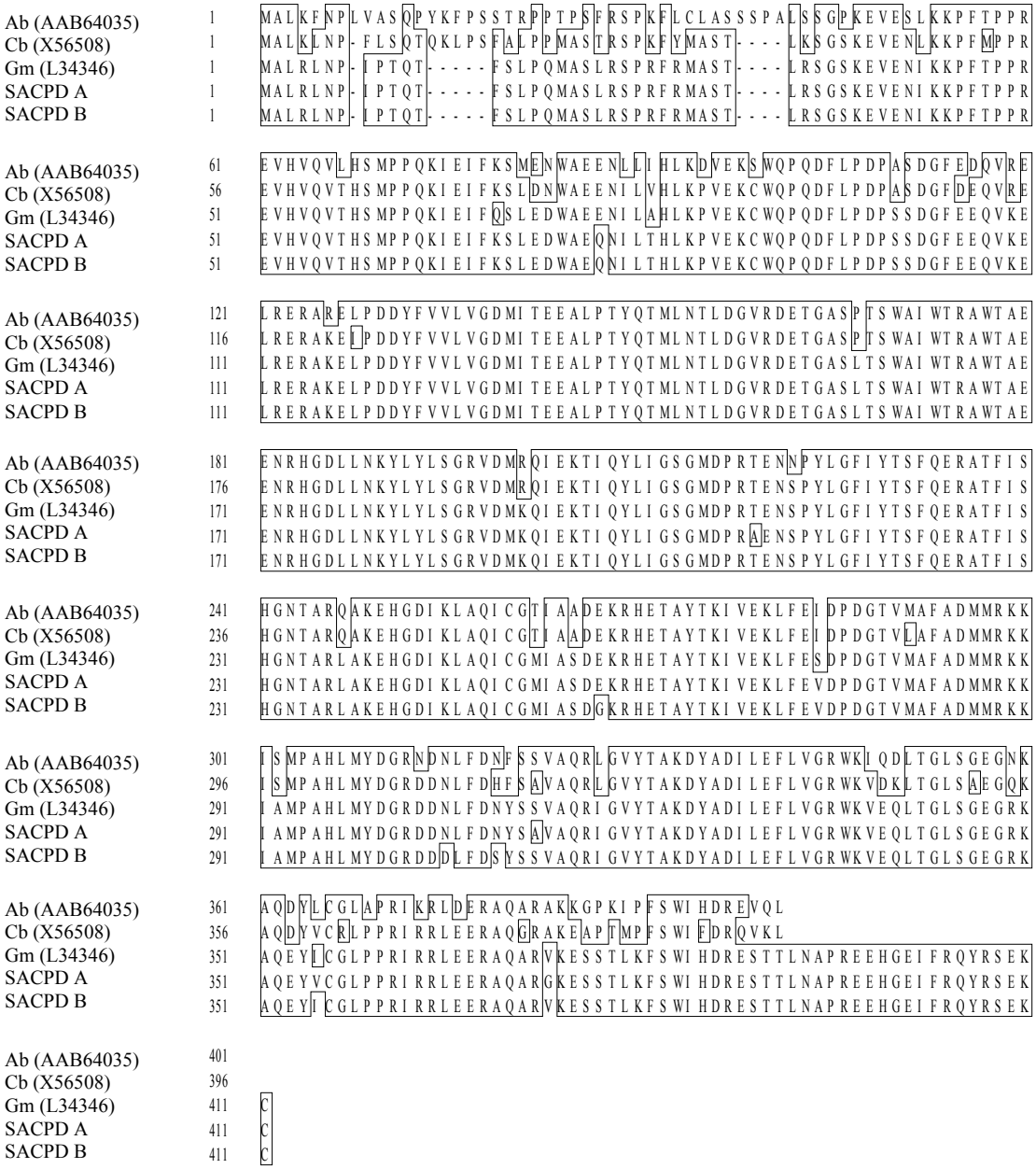

Figure 1B. Alignment of *Glycine max* cultivar Dare SACPD-A and SACPD-B protein sequences

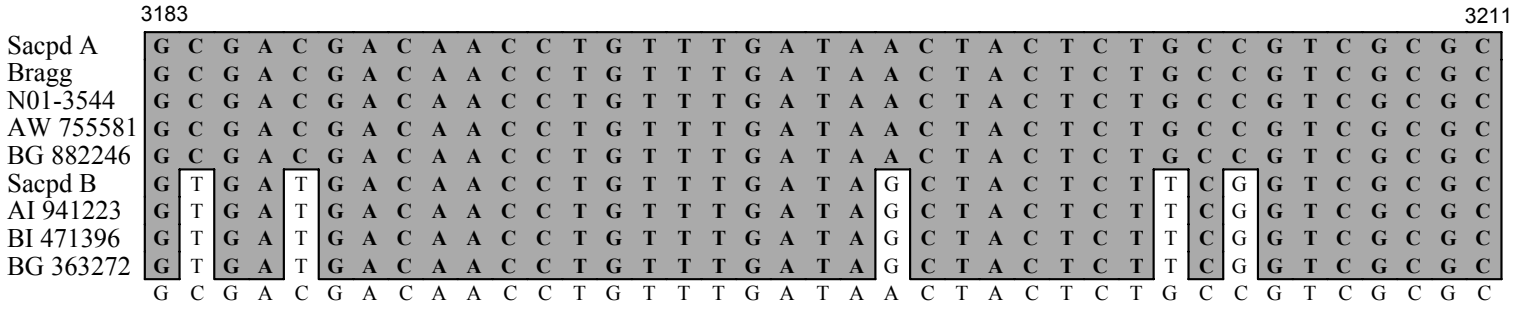

Figure 2A. SACPD exon 3 nucleotide (Blastn) sequence alignment

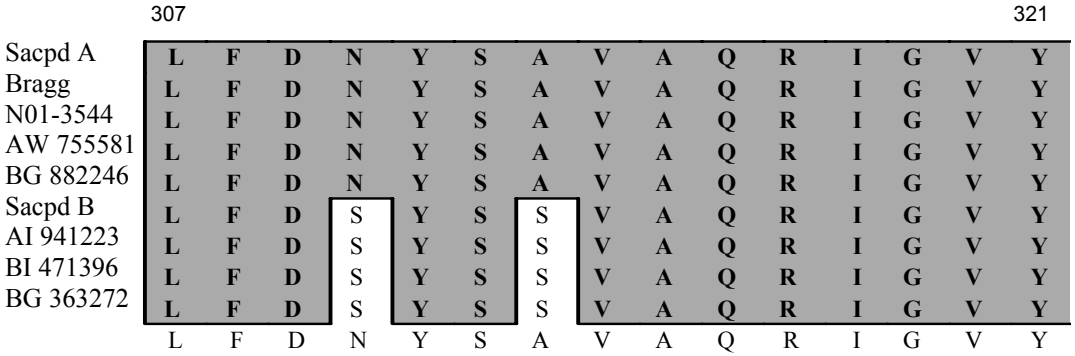

Figure 2B. SACPD exon 3 protein (Blastx) sequence alignment

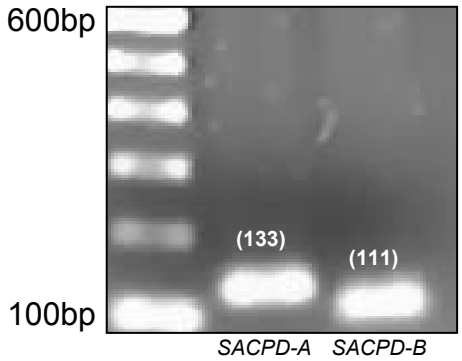

Figure 3. Ethidium bromide stained gel showing amplicons produced by PCR

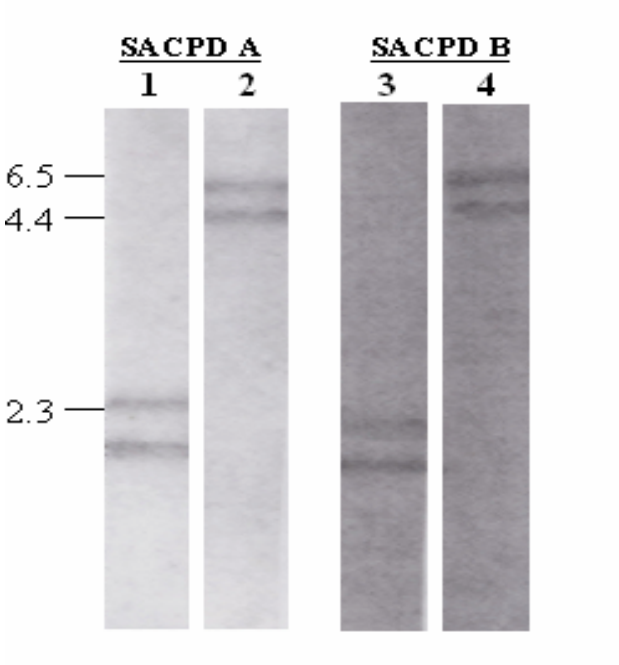

Figure 4. Southern blots of Dare soybean cultivar genomic DNA

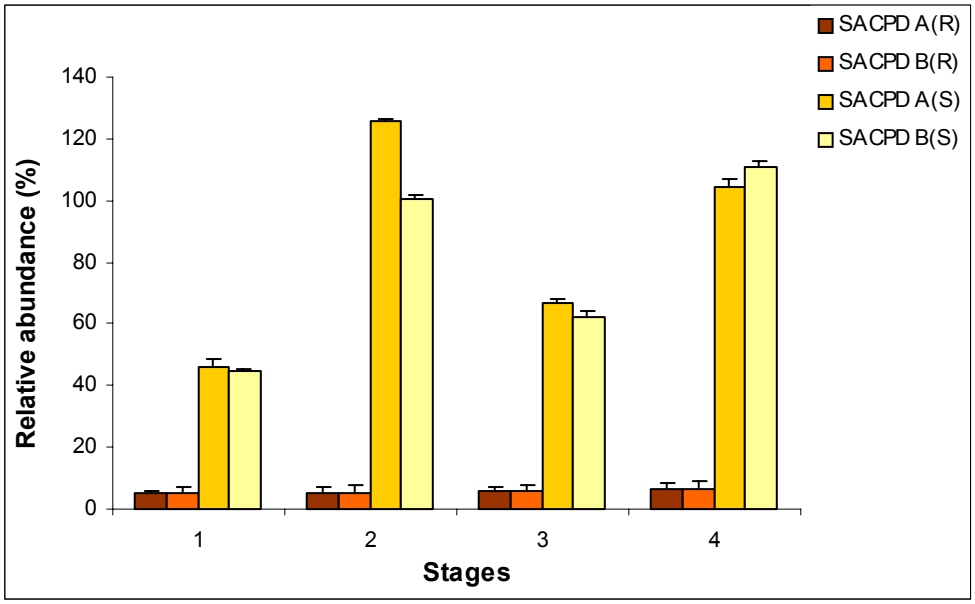

Figure 5. Relative expression of cultivar Dare Gm*SACPD-A* and Gm*SACPD-B* at four stages of soybean seed development (between R5 and R6) at 18, 23, 28, and 35 DAF.

Temperature modulates the expression of ∆9 stearoyl-ACP desaturase genes and the oleic/stearic acid content in soybean seeds

Running title: Temperature and stearoyl-ACP desaturase expression

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Abbreviations:	FAD	fatty acid desaturase
	SACPD	stearoyl acyl carrier protein desaturase
	FAME	fatty acid methyl ester
	RT-PCR	reverse transcription-polymerase chain reaction

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ABSTRACT

The Δ9 stearoyl-ACP desaturases (SACPDs) are important enzymes for the acclimation of organisms, including soybean, to low temperatures. Since these enzymes carry out important housekeeping functions such as lipid membrane biosynthesis they are under constitutive control, but may also be supplemented by fine regulation at both the transcriptional and post-transcriptional levels. We measured the transcript accumulation of *SACPD-A* and *-B* in developing soybean seeds across cold (22/18^{o}C) normal (26/22^{o}C), and warm (30/26^{o}C) temperatures by real-time RT-PCR. Transcript accumulation of *SACPD-A* and *-B* decreased by up to 69% with increasing temperature in cultivars Dare, A6 (a high stearate line), and N01-3544 (a mid-oleic line). Stearic acid content of A6 was inversely related to the level of *SACPD-A* and *-B* expression at the warm and cold temperatures, as might be expected, but the stearic acid content of N01-3544 and Dare did not appear to be affected by temperature despite dynamic changes in the expression levels of *SACPD* in these lines. This suggests that in these two lines stearate does not accumulate with higher temperature because it may be metabolically reclaimed as it is synthesized. Similarly, but unexpectedly, the oleic acid content of all three lines was inversely related to the levels of *SACPD* expression at the warm and cold temperatures. This suggests that transcription control of *SACPD* may not be a crucial factor for regulating oleic acid content in soybean.

INTRODUCTION

Soybean [*Glycine max* (L.) Merr.] is the largest oilseed crop produced and consumed worldwide, accounting for 56 % of the World Oilseed Production in 2003 (Soy Stats 2004). Oilseeds provide high quality protein for livestock and poultry feed, and the oil is used extensively in the cooking and food manufacturing industries around the world. Soybean producers have targeted improved oil traits as a priority area of research to enhance the market share of U.S. soybean. Soybean seed is approximately 18% oil, and standard commodity soybean oil a mixture of five fatty acids; palmitic (11%), stearic (4%), oleic (22%), linoleic (53%), and linolenic (8%). These fatty acids differ greatly in melting points, oxidative stabilities, and chemical functionalities (Cahoon 2003). Oils high in oleic acid possess increased oxidative stability which negates the need for hydrogenation and eliminates the production of trans-fats that are a major health concern.

Soluble Δ9 stearoyl-ACP desaturases (SACPDs) are found in all plant cells and are essential for the biosynthesis of unsaturated membrane lipids. This enzyme introduces the first double bond into stearoyl-ACP (18:0-ACP) between carbons 9 and 10 to produce oleoyl-ACP (18:1$^{\Delta 9}$-ACP). SACPD is unique to the plant kingdom since all other known desaturases are integral membrane proteins (Ohlrogge and Browse 1995). In addition, SACPD occupies a key position in C18 fatty acid biosynthesis since perturbation of SACPD gene expression and/or enzyme activity may modulate the relative levels of both stearic and oleic acid in soybean oil. Down-regulated SACPD expression or enzyme activity could produce oil with greater

stearic acid, while increased expression of SACPD or enzyme activity could produce oil with greater oleic acid content.

The composition of saturated and unsaturated fatty acids of both membrane and storage lipids varies depending on environmental temperature (Heppard *et al*. 1996). There is a general inverse relationship between unsaturation of fatty acids and growth temperature; unsaturated fatty acids increase with decreasing temperature in membranes as well as seed storage lipids (Neidleman, 1987; Rennie and Tanner 1989; Thompson 1993). A direct effect of temperature on desaturase enzyme activity has been shown in developing soybean seeds (Cheesbrough 1989). He found that SACPD enzyme activity increased with decreasing temperature. There are also reports suggesting the existence of cold-inducible ω_3 and Δ_{12} desaturase genes in soybean (Kinney 1994) and another identifying an isolated soybean Δ_{12} fatty acid desaturase (FAD) gene that is not regulated by low temperature (Heppard *et al*. 1996). The leaves of transgenic tobacco plants with additional desaturase genes showed significant increase in chilling tolerance compared to their wild-type counterparts (Kodama *et al*. 1994, 1995; Ishizaki-Nishizawa *et al*. 1996).

In this study, we determined the effect of temperature on the expression of the two previously discovered Δ9 stearoyl-ACP desaturase genes (Byfield and Upchurch 2005) in soybean seeds at four stages (R5-R6) of development. For this purpose, total RNA was extracted from seeds produced under three controlled air temperature regimes. Transcript accumulation of *SACPD-A* and *-B* was quantified using gene-specific desaturase primers and real-time RT-PCR. We then sought to determine

what relation, if any, exists between the observed modulation of *SACPD* transcript accumulation by temperature and the resultant stearic/oleic acid content of the seed.

MATERIALS AND METHODS

Cultivars and Growth Conditions

Glycine max lines were chosen based on their extreme fatty acid composition at maturity. A6, a maturity group 0 mutant line (Hammond and Fehr 1983), was chosen for its high (22%) stearic acid, and N01-3544 (Burton *et al*. 2005), a maturity group IV, for its high (52%) oleic acid content. Cultivar Dare, a maturity group V with 3% stearic and 21% oleic acid, was selected as the unimproved variety for the purpose of comparison. Plants were grown under controlled air temperature environments at the Southeastern Plant Environment Laboratory (Phytotron) at North Carolina State University, Raleigh, NC. Pre-germinated seeds were planted in 10" pots containing standard mix (1/3 peat-lite and 2/3 gravel) and five plants of each variety were grown in a random arrangement in each of three chambers. Chambers were initially maintained at 26/22^{o}C day/night (D/N) temperature with 14 hours of incandescent light and 10 hours of darkness with a one hour light interruption period. Plants were watered twice daily with deionized water and nutrient solution was supplied thrice per week. On day 15, the light interruption was stopped and chambers were reset to D/N=12/12 hours to induce flowering. On day 36, temperatures were changed in two chambers; one to 22/18^{o}C (cold) D/N=12/12 and the other to 30/26^{o}C (warm) D/N=12/12. These temperatures were maintained

to the end of the experiment. Seeds were harvested at four predetermined stages between R5 (beginning of seed set) and R6 (mature bean) coinciding with 18, 23, 28 and 35 days after flowering (DAF), respectively. Pods were harvested from at least three plants of each variety in each chamber, beans were then quickly removed, flash frozen in liquid nitrogen, and stored at -80^{o}C.

RNA Isolation and Real-time RT-PCR

Seed stage samples were pooled and total RNA was isolated from 100 mg of frozen powdered tissue using the Qiagen RNeasy Plant Mini kit (Valencia, CA) following the manufacturer's protocol. RNA extraction was repeated on two additional pooled samples and all samples were DNase treated with Ambion DNA-free (Houston, TX) according to the manufacturer's protocol. RNA concentrations were determined using a DU-640 spectrophotometer (Beckman Coulter, Fullerton, CA) set at absorbance 260 nm. Aliquots were diluted to 50ng/µl in RNase-free water and stored at -80^{0}C until use. To verify RNA integrity, 500ng of total RNA of each sample was visualized on a 1% agarose gel following electrophoresis and staining with ethidium bromide.

Real-time reverse transcriptase PCR was carried out according to the protocol outlined by Byfield *et al*. (2005) using the same *SACPD-A* and *SACPD-B* gene-specific primers. Negative control reactions did not produce products. Transcript copy numbers were initially determined per µg of total RNA isolated. Copy number was then normalized to the house keeping gene, soybean actin (Moniz de Sa and Drouin 1996).

Analysis of Fatty Acid Content

Fatty acid methyl esters (FAMEs) of mature soybean seed samples were prepared using acid methanolysis. Tissue samples used for RNA extraction were also the source material for fatty acid analysis. Powdered seed tissue was heated to and held at 85°C for 90 min. in a 5% HCl-95% methanol solution. FAME was partitioned 2x into hexane and transferred to 2ml vials for analysis. The FAMEs were separated by gas chromatography using an HP 6890 GC (Agilent Technologies, Inc., Wilmington, DE) equipped with a DB-23 30 m x 0.53 mm column (same source). Operating conditions were 1 µl injection volume, a 20:1 split ratio, and He carrier gas flow of 6 ml min^{-1}. Temperatures were 250^0C, 200^0C, and 275^0C for the injector, oven and FID, respectively. Chromatograms were analyzed using HP ChemStation software.

RESULTS

SACPD-A and *-B* gene expression across temperatures

Transcript accumulation of *SACPD-A* and *-B* and the 18:1/18:0 ratio was determined for stages 1-4 of seed development across cold (22/18^{o}C), normal (26/22^{o}C), and warm (30/26^{o}C) temperatures. Table 1A, B, and C show stage 1-3 data for varieties A6, the high stearate line, cultivar Dare, and N01-3544, the mid-oleic line, respectively. *SACPD-A* and *-B* transcript accumulation increased from stage 1 to 3 in each of these varieties, with highest accumulation at the cold and lowest accumulation at the warm temperature. Generally, the transcript accumulation of both *SACPD-A* was similar to that of *SACPD-B* over developmental stages. Figure 1 shows data for stage 4 (35 DAF) of seed development. At this stage, transcript accumulation of *SACPD-A* and *-B* decreased by approximately 69% with increasing temperature from 22/18^{o}C to 30/26^{o}C in seeds of Dare. The negative correlation between *SACPD* transcript accumulation and increasing temperature was true for the other two lines investigated. Transcript accumulation of both *SACPD-A* and *-B* was also similar in pattern across temperatures with respect to variety but included some differences that were significant. For example, at the normal temperature *SACPD-A* was highest and *SACPD-B* lowest, in A6. The highest level of expression for *SACPD-A* and *-B* was found in Dare at the cold temperature, while at the warm temperature A6, the high stearic line, showed highest expression of *SACPD-A* and *-B*. Overall, the mid-oleic line N01-3544 exhibited the lowest levels of *SACPD* expression across temperatures.

Temperature effect on stearic (18:0) and oleic (18:1) acid content

Fatty acid methyl esters (FAMEs) were determined for all four stages of seed development with tissue from the same pooled samples used for *SACPD* gene expression analysis (Table 1A, B, and C; Figure 1). From stage 1-3, the 18:1/18:0 ratio generally increased with increasing *SACPD* transcript accumulation for varieties Dare and N01-3544, but an inverse relationship was observed with A6 transcript accumulation and the 18:1/18:0 ratio. Figure 2 shows the levels of stearate (18:0) and oleate (18:1) for stage 4 seeds at the three temperatures under investigation. Not surprisingly, A6 had the highest level of stearate overall while N01-3544 had the highest level of oleate. The greatest effect of temperature on stearic acid content was observed in the A6 line where it increased greater than two-fold in response to the warmer temperatures. Dare and N01-3544 each maintained a nearly constant and, by comparison, low amount of stearic acid in stage 4 across all temperatures.

Oleic acid content showed a positive correlation to increasing temperature in stage 4 seeds of all three varieties (Figure 2). The greatest increase in 18:1 concentration from cold to warm temperature was seen in Dare, followed by A6, then N01-3544. Greater variation in the response of stearate to temperature (cold to warm) was detected in stages 1-3 (Table 1). For Dare and N0I-3544, 18:0 increased in stage 2 seeds, but at stage 1 and 3 these lines exhibited either no change or a reduction in stearate content. Oleate content increased with increasing temperature in all lines in stages 1-4. N01-3544 showed a 10.3% increase in 18:1 from cold to

warm temperature (stage 1) while Dare showed only a 0.5% increase in 18:1 at stage 2.

DISCUSSION

Desaturases are known to be important enzymes in the acclimation of organisms to low temperatures. The regulation of fatty acid desaturases may vary depending on tissue localization and physiological function. Increased desaturase enzyme activity may be met by up-regulation of gene expression or induction of other desaturases (Murphy & Piffanelli 1998). The ∆9 stearoyl-ACP desaturase which carries out important housekeeping functions such as lipid membrane biosynthesis, is under constitutive control, but may also be supplemented by fine regulation at both the transcriptional and post-transcriptional levels.

We found that overall, stearoyl-ACP desaturase (-*A* and -*B*) gene expression increased with decreasing temperature in the seeds of three soybean lines examined. Previous reports implicated fatty acid desaturases in the response of soybean tissue cultures to high and low growth temperatures (MacCarthy *et al*. 1980a, 1980b). A recent report demonstrated that ∆9 desaturase gene expression responds to cold acclimation in young potato leaves (Vega *et al*. 2004). Results showed that the expression of ∆9 desaturase increased significantly during cold acclimation in a cold-tolerant species of potato (*S*. *commersonii*), but not in a cultivated nonacclimating species, despite the presence of greater constitutive ∆9 desaturase gene expression. In *Arabidopsis*, an up-regulation in desaturase

expression after a downward shift in temperature was shown to cause an increase in desaturation (Gibson *et al*. 1994). It is likely that this response is general and necessary to preserve membrane integrity during cold stress.

Previous studies have shown that the stearic/oleic fatty acid content of soybean seed is modulated by temperature, thus fatty acid composition and growth temperature are linked. The work of Rennie and Tanner (1989) demonstrated that the 18:0 content in A6 was a temperature sensitive trait and positively correlated with increasing growth temperature. Wilson and Burton (1993) found a positive correlation between the stearic acid in triacylgylcerol (TG) from A6 seeds and growth temperature. In their study, the decline in 18:0 content in A6 seeds grown at low temperature (22/18^{o}C) was attributed to an actual loss of 18:0, possibly as a function of the effect of temperature on 18:0-ACP desaturase enzyme activity.

We found that the stearic acid content of A6 had the most dramatic response of the three soybean lines to temperature manipulation. Stearic acid content increased at the warmer temperature for all stages of A6, but for Dare and N01-3544 the percentage change in 18:0 was slight and net negative. Conversely, growth at the cold temperature resulted in the most dramatic reduction (48%) of stearic acid content in A6. This response of stearic acid content in A6 to cold temperature agrees with the results of Wilson and Burton (1993). We found that the oleate (18:1) concentration increased at the higher growth temperature compared to normal across all varieties and stages, with Dare showing the most dramatic (32 %) increase in oleic acid content at the warm temperature. The increase in oleate content at the higher temperature was of a smaller magnitude for the A6 (11 %) and

N01-3544 (2 %). This positive correlation corroborates a number of previous studies that have shown an increase in oleic acid concentration with increasing temperature (Thomas *et al*. 1986, 2003; Rennie and Tanner 1989; Gibson and Mullen 1996).

The stearic acid content of A6 was inversely related to the levels of *SACPD-A* and *-B* expression at the warm and cold temperatures, as one might expect, but the stearic acid content of N01-3544 and Dare did not appear to be affected by temperature despite the dynamic changes in the expression levels of *SACPD* in these lines. This suggests that in these two lines stearate does not accumulate with higher temperature because it may be metabolically reclaimed as it is synthesized. Similarly, but unexpectedly, the oleic acid content of all three lines was inversely related to the levels of *SACPD* expression at the warm and cold temperatures. This suggests that transcription control of *SACPD* may not be a crucial factor for regulating oleic acid content in soybean and that oleic acid content is more likely determined by the relative expression and/or activity of another desaturase such as the ω6 desaturases encoded by the seed-specific FAD2-1 genes.

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Table 1A. Changes in actin normalized SACPD gene expression (i) and the oleic/stearic acid ratio (ii) in response to temperature in A6.

(i)

	Stage 1			Stage 2			Stage 3		
	A	**B**	Total	**A**	**B**	Total	**A**	**B**	Total
Warm	48.8	28.5	77.3	51.8	53.8	105.6	58.7	66.7	125.4
Normal	46.7	46.1	92.8	135.8	103.4	239.4	69.0	80.9	149.9
Cold	78.0	75.7	153.7	110.1	122.4	232.5	109.9	128.7	238.6

(ii)

	1	**2**	**3**
Warm	0.79	1.05	0.73
Normal	1.13	0.70	0.70
Cold	0.83	2.38	1.16

Table 1B. Changes in actin normalized SACPD gene expression (i) and the oleic/stearic acid ratio (ii) in response to temperature in Dare.

(i)

	Stage 1			Stage 2			Stage 3		
	A	**B**	Total	**A**	**B**	Total	**A**	**B**	Total
Warm	33.9	37.7	71.6	45.7	56.6	102.3	54.2	62.2	116.4
Normal	46.8	44.6	91.4	127.4	100.6	228.0	67.8	62.2	130.0
Cold	79.8	86.4	166.2	123.2	134.5	257.7	135.5	144.5	280.0

(ii)

	1	**2**	**3**
Warm	1.29	3.85	5.88
Normal	1.23	3.12	5.00
Cold	0.90	4.55	5.00

Table 1C. Changes in actin normalized SACPD gene expression (i) and the oleic/stearic acid ratio (ii) in response to temperature in N01-3544.

(i)

	Stage 1			Stage 2			Stage 3		
	A	**B**	Total	**A**	**B**	Total	**A**	**B**	Total
Warm	56.4	38.0	94.4	59.1	63.1	122.2	52.4	60.9	113.3
Normal	45.4	43.6	89.0	112.6	95.4	208.0	89.8	89.6	179.4
Cold	64.4	85.4	149.8	118	121.8	239.8	108.4	129.9	238.3

(ii)

	1	**2**	**3**
Warm	2.21	5.36	10.86
Normal	1.23	3.83	6.31
Cold	0.88	5.38	7.70

Legends

Figure 1. Relative expression of ∆9 stearoyl-ACP desaturase A and B genes in stage 4 soybean seeds across temperatures. Calculations were based on three sets of samplings, each analyzed in duplicate and vertical bars represent standard error of the mean. Expression levels were normalized to the soybean actin (Soy57) housekeeping gene.

Figure 2. Changes in stearic (18:0) and oleic (18:1) acid composition of stage 4 soybean seeds as affected by temperature. Plants were grown at the following day/night temperatures and harvested approximately 35 DAF; cold (22/18^{o}C), normal (26/22^{o}C), and warm (30/26^{o}C).

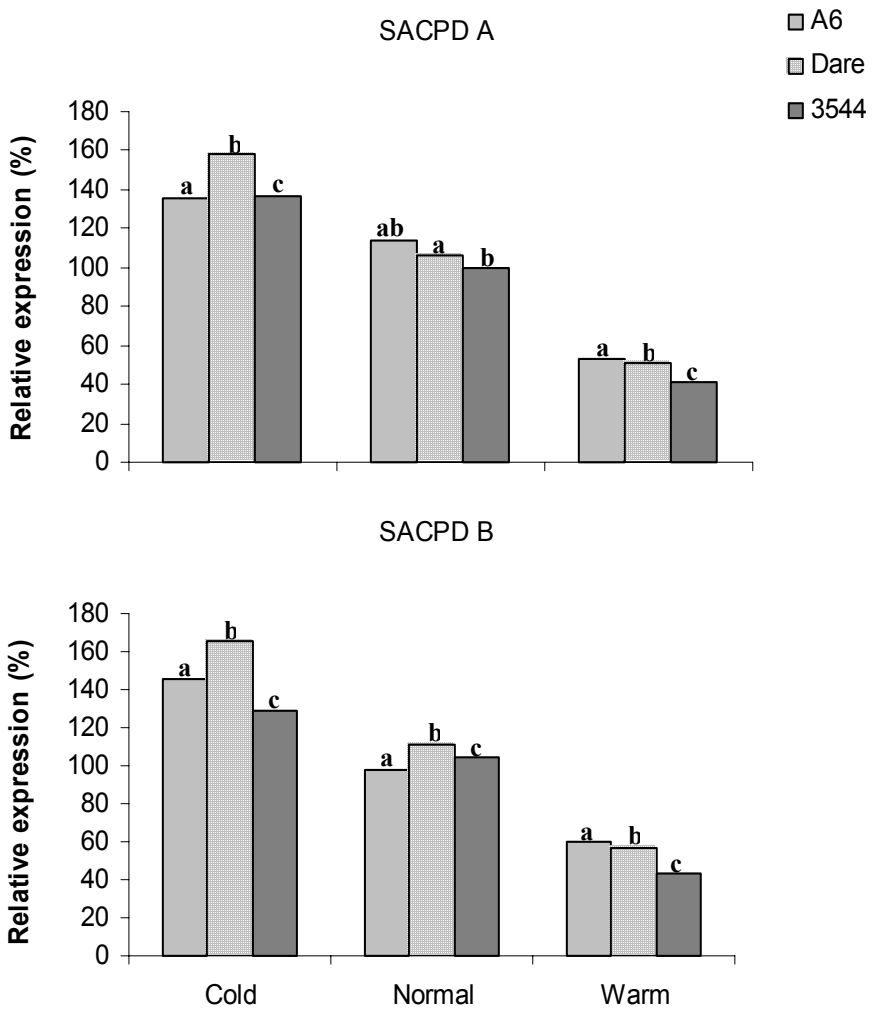

Figure 1. Relative expression of ∆9 stearoyl-ACP desaturase A and B genes in stage 4 soybean seeds across temperatures.

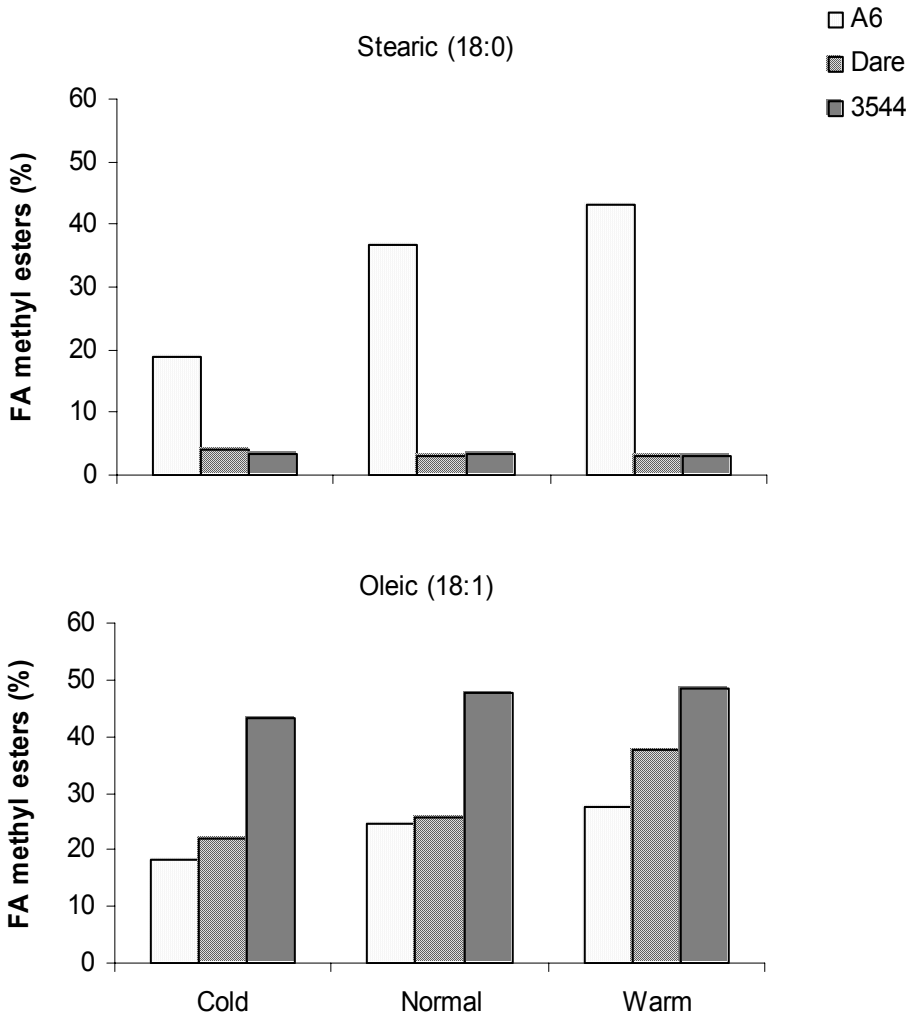

Figure 2. Changes in stearic (18:0) and oleic (18:1) acid composition of stage 4 soybean seeds as affected by temperature

Effect of temperature on microsomal ω-6 desaturase gene expression and linoleic/oleic acid content in soybean seeds

Running title: Temperature and omega-6 fatty acid desaturase expression

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ABSTRACT

The microsomal ω-6 desaturase (FAD2) enzyme catalyzes the first extra-plastidial desaturation, converting oleic acid (18:1) to linoleic acid (18:2). Environmental temperature modulates the 18:1/18:2 ratio of membrane and storage lipids through effects on desaturase enzyme activity and possibly gene expression. We measured changes in steady state transcript levels of the seed-specific FAD2-1A and FAD2-1B genes in developing soybean seeds from plants grown at 22/18°C (cold), 26/22°C (normal), and 30/26°C (warm) temperatures, and analyzed associations between temperature, FAD2-1 gene expression, and oleic/linoleic acid levels. FAD2-1A and FAD2-1B gene expression was comparable at the normal temperature, but a change in growth temperature to either side of the norm resulted in increased expression of FAD2-1B over FAD2-1A, slight at the warm temperature, but more pronounced at the cold temperature. The response to temperature across varieties was also more pronounced for FAD2-1B compared to FAD2-1A. The increase in FAD2-1B transcript accumulation with decreasing temperature was associated with increasing 18:2 content in two of the three varieties.

INTRODUCTION

Soybean [*Glycine max* (L.) Merr.] is one of the world's most important oilseed crops. Soybean seed is approximately 18% oil and standard commodity soybean oil contains approximately 22% oleic (18:1) and 53% linoleic (18:2) acid. The stability of soybean oil could be improved by increasing the oleic acid content through manipulation of genes integral to the C_{18} desaturase pathway (Wilson, 2004). Soybean with high 18:1 content has potential for use as soy diesel and cosmetic products. The microsomal ω-6 desaturase-catalyzed pathway is the primary route of polyunsaturated lipid production in plants. This membrane-bound enzyme catalyzes the first extra-plastidial desaturation and converts oleic acid esterified to phosphatidylcholine to linoleic acid. Microsomal ω-6 desaturase (FAD2) genes showing tissue-specific expression have been identified in cotton (Liu et al., 1997), as well as sunflower (Martinez-Rivas et al., 2001). *Arabidopsis thaliana* has a single FAD2 gene (Okuley et al., 1994). Two different ω-6 desaturase genes, FAD2-1 and FAD2-2, were identified in soybean (Heppard *et al*., 1996). The FAD2-1 gene is strongly expressed in developing seeds while the FAD2-2 is constitutively expressed in both vegetative tissue and developing seeds. The seed-specific FAD2-1 gene is likely to play a major role in the conversion of oleic acid to linoleic acid within storage lipids during seed development. Recently, two seed-specific isoforms (A and B) of the original FAD2-1 gene, differing in only 24 amino acid residues were identified in soybean. Heterologous expression in yeast revealed that FAD2-1A is less stable than FAD2-1B in cultures maintained at elevated growth temperatures (Tang et al.,

2005). Moreover, results suggested that FAD2-1 enzyme activity is controlled at the post-translational level.

Environmental temperature potentially dictates the saturated/unsaturated fatty acid ratio of membrane and storage lipids (Heppard *et al*., 1996) with polyunsaturated fatty acids tending to increase with decreasing temperature (Neidleman, 1987; Rennie and Tanner, 1989; Thompson, 1993). Heppard et al. (1996) found that linoleic acid level increased as temperature decreased in soybean seed 26 DAF, but that this change was not accompanied by any corresponding change in FAD2-1 transcript accumulation as determined by RNA gel blot analysis. A direct effect of temperature on desaturase enzyme activity has been shown in developing soybean seeds (Cheesbrough, 1989). In experiments using soybean cell suspension cultures, the activities of both oleoyl (ω-6) and linoleoyl (ω-3) desaturases showed significant decrease in activity in response to increasing growth temperatures.

In the present study, we amplified and sequenced FAD2-1 genes from genomic DNA of a number of soybean varieties using primers designed to the *Glycine max* FAD2-1 microsomal omega-6 desaturase mRNA (Heppard *et al*., 1996). This aspect of our work resulted in the production of PCR primers specific for FAD2-1A and FAD2-1B amplification designed for real-time RT-PCR analysis of transcript accumulation. Our investigation was aimed at applying this method to determine whether transcript accumulation levels of these genes respond to changes in temperature in developing soybean seeds. Fatty acid composition of the seeds was

also analyzed to identify possible links between temperature, desaturase expression, and oleic/linoleic acid levels.

RESULTS AND DISCUSSION

Transcript levels of FAD2-1A and FAD2-1B across temperatures

Growth temperature is known to modulate desaturase activity and stability as well as fatty acid content in oilseed plants. Cheesbrough et al. (1989) found that ω-6 desaturase (FAD2) enzyme activity increased with decreasing growth temperature. A recent study suggests that of the two soybean isoforms of FAD2-1 (A and B), FAD2-1A is less stable than FAD2-1B especially when expressed in yeast cultures maintained at high temperatures (Tang *et al*., 2005). Here we show that the transcript accumulation of both FAD2-1 genes also changes in response to growth temperature in three soybean varieties. Table 1A, B, and C shows the transcript accumulation of *FAD2-1A* and *FAD2-1B* and the 18:2/18:1 ratio for seed stages 1-3 across cold (22/18^{o}C), normal (26/22^{o}C), and warm (30/26^{o}C) temperatures for the mid-oleic line N01-3544, the standard cultivar Dare, and the high linoleic line N99-3170. Accumulation of total *FAD2-1A* + *FAD2-1B* transcripts generally increased in seeds of all three varieties grown at the normal and cold temperature, but decreased at the warm temperature from stage 1-3. For stages 1 to 2 at the warm temperature, transcript accumulation of both FAD2-1 genes decreased by about half in each variety. At the normal temperature, transcript accumulation of *FAD2-1A* increased 9 to 17 times, while *FAD2-1B* accumulation increased only slightly from stage 1 to 2.

At the cold temperature, transcript accumulation of *FAD2-1A* doubled, while *FAD2-1B* accumulation decreased from stage 1 to 2. At stage 2 through 3, *FAD2-1A* and *-B* transcript accumulation was similar in abundance at the warm and normal temperatures, but *FAD2-1B* accumulation was 1.5-2 times higher than *FAD2-1A* at the cold temperature. Figure 1 shows *FAD2-1A* and *-B* expression levels at stage 4. At this stage, *FAD2-1A* and *FAD2-1B* transcript accumulation is similar at the normal (26/22^{o}C) temperature in all three varieties. Cold or warm growth temperature however, resulted in increased expression of *FAD2-1B* over *FAD2-1A*, slight at the warm temperature, but more pronounced at the cold temperature in all three varieties. This finding may reflect the relative importance of *FAD2-1B* expression for determining seed 18:2 levels at the cold temperature at later seed stages. Variations of *FAD2-1B* transcript accumulation were observed among the three soybean varieties at all three growth temperatures at stage 4, but *FAD2-1A* exhibited varietal differences only at the cold temperature (Fig.1). The most extreme differences in *FAD2-1* transcript accumulation were seen among the soybean varieties at the cold temperature, with Dare showing the highest *FAD2-1A* and *-B* accumulation. An earlier report (Heppard et al., 1996) detected no change in *FAD2-1* transcript accumulation in response to changing temperature. It should be noted that, at the time, the existence of the two FAD2-1 genes, A and B was unknown.

Temperature Effect on Fatty Acid Composition

Both monounsaturated and polyunsaturated fatty acids in soybean oil are known to decrease with decreasing temperature (Wolf *et al*., 1982). Heppard et al. (1996),

also observed this general trend with 18:1, but found no corresponding changes in *FAD2-1* transcript accumulation in seeds produced at warm or cold temperatures. Table 1A, B, and C show the ratio of linoleic to oleic acid accumulation for seed stages 1, 2, and 3 for the three soybean varieties examined. At the warm growth temperatures all three varieties showed declining 18:2/18:1 ratios through these stages. At the normal growth temperature, the high linoleic acid line N99-3170 and the standard cultivar Dare showed declining ratios, but the mid-oleic N01-3544 line showed a ratio increase. At the cold growth temperature, all three soybean varieties showed increasing ratios (decreased 18:1 content) through these growth stages. Our results suggest that at these earlier developmental stages warm temperature favor 18:1 production at the expense of 18:2, and cold temperature favors 18:2 at the expense of 18:1. Results for stage 4 beans also indicate a general decrease in oleic (18:1) with decreasing temperature in all three varieties (Fig. 2). This trend was observed in other temperature studies (Rennie and Tanner, 1989; Thomas *et al*., 2003). Linoleic acid composition (Fig. 2) showed more variation across temperature among the soybean varieties analyzed at stage 4. With the mid-oleic line N01-3544 and cultivar Dare, 18:2 content increased with decreasing temperature. This finding relates well to the increase in *FAD2-1B* and *-A* transcript accumulation we observed in stage 4 seeds of these two varieties subjected to decreasing temperatures. With the high linolenic acid line N99-3170, 18:2 content decreased as temperature decreased. This finding while in agreement with previous temperature studies, is difficult to reconcile with the increase in *FAD2-1A* and *-B* transcript accumulation that we observed in the N99-3170 line at cold temperature.

Perhaps FAD2-1 message stability or post translational factors are of considerably more importance in governing the response of 18:2 levels to temperature in N99-3170 line.

MATERIALS AND METHODS

Plant Materials

Two soybean [*Glycine max* (L.) Merr.] lines were chosen for study based on elevated oleic or linoleic acid content at maturity. These were N01-3544, a maturity group IV mid-oleic acid line (52% 18:1, 33% 18:2) and N99-3170, a maturity group VI high linoleic acid line (24% 18:1, 64% 18:2). Cultivar Dare, a maturity group V cultivar (21% 18:1, 58% 18:2) was selected as the unimproved variety for comparison. Desaturase activity and lipid synthesis are highest during the early developmental stages of R5 through R6 in developing seeds. Therefore, seeds for analysis were harvested at 18, 23, 28, and 35 DAF during this period.

Growth Conditions

Plants were grown in semi-controlled environments at the Southeastern Plant Environment Laboratory (Phytotron) at North Carolina State University, Raleigh, NC. Germinated seeds were planted in 25 cm pots containing a one-third peat-lite and two-thirds gravel mix. Five plants of each variety were grown in a random arrangement in each of three chambers. Chambers were initially maintained at 26/22°C day/night (D/N) temperature with 14/10 hr photoperiod. Day illumination

was 200μmol $m^{-2}s^{-1}$. The dark cycle of day 1-14 was interrupted for one hour to synchronize plant growth. The dark interruption illumination was 150μmol $m^{-2}s^{-1}$. Plants were watered twice daily with deionized water and thrice weekly with nutrient solution. On day 15, the light interruption was stopped and chambers were reset to 12/12 hr photoperiod to induce flowering. On day 36, temperatures were changed in two chambers; one to 22/18°C (cold) and the other to 30/26°C (warm) without changing the existing photoperiod. These environmental conditions were maintained to the end of the experiment. Seeds were harvested at four stages between R5 (beginning of seed set) and R6 (mature bean) that coincided with 18, 23, 28 and 35 days after flowering (DAF). Pods were harvested from at least three plants of each variety in each chamber, beans were quickly removed, flash frozen in liquid nitrogen, and stored at -80°C.

RNA Isolation

Total RNA was isolated from 100 mg of frozen powdered tissue using the RNeasy Plant mini kit (Qiagen, Valencia, CA) following the manufacturer's protocol. A total of three separate samples were used and all were treated with DNA-free (Ambion, Houston, TX) according to the manufacturer's protocol. Aliquots of RNA were diluted to 50 ng/μl in RNase-free water and stored at -80°C until use. To verify RNA integrity, 500 ng of total RNA of each sample was electrophoresed on a 1% agarose gel stained with ethidium bromide.

Real-time RT-PCR

Real-time reverse transcriptase PCR was performed with the iCycler (Bio-Rad, Hercules, CA) using the SYBR Green RT-PCR kit (Qiagen, Valencia, CA). An original set of FAD2-1 primers (5'CACCTACTTCCACCTCCTTC3' and 5'CACCCTCAATACCTTCAAAACA3') were designed based on published sequence for *Glycine max* FAD2-1 microsomal omega-6 desaturase mRNA (Genbank accession no. L43920, Heppard et al., 1996). Sequence analyses of initial FAD2-1 amplifications using genomic DNA from a number of soybean lines lead to the identification of two FAD2-1 genes similar to work published by Tang et al., (2005). The following gene-specific primer pairs, FAD2-1A (5'CCAATGGGTTGATGATGTTG3' and 5'GTTGTTTAAGTACTTGGAAA3') and FAD2-1B (5'TTGACCGTTCACTCAGCAC3' and 5'GGTTGTTCAGGTACTTGGTGT3') were then designed to amplify approximately 178 and 154 bps, respectively across a variable region that distinguishes between the two. Duplicate reactions were done for each of the three samples for each cultivar. A typical reaction contained 12.5 µl of 2X SYBR Green PCR master mix, 250 nM each primer, 0.25 µl RT mix, 250 ng total RNA, and nuclease-free water to 25 µl. The reactions were performed in 96-well plates (0.2 ml tube volume) sealed with optical tape. Conditions for reverse transcription and amplification was 50°C for 30 min followed by 95°C for 15 min then 45 cycles of 30s at 94°C, 60s at 56.5°C and 60s at 75°C. A melt curve analysis over a 10°C temperature gradient at 0.05°Cs^{-1} from 78 to 88°C was done after amplification to verify a single product in each reaction.

Analysis of Transcript Accumulation

Steady state transcript levels for FAD2-1 genes were mathematically determined by comparison of individual cycle threshold (Ct) values with a standard curve generated from serial dilutions of an identical PCR product using soybean genomic DNA as template. Targets (copy number), were determined per microgram of total RNA isolated and significant differences in expression levels were statistically determined by student t-tests at $p = 0.05$ level.

Analysis of Fatty Acid Content

Fatty acid analyses were done on identical tissue samples as those used for RNA extraction. Fatty acid methyl esters (FAMEs) were prepared using acid methanolysis. Briefly, powdered seed tissue was heated at 85°C for 90 min in a 5% HCl-95% methanol solution. FAME was partitioned 2x into hexane and transferred to 2 ml vials for analysis. The FAMEs were separated by gas chromatography using an HP 6890 GC (Agilent Technologies, Inc., Wilmington, DE) equipped with a DB-23 30 m x 0.53 mm column (same source). Operating conditions were 1 µl injection volume, a 20:1 split ratio, and He carrier gas flow of 6 ml min^{-1}. Temperatures were 250°C, 200°C, and 275°C for the injector, oven and FID, respectively. Chromatograms were analyzed using HP ChemStation software.

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FIGURE CAPTIONS AND LEGENDS

Figure 1. Expression of microsomal ω-6 fatty acid desaturase FAD2-1 genes in stage 4 soybean seeds grown at various temperatures. A, Cold (22/18^{o}C); B, Normal (26/22^{o}C); C, Warm (30/26^{o}C). Calculations are based on the average of duplicate amplification reactions from three sets of samples. Means followed by identical letters indicate expression levels that are not significantly different at p=0.05.

Figure 2. Oleic (18:1) and linoleic (18:2) acid content of stage 4 soybean seeds grown at cold (22/18^{o}C), normal (26/22^{o}C), and warm (30/26^{o}C) temperatures and harvested approximately 35 days after flowering (DAF). Fatty acid methyl esters (FAME) were analyzed by gas chromatography.

Table 1A. Changes in actin normalized FAD2-1 gene expression (i) and the linoleic/oleic acid ratio (ii) in response to temperature in N01-3544.

(i)

	Stage 1			Stage 2			Stage 3		
	A	**B**	Total	**A**	**B**	Total	**A**	**B**	Total
Warm	157.7	126.5	284.2	49.4	62.1	111.5	62.0	86.1	148.1
Normal	10.7	106.3	117.0	127.7	112.9	240.6	112.1	111.2	223.3
Cold	43.4	214.0	257.4	102.5	182.9	285.4	111.1	185.0	296.1

(ii)

	1	**2**	**3**
Warm	1.85	1.39	1.15
Normal	0.37	1.96	2.27
Cold	0.27	1.52	1.45

Table 1B. Changes in actin normalized FAD2-1 gene expression (i) and the linoleic/oleic acid ratio (ii) in response to temperature in Dare.

(i)

	Stage 1			Stage 2			Stage 3		
	A	**B**	Total	**A**	**B**	Total	**A**	**B**	Total
Warm	152.1	127.6	279.7	41.4	64.4	105.8	66.1	88.0	154.1
Normal	15.1	109.0	124.1	137.8	125.9	263.7	68.0	101.3	169.3
Cold	53.7	261.3	315.0	96.1	193.3	289.4	100.9	218.1	319.0

(ii)

	1	**2**	**3**
Warm	2.44	2.22	2.00
Normal	2.70	2.78	2.56
Cold	2.08	2.27	2.17

Table 1C. Changes in actin normalized FAD2-1 gene expression (i) and the linoleic/oleic acid ratio (ii) in response to temperature in N99-3170.

(i)

	Stage 1			Stage 2			Stage 3		
	A	**B**	Total	**A**	**B**	Total	**A**	**B**	Total
Warm	137.5	105.5	243.0	46.4	63.2	109.6	66.1	89.4	155.5
Normal	7.8	105.7	113.5	138.7	122.3	261.0	112	111.1	223.1
Cold	44.6	245.0	289.6	88.2	191.8	280.0	97.7	189.2	286.9

(ii)

	1	**2**	**3**
Warm	2.22	1.79	1.75
Normal	2.70	2.70	2.63
Cold	ND	2.50	2.70

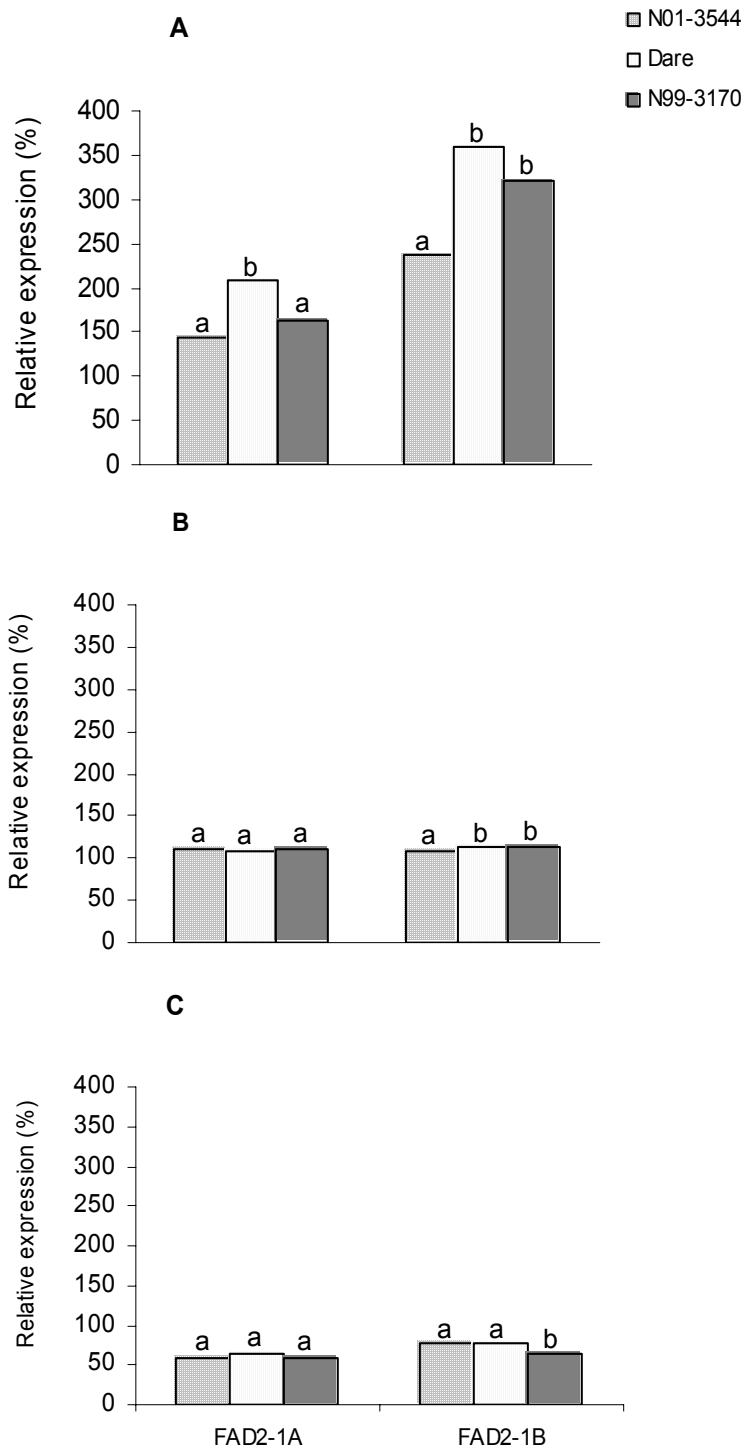
A
N01-3544
Dare
N99-3170
Relative expression (%)
400
350
300
250
200
150
100
50
0
a
b
a
a
b
b
B
Relative expression (%)
a
a
a
a
b
b
C
Relative expression (%)
a
a
a
a
a
b
FAD2-1A
FAD2-1B

Figure 1. Expression of microsomal ω-6 fatty acid desaturase FAD2-1 genes in stage 4 soybean seeds grown at various temperatures

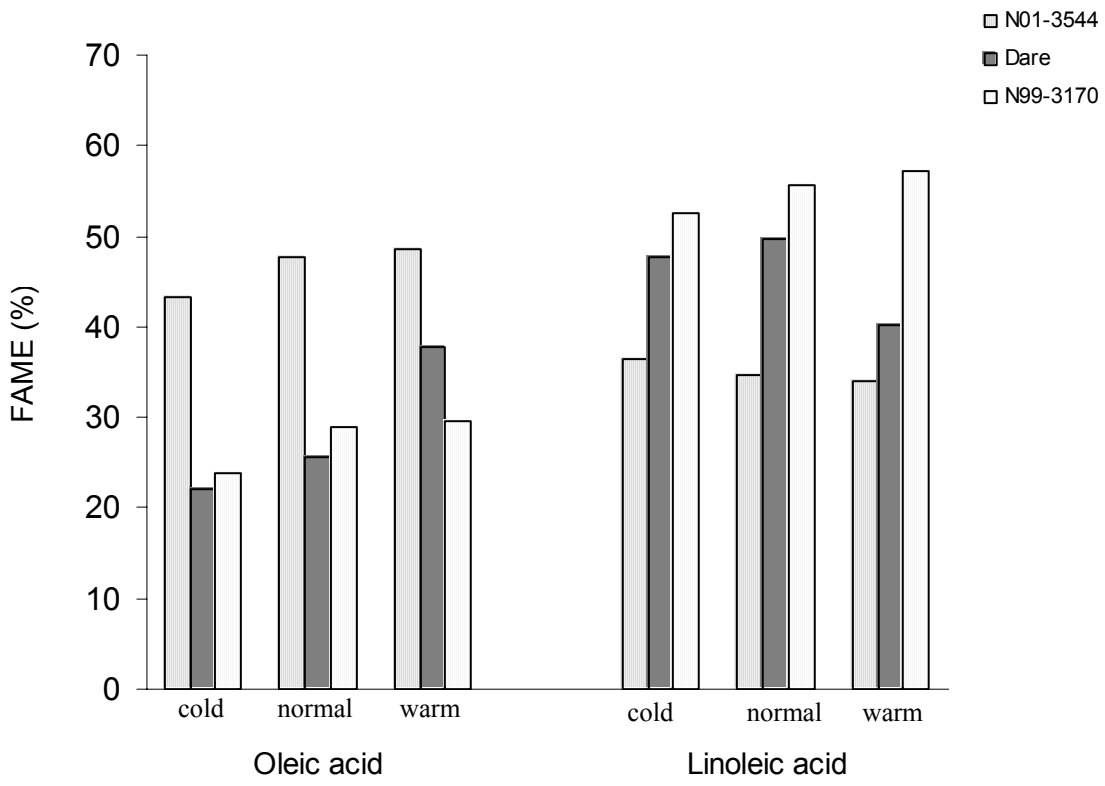

Figure. 2. Oleic (18:1) and linoleic (18:2) acid content of stage 4 soybean seeds grown at cold (22/18^{o}C), normal (26/22^{o}C), and warm (30/26^{o}C) temperatures and harvested approximately 35 DAF

Effect of temperature on omega-3 fatty acid desaturase gene expression and linoleic/linolenic acid content in soybean seeds

Running title: Temperature and omega-3 desaturase expression

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Research area: Environmental Stress and Adaptation

ABSTRACT

Microsomal ω-3 fatty acid desaturases (FAD3s) catalyze the insertion of a third double bond into the linoleic (18:2) acid precursor to produce linolenic (18:3) acid. Four independent microsomal ω-3 desaturase genes (Gm*FAD3A*/Gm*FAD3-1a*, Gm*FAD3B*/Gm*FAD3-1b*, Gm*FAD3C*/Gm*FAD3-2a*, and Gm*FAD3-2b*) showing tissue specificity have been identified in soybean. We analyzed the transcript accumulation of *GmFAD3A*, *B*, and *C* in response to temperature in developing seed of three soybean varieties. The three omega-3 fatty acid desaturase genes exhibited the highest levels of transcript accumulation at the cold temperature, with *FAD3A* levels 1.3 to 1.8 fold higher than *3B* and *3C* at stage 4 of seed development. At this stage, increased Gm*FAD3A* transcript accumulation was accompanied by an increase in 18:3 in all three soybean varieties examined. The differences in steady state mRNA levels we observed could be due to changes in transcription rate or mRNA stability. In light of this, further studies to measure desaturase activity under specific temperature regimes are needed in order to clarify the linkages of transcript level to enzyme activity and the 18:2/18:3 content in developing seed.

INTRODUCTION

Fatty acid desaturases (FADs) are essential for a number of physiological processes in plants. The fluidity of membrane lipids as well as the physical properties and nutritional value of many plant storage lipids are determined by desaturases. Omega-3 fatty acid desaturases (FAD3s) catalyze the insertion of a third double bond into linoleic (18:2) acid precursors to produce linolenic (18:3) acid. Seed linolenic acid levels have been shown to be determined by microsomal ω-3 desaturases (Yadav et al., 1993). A recent study identified two FAD3 genes that control the level of linolenic acid in flax (Vrinten et al. 2005). The level of Lu*FAD3A* and Lu*FAD3B* transcripts peaked at about 20 DAF and a dramatic reduction in the level of both transcripts occurred in the low-linolenic acid flax line solin. Three independent microsomal ω-3 desaturase genes (Gm*FAD3A*, Gm*FAD3B* and Gm*FAD3C*) have been identified in soybean (Bilyeu et al., 2003). They determined that the low-linolenic acid breeding line A5 contained two of these three genes, but lacked the Gm*FAD3A* sequence. More recently, Anai et al. (2005) identified four ω-3 desaturase genes as a result of an examination of low linolenic acid mutants of soybean. Three of the four genes corresponded to the three previously reported by Bilyeu et al. (2003). Currently, genetic approaches are being employed to reduce linoleic (18:2) and linolenic (18:3) acids while increasing oleic acid (18:1), to improve the oxidative stability of soybean oil.

Although several natural gene mutations have been discovered in soybean that potentially allow breeders to tailor fatty acid composition in the oil, including 18:2 and 18:3, (Wilson 2004), there are also important environmental factors that influence

fatty acid composition, particularly temperature. A general inverse relationship exists between polyunsaturation of fatty acids and growth temperature; polyunsaturated fatty acids increase with decreasing temperature in membranes as well as seed storage lipids (Neidleman 1987; Rennie and Tanner 1989; Thompson 1993). The mechanism of low-temperature adaptation in plants probably involves both transcriptional and/or post-translational regulation of the desaturases involved in polyunsaturate formation. Changes in growth temperatures have been shown to affect linoleoyl desaturase enzyme activity in soybean seeds from pods cultured in vitro (Cheesbrough 1989). The study showed a 98% decrease in linoleoyl desaturase activity at 25^{o}C compared to 20^{o}C. In another temperature study involving soybean seed, linolenic acid levels declined with increasing temperature as did the transcript of a gene involved in normal seed development (Thomas et al., 2003). The expression of the soybean FAD3s was been found to be tissue specific based on an analysis of the steady state mRNA levels (Bilyeu et al., 2003). Gm*FAD3A* had the highest relative expression in seeds.

Previously, we showed that the expression of desaturases upstream of the FAD3s were significantly affected by temperature in some soybean varieties and that these effects impacted the fatty acid content of developing soybean seed (Byfield et al., 2005). The present investigation was conducted to determine the changes in ω-3 fatty acid desaturase transcript accumulation in developing soybean seeds changes in response to changes in temperature, and how these changes are associated with changes in linoleic/linolenic acid content.

RESULTS AND DISCUSSION

Temperature and omega-3 desaturase gene expression

The steady state mRNA transcript levels for the three soybean FAD3 genes were determined by real-time RT-PCR. Table 1A, B, and C shows the transcript accumulation normalized to soybean actin of *FAD3A*, *B*, and *C* at the warm (30/26^{o}C), normal (26/22^{o}C), and cold (22/18^{o}C), temperatures in the three soybean varieties with their corresponding linolenic/linoleic ratios for seed developmental stages 1 through 3. The sum of *FAD3A*, *B*, and *C* accumulated transcripts declined from stage 1 to 3 (2 to 3 in some cases) in all three soybean varieties across all three temperatures. *FAD3* transcript accumulation was always highest at the cold temperature. A parallel trend was observed with the 18:3/18:2 ratio which also declined from seed stage 1 to 3 (2 to 3 in some cases) in all three soybean varieties across all three temperatures. The 18:3/18:2 ratios were highest at stage 3 in seeds grown at the cold temperature. These trends were observed at seed stage 4, 35 DAF (Fig. 1). All three omega-3 fatty acid desaturase genes exhibited the highest levels of transcript accumulation at the cold temperature. The *FAD3A* transcript showed the highest accumulation (132.6-176.7%) in the three varieties investigated at this temperature, while *FAD3B* and *FAD3C* transcript levels were between 8 and 68% lower by comparison. At normal and warm temperatures, the mean relative expression of all three FAD3 genes were comparable (not significantly different, p=0.05) and no significant varietal differences were detected. There were varietal differences, though not significant, in the expression of *FAD3A* at the cold temperature with Dare showing the highest expression. Varietal differences were

significant for *FAD3B* and *FAD3C* at the cold temperature. Dare and the high linoleic line N99-3170 had significantly higher *FAD3B* transcript accumulation than the high linolenic line N83-375 at the cold temperature. For *FAD3C* transcript accumulation, Dare showed the largest increase (115.6%) in expression level in response to the cold temperature, followed by N99-3170 (104.9%) and N83-375 (74.5%). An examination of the levels of total *FAD3* transcript accumulation for stage 1 through 4 shows that transcript accumulation was inversely related to growth temperature. Thus, our findings not only show that cold temperature elevates the level of *FAD3A*, *FAD3B* and *FAD3C* expression in the seeds of the soybean varieties investigated, but also confirm the previous study (Bilyeu et al., 2003) showing *FAD3A* to be more highly expressed than *3B* and *3C* at stage 4 of seed development. Our data also complement the results of Cheesbrough (1989) who showed that the activity of linoleoyl desaturase was greater at lower temperatures.

Temperature and linoleic/linolenic acid content

Identical seed samples were used for both steady state transcript and FAME analyses. Figure 2 shows the distribution of linoleic (18:2) and linolenic (18:3) acid in stage 4 seeds across the three experimental temperatures. Previous research has shown that at normal growth temperature, 18:2 increases linearly throughout seed development while 18:3 decreases until 60 DAF before increasing during the final stages of seed maturation (Simmons and Quackenbush 1954, Hirayama and Hujii 1965). As expected, we found that the overall level of 18:3 was considerably lower than 18:2 at all temperatures in the seeds of all three varieties. The 18:2

content of the high linoleate line N99-3170 decreased 4.8% as temperature decreased from warm to cold. In contrast, the 18:2 content of the other two varieties increased by 7% for Dare, and remained relatively unchanged for N83-375, the high linolenate line, as temperature decreased from warm to cold. Since many reports have documented that polyunsaturated fatty acid levels in membrane and storage lipids tend to increase with decreasing temperature, the behavior of N99-3170 seems exceptional with respect to 18:2 accumulation and decreasing temperature. We found that 18:3 levels increased as temperature decreased from warm to cold for all three varieties. Dare showed an increase of 5.2%, followed by N83-375 (5.1%), and N99-3170 (4.5%). This inverse correlation was also evident in stage 2 and 3 seeds (Table 1). Leaves of *Brassica napus* also showed significant increase in unsaturated fatty acid with decreasing growth temperature (Williams et al., 1996) which is consistent with our observations reported here.

We have shown that temperature does affect the steady state transcript levels of the omega-3 fatty acid desaturase genes in soybean seeds. Transcript levels of Gm*FAD3A* increased with decreasing temperature and this response was accompanied by an increase in 18:3 in all three soybean varieties examined. A subsequent decrease in 18:2 occurred in one (Dare) of the varieties. The differences in steady state mRNA levels we observed could be due to changes in transcription rate or mRNA stability. In light of this, further studies to measure desaturase activity under specific temperature regimes are needed in order to clarify the linkages of transcript level to enzyme activity and the 18:3/18:2 content in developing seed.

MATERIALS AND METHODS

Plant Materials

Glycine max varieties were selected based on linoleic/linolenic acid content at seed maturity. N99-3170 is a high linoleate line (64%) from maturity group VI and N83-375 a high in linolenate (9%) from maturity group V. Cultivar Dare, also a maturity group V with 58% linoleic and 7% linolenic acid was chosen as the unimproved variety for purposes of comparison. Seeds of the three soybean varieties were given to us by J.W. Burton, ARS Soybean & Nitrogen Fixation Unit, N.C. State University, Raleigh, N.C.

Growth Conditions

Plants were grown in semi-controlled environments at the Southeastern Plant Environment Laboratory (Phytotron) at North Carolina State University, Raleigh, NC. Germinated seeds were planted in 25cm pots containing a one-third peat-lite and two-thirds gravel mix. Five plants of each variety were grown in a random arrangement in each of three chambers. Chambers were initially maintained at 26/22^{o}C day/night (D/N) temperature with 14010 hr photoperiod. Day illumination was 200µmol $m^{-2}s^{-1}$. The dark cycle of day 1-14 was interrupted for one hour to synchronize plant growth. The dark interruption illumination was 150µmol $m^{-2}s^{-1}$. Plants were watered twice daily with deionized water and thrice weekly with nutrient solution. On day 15, the light interruption was stopped and chambers were reset to 12/12 hr photoperiod to induce flowering. On day 36, air temperatures were changed in two chambers; one to 22/18^{o}C (cold) D/N=12/12 and the other to

30/26^{o}C (warm) without changing the existing photoperiod. These environmental conditions were maintained to the end of the experiment. Seeds were harvested at four predetermined stages between R5 (beginning of seed set) and R6 (mature bean) that coincided with 18, 23, 28 and 35 days after flowering (DAF). Pods were harvested from at least three plants of each variety in each chamber, beans were quickly removed, flash frozen in liquid nitrogen, and stored at -80^{o}C.

RNA Isolation and Real-time RT-PCR

Total RNA was isolated from 100 mg of frozen powdered seed tissue using the Qiagen RNeasy Plant Mini kit (Valencia, CA) following the manufacturer's protocol. A total of three separate samples were used and all were DNase treated with Ambion DNA-free (Houston, TX) according to the manufacturer's protocol. Aliquots of RNA were diluted to 50ng/µl in RNase-free water and stored at -80^{o}C until use. To verify RNA integrity, 500ng of total RNA of each sample was visualized on a 1% agarose gel following electrophoresis and staining with ethidium bromide. The total RNA isolated from soybean seeds was used as template for real-time reverse transcriptase PCR. Reaction conditions were as previously outlined (Byfield et al. 2005) using the following FAD3 gene-specific primers from Bilyeu et al. (2003) and the actin primers from Byfield et al. (2005): Gm*FAD3A* (AGCGACACAAGCAGCAAAAT and GTCTCGGTGCGAGTGAAGGT), Gm*FAD3B* (TCCACCCAGTGAGAGAAAA and AGCACTAGAAGTGGACTAGTTATGAAT),Gm*FAD3C* (GCTGGGAGAAGAACACATTGAG and CCCAAAACATTGTGCCTTG) and actin

housekeeping gene (GAGCTATGAATTGCCTGATGG and CGTTTCATGAATTCCAGTAGC). Annealing temperatures were 60^{o}C for *FAD3A*, *3C*, Actin and 56.5^{o}C for *FAD3B*. PCR reactions with the FAD3 primer sets produced the following amplicons: Gm*FAD3A*, 183 bp; Gm*FAD3B*, 104 bp; Gm*FAD3C*, 143 bp. Negative controls did not result in any products. Transcript copy numbers, initially determined per µg of total RNA were normalized as a percent of the soybean actin gene expression level.

Analysis of Fatty Acid Content

The same powdered tissue samples were used for both RNA extraction and fatty acid analysis. Fatty acid methyl esters (FAMEs) were prepared using acid methanolysis as outlined previously (Byfield et al. 2005). Chromatograms were analyzed using HP ChemStation software.

ACKNOWLEDGMENTS

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Legends

Figure 1. Relative expression of ω-3 fatty acid desaturase genes GmFAD3A, FAD3B, and FAD3C in stage 4 soybean seeds across cold (22/18^{o}C), normal (26/22^{o}C), and warm (30/26^{o}C) temperatures. Three sets of samples were analyzed in duplicates. Means followed by identical letters are not significantly different at p=0.05.

Figure 2. Linoleic (18:2) and linolenic (18:3) acid content of stage 4 soybean seeds grown at cold (22/18^{o}C), normal (26/22^{o}C), and warm (30/26^{o}C) temperatures and harvested approximately 35 DAF. Fatty acid methyl esters (FAME) were analyzed by gas chromatography.

Table 1A. Changes in actin normalized FAD3 gene expression (i) and the linolenic/linoleic acid ratio (ii) in response to temperature in N99-3170.

(i)

	Stage 1				Stage 2				Stage 3			
FAD3	**A**	**B**	**C**	**Total**	**A**	**B**	**C**	**Total**	**A**	**B**	**C**	**Total**
Warm	69.5	72.5	150.2	292.2	59.7	62.1	61.0	182.8	52.9	60.8	43.8	157.5
Normal	42.8	63.8	52.3	158.9	94.2	123.4	59.1	276.7	61.5	99.9	45.3	206.7
Cold	34.1	260.1	3.4	451.1	108.6	119.8	79.1	307.5	114.6	180.3	80.4	375.3

(ii)

	1	**2**	**3**
Warm	0.35	0.15	0.08
Normal	0.28	0.20	0.09
Cold	ND	0.19	0.16

Table 1B. Changes in actin normalized FAD3 gene expression (i) and the linolenic/linoleic acid ratio (ii) in response to temperature in Dare.

(i)

	Stage 1				Stage 2				Stage 3			
FAD3	**A**	**B**	**C**	**Total**	**A**	**B**	**C**	**Total**	**A**	**B**	**C**	**Total**
Warm	98.9	97.7	114.0	310.6	76.6	68.1	62.9	207.6	65.5	62.2	50.6	178.3
Normal	63.8	56.5	66.4	186.7	116.1	114.5	59.4	290.0	82.8	69.7	59.4	211.9
Cold	49.3	243.4	4.3	497.3	135.2	150.0	90.7	497.6	147.6	200.8	97.9	390.2

(ii)

	1	**2**	**3**
Warm	0.50	0.25	0.18
Normal	0.95	0.38	0.19
Cold	0.65	0.32	0.28

Table 1C. Changes in actin normalized SACPD gene expression (i) and the acid linolenic/linoleic ratio (ii) in response to temperature in N83-375.

(ND= not determined)

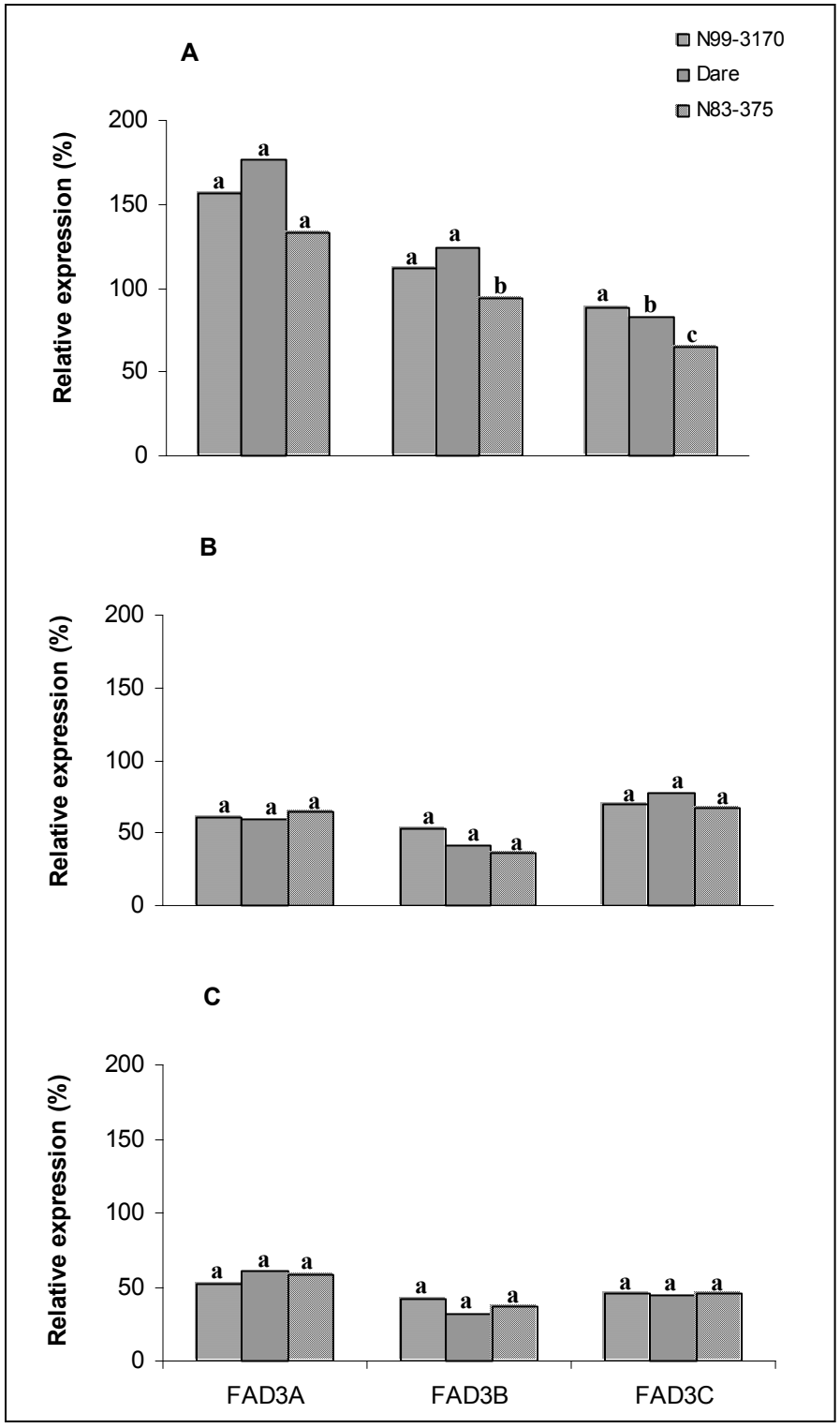
(i)

	Stage 1				Stage 2				Stage 3			
FAD3	**A**	**B**	**C**	**Total**	**A**	**B**	**C**	**Total**	**A**	**B**	**C**	**Total**
Warm	98.3	96.7	138.5	333.5	81.7	72.3	65.6	219.6	65.8	63.6	51.3	180.7
Normal	61.4	56.8	58.9	177.1	110.5	108.8	72.4	291.7	77.5	91.6	48.6	217.7
Cold	68.8	232.7	0.7	302.2	134.5	150.1	101.4	386.0	146.8	203.6	91.4	441.8

(ii)

	1	**2**	**3**
Warm	0.59	0.27	0.17
Normal	0.72	0.40	0.19
Cold	ND	0.35	0.26

Figure 1. Relative expression of ω-3 fatty acid desaturase genes in stage 4 soybean seeds across cold (22/18^{o}C), normal (26/22^{o}C), and warm (30/26^{o}C) temperatures


A
N99-3170
Dare
N83-375
Relative expression (%)
200
150
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a a a
a a b
a b c
B
Relative expression (%)
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a a a
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Relative expression (%)
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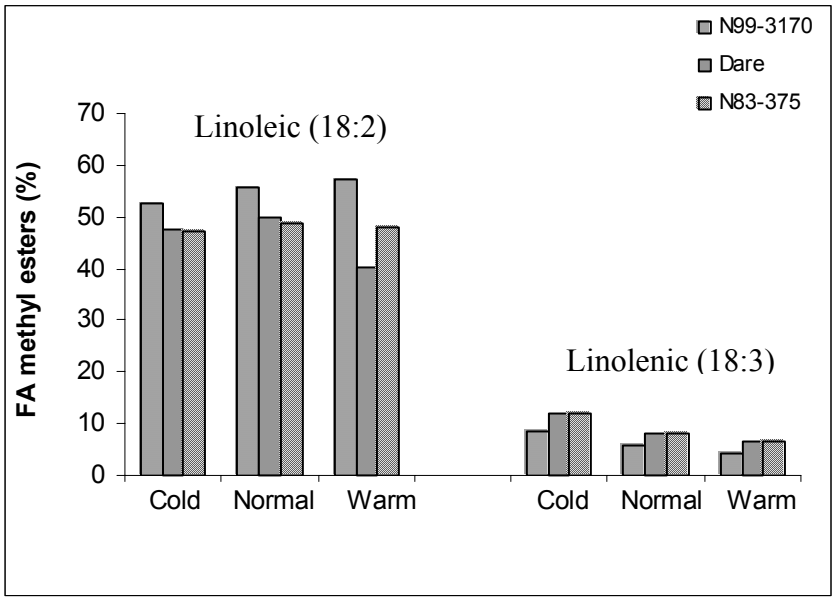

Figure 2. Linoleic (18:2) and linolenic (18:3) acid content of stage 4 soybean seeds grown at cold (22/18^{o}C), normal (26/22^{o}C), and warm (30/26^{o}C) temperatures and harvested approximately 35 DAF

APPENDICES

APPENDIX A: Heterologous Expression of Soybean Stearoyl-ACP Desaturase (SACPD) Genes in *Escherichia coli*

The Δ-9 stearoyl-acyl-carrier-protein desaturase (SACPD) gene encodes a soluble enzyme that converts stearic (18:0) to oleic (18:1) acid through the insertion of a double bond at the C_9 position. Previously, sequence analysis of SACPD genes amplified from the genomic DNA of a number of soybean lines revealed the existence of two such genes, designated *SACPD-A* and *SACPD-B* (Chapter 2, this study). Further investigation showed that both genes were present in all fifty-one lines used in the study and structurally both genes consisted of three exons and two introns. Exon three contained amino acid variability that provided a clear distinction between the two genes. It was thought that a biochemical analysis of the proteins encoded by these two SACPD genes (Figure 1) might reveal whether this amino acid variability has any relation to enzyme activity. The ability to clone fragments of plant DNA into bacterial plasmid expression vectors has provided an important tool for conducting extensive studies on plant genes and proteins. This investigation was designed to clone both SACPD genes from soybean into an expression vector, and after transforming appropriate *E. coli* cells, produce both SACPD proteins *in vivo*. The relative enzyme activity of SACPD-A and -B and the effect of temperature on that activity will then be measured using an *in vitro* assay with either crude protein extracts or purified enzyme.

MATERIALS AND METHODS

SACPD full length message

Total RNA was isolated from *Glycine max* cultivars A6 and Dare using Plant RNeasy Mini kit (Qiagen, Valencia, CA) and cDNA synthesized with Omniscript RT kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. The following primers were designed to SACPD exon 2 and 3 (Figure 2) region as determined by sequence analysis from previous experiments (Byfield et al., 2005); 5'GAGGTTGAAAATATTAAGAAGCC3' and 5'TCAACATTTCTCAGAAAGG3'. These primers were used to PCR amplify full length messages of both SACPD A and SACPD B genes from the cDNA pool. Amplification reactions contained 2.5 µl RT product, 2 µM each primer, 250 µM dNTPs, 1 unit Taq DNA polymerase (Fisher, Atlanta, GA), and 1X PCR buffer (300 mM Tris-HCl, 75 mM $(NH_4)_2SO_4$, 7.5 mM $MgCl_2$, pH 8.5) in a total volume of 25µl. Reaction conditions were 94^0C for 2 min (1 cycle), then 94^0C for 1 min, 58.7 ^{0}C for 2 min, 72^0C for 3 min (40 cycles), then 72^0C for 7 min. Amplicons were cloned into the sequencing vector pCR 2.1 using the TOPO TA cloning kit (Invitrogen, Carlsbad, CA). Recombinants were analyzed by PCR in separate reactions using gene-specific primers to the variable region of exon 3 in SACPD A and SACPD B as outlined previously (Byfield et al., 2005). Both strands of PCR clones representing SACPD A and SACPD B from each line were sequenced (Iowa State University Biotechnology Center, Ames, IA). In order to obtain the *SACPD A* full length messages, the message pool was first digested with *Sau 96I* prior to cloning. This enriched for *SACPD-A* as an internal *Sau 96I* restriction site was specific to *SACPD-B*.

A second set of primers were designed to add *NcoI* and *EcoRI* restriction sites on 5' and 3' ends respectively, of the *SACPD-A* and *-B* full length messages. These primers, FNcoI-5'GCCGTTCCATGGGAGGTTGAAAATATTAAGAAGCC3' and REcoRI-5'GCCGAATTCTTAGAGTAGTACTTCCCTGTTCATGAATCC3' amplified a fragment of approximately 1280 base pairs in a PCR reaction with recombinant clone (pCR2.1+ message insert) as template (Figure 3). The amplification reaction was set up as described above with the following modifications. The DNA template was 4µl of the recombinant clone, total reaction volume was 50µl, annealing temperature was 75^{o}C, and there were 35 amplification cycles.

Directional Cloning of SACPD-A and SACPD-B transcripts into expression vector pTrc 99A

The PCR product of the full length messages with added restriction sites were digested with *NcoI* then *EcoRI* in separate 50µl reactions. Similar reactions were set up with the expression vector pTrc 99A (Amersham Pharmacia Biotech, GenBank Accession Number U13872). Resulting DNA was ethanol precipitated after each digestion and the final product visualized on a 0.8% agarose gel (Figure 4). A 1:3 vector to insert ligation reaction was set up in a total volume of 10µl and incubated overnight at 4^{o}C. 'Topo 10' (Invitrogen, Carlsbad, CA) chemically competent *E. coli* cells were transformed with 3µl of the ligation reaction as per manufacturer's protocol. Transformants were allowed to grow on LB medium with ampicillin (50mg/ml) and 0.3% glucose. Plasmids with the correct inserts were identified by isolation of 5ml overnight cultures using QIAprep Spin Miniprep kit (Qiagen,

Valencia, CA) followed by restriction digests with NcoI and EcoRI to recover the inserts. Clones were confirmed by diagnostic PCR (Figure 5) using the previous FNcoI primer and pTrcR-5'GGGACCACCGCGCTACTGCCG3', the latter being anchored in the expression vector (Figure 6) and confirms orientation.

Induction of SACPD A and B

Expression vector containing the correct *SACPD* messages were used to transform BL21-Codon Plus® (DE3) RIPL competent cells (Stratagene, La Jolla, CA) as per manufacturer's instructions. An empty vector null transformation was also set done. Overnight cultures of transformants were set up and following plasmid isolations with QIAprep Spin Miniprep kit (Qiagen, Valencia, CA), restriction digests were used to confirm the presence of the insert as described above (Figure 7). *SACPD-A* and *SACPD-B* specificity was also confirmed by PCR using gene-specific primers as outlined above. An overnight culture of *SACPD* in BL21-RIPL background was prepared in LB containing ampicillin (50mg/ml) and chloramphenicol (34mg/ml), and 50µl of this culture was used to inoculate 1ml of LB with no selection antibiotics. Duplicate cultures were incubated for 2 hours at 37^{o}C with shaking at approximately 250 rpm. Before induction, 1ml of each culture was removed and kept on ice for SDS-PAGE analysis. The remaining culture was induced by the addition of IPTG (to a final concentration of 1mM) and allowed to grow for an additional 2 hours. At the end of the incubation period another 1ml of culture was removed for SDS-PAGE analysis.

SDS-PAGE Analysis

Aliquots (20µl) of each induced and uninduced culture were mixed with 2x SDS gel sample buffer in a microcentrifuge tube and heated to 95^{o}C for 5 min. Samples were placed on ice then loaded on a 12% Tris-HCl minigel (BioRad, Hercules, CA) and electrophoresed at 200 volts (not exceeding 50 mA) for about 2 hours. The protein gel was stained with Coomassie Blue and destained (Figure 8).

CURRENT RESULTS

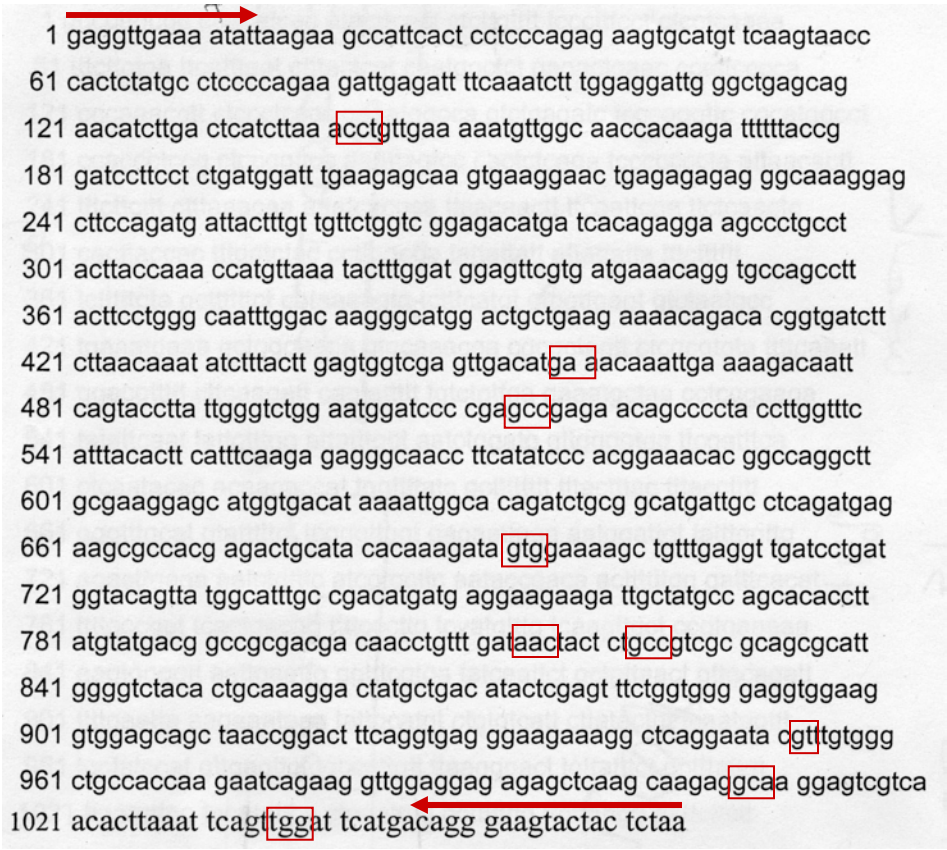

Figure 1. Nucleotide sequence of *SACPD-A* and *-B* cDNA. Arrows indicate location of primers and boxes identify regions of variable sequences.

Figure 2. Amino acid sequence of SACPD-A and -B mature peptides. Arrows indicate amino acid variations.

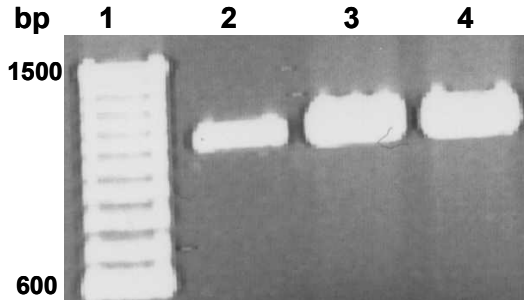

Figure 3. PCR amplification of *SACPD-A and -B* coding sequence with added restriction sites. Recombinant cloning vector (pCR2.1 = insert) was used as DNA template. MW marker, 100bp ladder (lane1), *SACPD-A* from A6 line (lane 2), *SACPD-B* from A6 (lane 3), *SACPD-B* from cultivar Dare (lane 4). Approximate size of insert 1280bp represents a shortened message fragment.

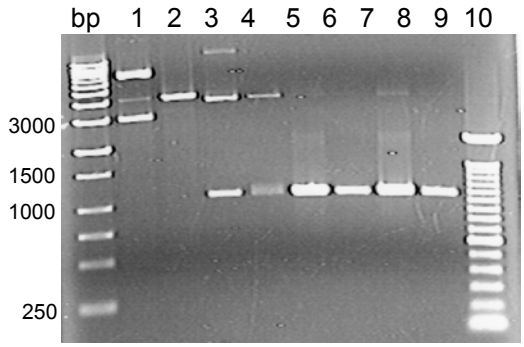

Figure 4. Restriction analysis of *SACPD-A* and *-B* reamplified insert. PCR fragments were digested with *Nco*I and *Eco*RI separately and ethanol precipitated after each digest. Lanes: 1- Kb DNA ladder, 2-3 pTrc 99A, 4-5 *SACPD-A* (A6), 6-7 *SACPD-B* (A6), 8-9 *SACPD-B* (Dare), 10- 100bp DNA ladder. Undigested and digested products are loaded in adjacent lanes.

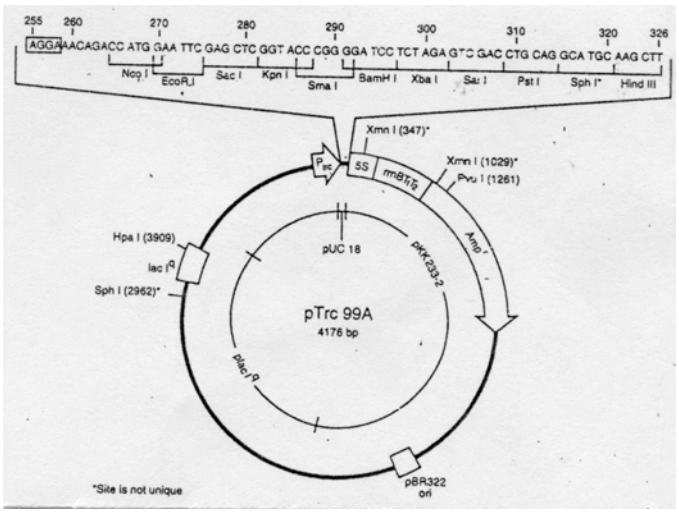

Figure 5. pTrc 99A Expression Vector. GenBank Accession Number U13872. Fragment encoding SACPD message was inserted between NcoI and EcoRI restriction sites. (Reproduced from Amersham Pharmacia Biotech Catalog, p. 493.)

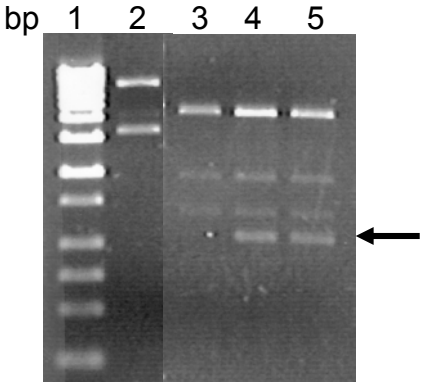

Figure 6. Diagnostic digests used to verify *SACPD* inserts in pTrc 99A in the BL21-RIPL *E. coli* background. Products were visualized on a 0.8% agarose gel following digestion of isolated plasmids. Lanes: 1- Kb DNA ladder, 2- pTrc (uncut), 3- pTrc-null (digest), 4- pTrc-*SACPD-A* (A6) (digest), 5- pTrc-*SACPD-B* (Dare) (digest). Arrows indicate insert and larger bands represent other plasmid DNA present in BL21-RIPL cells to facilitate expression of proteins that have codon bias problems in conventional *E. coli* systems.

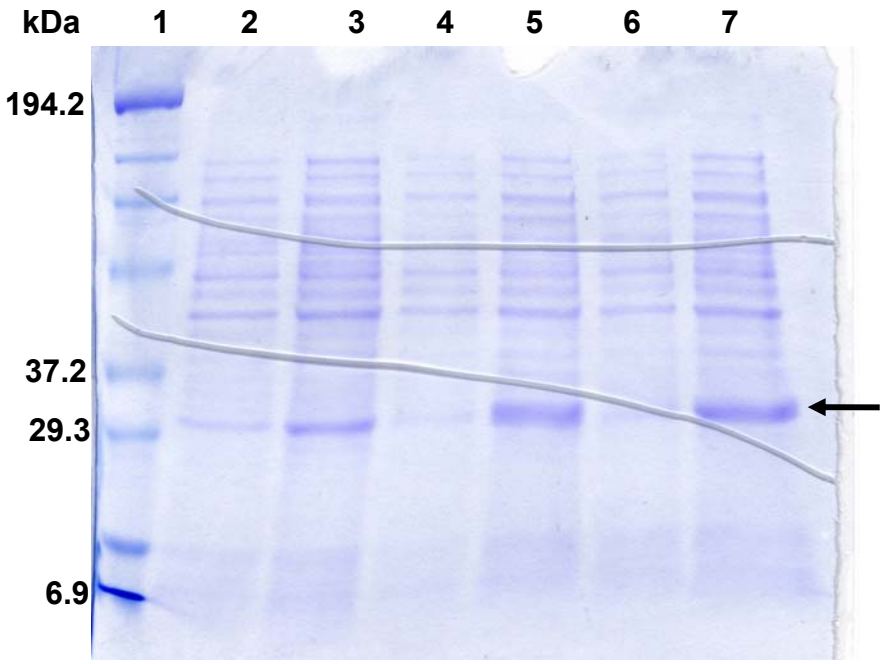

Figure 7. SDS-PAGE of SACPD-A and -B following heterologous expression in *E. coli* and coomassie blue staining. Lanes: 1- Broad range standard, 2-3 null vector, 4-5 SACPD-A (A6), 6-7 SACPD-B (Dare). Protein extracts from control and induced samples are loaded in adjacent lanes. Arrows identify thicker bands (doublets) which indicate expressed truncated SACPD proteins at approximately 33 kDa.

DISCUSSION AND FUTURE WORK

Thus far we have been able to successfully clone *SACPD-A* and *-B* coding sequences from two soybean lines (A6 and Dare), into an appropriate vector and induce their protein expression in *E. coli*. The next step involves the preparation of active crude protein extracts or purified SACPD enzyme solutions. An in vitro SACPD assay with stearoyl-ACP as substrate will be used to determine whether there are any differences in the enzyme activity of SACPD-A and SACPD -B and how temperature affects activity.

Acyl-ACP Desaturase Assay

Protein will be isolated from control and induced samples of each cell culture using an appropriate method. If possible, the crude extract will be used in the enzyme assays as outlined in Cahoon et al (1994). The enzyme assay requires the stearoyl-ACP substrate that is not available commercially and will probably have to be synthesized or donated. Assays are performed in a total volume of 150µl in loosely capped 13x100 mm glass tubes. Reactions are started with the addition of 20-25µg of total protein and incubated at room temperature with shaking at 100 rpm. Assays are terminated with the addition of 850µl of 2.34M sodium hydroxide and carrier fatty acids. Stearoyl- ACP Δ9 desaturase has been purified previously (Hoffman et al., 1995, Cahoon et al., 1997, Whittle et al., 2005) and some level of protein purification may be required here.

A typical assay reaction is outlined below:

1.25 mM NADPH in 100mM Tricine, pH 8.2

3.3 mM ascorbate

0.7 mM dithiothreitol

8000 units bovine liver catalase

5 µg BSA (Fraction V)

20 µg spinach ferredoxin

80 milli units spinach ferredoxin:NADPH reductase

33 mM PIPES, pH 6.0

1.25 mM acyl-ACP substrate [1-^{14}C]

Assay reactions are acidified and the resulting free fatty acids recovered by extraction with hexane and converted to methyl ester derivatives by methanolysis. Reaction products are then analyzed on 15% silver nitrate Thin Layer Chromatography (TLC) plates at -20^{o}C. Radioactivity is detected by autoradiography and quantified by liquid scintillation counting of TLC scrapings in a non-aqueous complete mixture.

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Cahoon, E.B., Lindqvist, Y., Schneider, G., Shanklin, J., (1997). Redesign of soluble fatty acid desaturases from plants for altered substrate specificity and double bond position. Proc. Natl. Acad. Sci. USA 94: 4872-4877.

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Whittle, E., Cahoon, E.B., Subrahmanyam, S., Shanklin, J., (2005). A Multifunctional Acyl-Acyl Carrier Protein Desaturase from *Hedera helix* L. (English Ivy) Can Synthesize 16- and 18-Carbon Monoene and Diene Products. J. Biol. Chem. 280: 28169-28176.

APPENDIX B: REAL-TIME REVERSE TRANSCRIPTASE-PCR

Table 1. Real-time RT-PCR thermocycle protocols

Gene	Actin/ SACPD A & B	FAD2-1A & B	FAD3A & 3C	FAD3B
Primers	2 µl	3 µl	2 µl	2 µl
Amp Cycle	x 45 94°C (15s) 60°C (30s) 78°C (30s)	x 45 94°C (30s) 56.5°C (60s) 75°C (60s)	x 45 94°C (15s) 60°C (30s) 78°C (30s)	x 45 94°C (30s) 56.5°C (60s) 75°C (60s)
Melt Curve Cycle	x 20	x 16	x 20	x 18
Melt Curve Start Temp	75°C	78°C	76°C	73°C

Table 2. Threshold (C_T) values and calculations

	Ct	Avg. Ct	Log SQ /(250ng)	Copy # /ug tot RNA	Log Copy # /ug tot RNA	Avg. Log Copy # /ug tot RNA		SD	SE	/Actin(%)
A6	37.4	37.92	2.82	2641	3.42	**3.26**	0.03	0.16	0.063	46.7
	38.3		2.54	1395	3.14		0.01			
	38.0		2.63	1725	3.24		0.00			
	37.3		2.85	2835	3.45		0.04			
	37.9		2.67	1852	3.27		0.00			
	38.6		2.45	1127	3.05		0.04			
Dare	36.3	38.52	3.16	5765	3.76	**3.08**	0.47	0.52	0.213	46.8
	36.8		3.00	4043	3.61		0.28			
	40.1		1.99	389	2.59		0.24			
	40.2		1.96	362	2.56		0.27			
	38.2		2.57	1497	3.18		0.01			
	39.5		2.17	595	2.77		0.09			
375	37.0	37.65	2.94	3508	3.55	**3.34**	0.04	0.17	0.067	47.8
	37.4		2.82	2641	3.42		0.01			
	37.4		2.82	2641	3.42		0.01			
	37.5		2.79	2460	3.39		0.00			
	38.2		2.57	1497	3.18		0.03			
	38.4		2.51	1299	3.11		0.05			
3170	36.9	38.45	2.97	3766	3.58	**3.10**	0.23	0.59	0.242	46.7
	37.3		2.85	2835	3.45		0.13			
	36.9		2.97	3766	3.58		0.23			
	37.8		2.70	1988	3.30		0.04			
	40.8		1.77	237	2.37		0.52			
	41.0		1.71	205	2.31		0.62			
3544	39.8	38.42	2.08	481	2.68	**3.11**	0.18	0.51	0.209	45.4
	40.8		1.77	237	2.37		0.54			
	37.7		2.73	2135	3.33		0.05			
	36.1		3.22	6644	3.82		0.51			
	38.2		2.57	1497	3.18		0.00			
	37.9		2.67	1852	3.27		0.03			

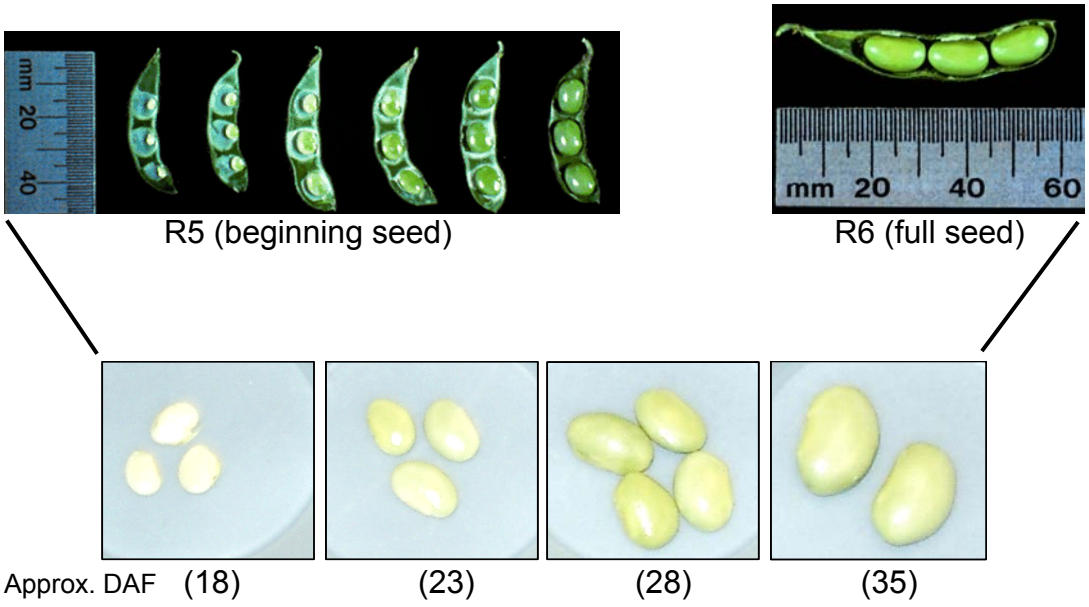

Figure 1. Pre-determined seed stages (1-4) between R5 and R6.

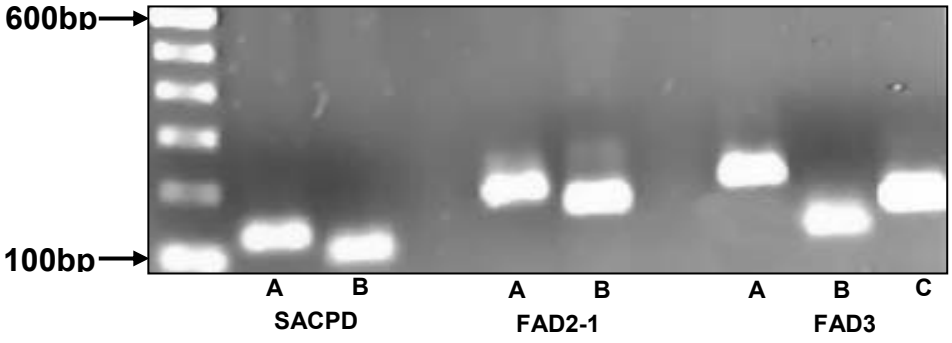

Figure 2. Ethidium bromide stained agarose gel showing amplicons for soybean desaturase genes produced by PCR.

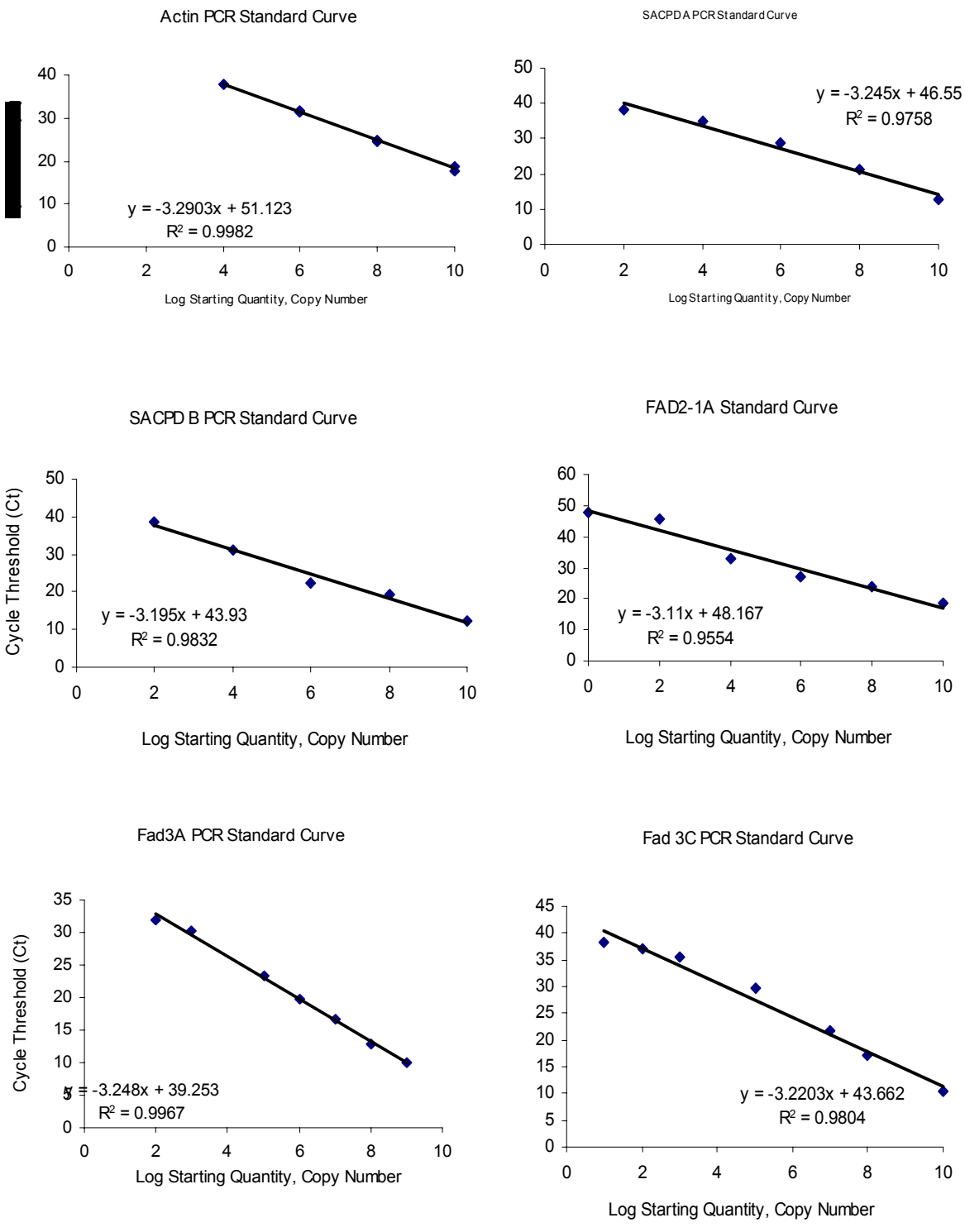

Figure 3. Standard curves generated by real-time RT-PCR for soybean desaturase genes.

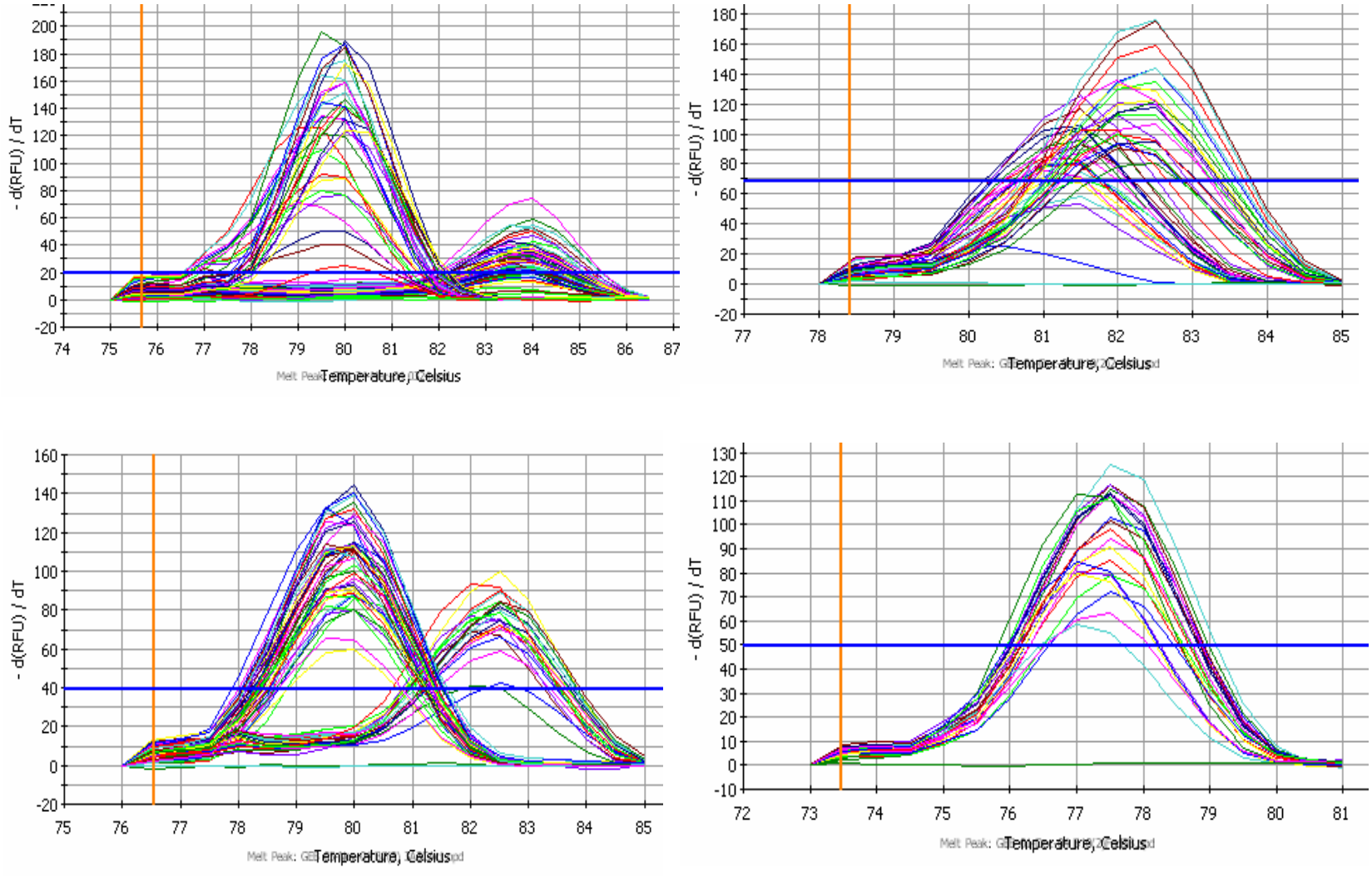

Figure 4. Melt curve analyses for real-time RT-PCR products. A; SACPD A and B, B; FAD2-1A and B, C; FAD3A and 3C, D; FAD3B

APPENDIX C: FATTY ACID RAW DATA

Table 1. Fatty Acid Methyl Ester (FAME) - Area %

Sample code	16:0	18:0	18:1	18:2	18:3
1130	26.2	12.8	9.2	28.0	23.8
1131	24.5	11.4	10.9	29.1	24.0
1133	33.6	13.3	12.0	24.9	16.2
1142	27.6	12.4	10.6	30.8	18.6
1143	24.4	10.3	11.0	34.8	19.4
1144	24.6	9.7	6.9	40.4	18.5
1700	24.3	19.2	16.2	25.1	15.1
1701	26.4	25.5	19.1	18.1	10.9
1702	24.5	21.2	16.9	23.8	13.7
1703	28.7	10.6	18.2	28.4	14.1
1704	34.0	13.0	13.6	24.7	14.8
1705	23.4	6.9	7.9	42.9	18.9
1706	29.2	11.4	18.1	25.7	15.7
1707	26.5	9.8	19.1	28.5	16.2
1709	11.9	7.2	21.2	42.2	17.6
1710	12.9	7.5	19.2	46.9	13.5
1712	25.8	10.4	22.0	30.5	11.3
1713	21.6	8.6	24.9	32.6	12.3
1714	22.7	7.7	12.2	46.7	10.6
1816	25.7	13.4	15.1	27.3	18.5
1818	22.8	8.8	10.9	29.5	28.0
1821	28.7	11.9	14.9	25.8	18.7
1822	26.0	11.1	14.7	27.5	20.7
1823	31.5	10.3	13.6	26.6	18.0
1824	17.7	10.3	22.2	35.3	14.5
1826	15.6	7.1	11.3	55.1	10.9
1827	27.7	12.2	16.6	28.9	14.5
1829	28.3	9.1	9.7	41.7	11.3
2860	16.2	22.4	16.2	30.7	14.5
2862	10.4	26.5	18.0	34.2	10.9
2863	19.0	5.3	17.2	39.8	18.7
2864	17.3	5.0	21.5	39.9	16.4
2865	16.2	5.3	9.5	53.0	16.0
2866	19.7	6.7	18.4	36.2	18.9
2867	16.9	5.5	20.1	41.9	15.6
2868	12.8	5.5	14.1	51.0	16.6
2869	11.4	6.5	21.0	47.8	13.3
2870	10.6	6.0	20.9	51.5	11.1
2871	11.2	6.7	16.0	59.1	7.0
2872	18.5	5.4	24.1	42.6	9.5
2873	21.5	6.9	21.0	40.9	9.7
2874	17.4	5.2	21.4	48.0	8.0

Table 1 (cont'd). Fatty Acid Methyl Ester (FAME) - Area %

Sample code	16:0	18:0	18:1	18:2	18:3
3805	9.9	35.1	19.0	27.1	8.9
3806	12.6	19.8	17.8	36.6	13.1
3807	10.0	28.9	20.9	31.9	8.3
3809	14.2	3.6	21.3	50.9	9.9
3810	14.1	4.4	19.5	52.6	9.3
3812	15.5	5.1	24.1	46.4	8.9
3813	14.5	5.2	14.4	55.8	10.1
3815	7.0	4.7	24.8	57.9	5.6
3816	6.1	4.2	21.0	63.0	5.7
3818	17.2	3.8	16.8	53.5	8.7
3819	15.6	3.1	27.4	47.0	6.9
4166	10.3	21.5	17.8	36.2	14.2
4167	10.6	16.4	18.4	39.8	14.8
4169	14.7	4.6	25.4	43.4	11.9
4170	13.9	3.5	18.8	51.9	11.9
4172	14.2	5.3	26.5	43.9	10.2
4173	12.6	4.5	18.9	50.6	13.4
4175	7.1	4.5	27.2	53.5	7.8
4176	12.3	6.2	20.5	51.4	9.5
4178	12.9	3.7	39.6	38.4	5.3
4179	11.2	3.1	46.9	34.6	4.2
4736	7.6	42.2	30.4	16.2	3.6
4737	8.0	43.8	24.8	18.8	4.7
4739	12.0	3.0	41.3	36.8	6.9
4740	12.3	3.3	34.2	43.6	6.5
4743	12.7	3.6	28.8	48.1	6.7
4745	6.0	3.9	29.2	56.3	4.6
4746	4.6	3.3	30.0	58.3	3.8
4748	12.0	3.1	40.4	41.1	3.3
4749	11.5	2.8	56.7	26.8	2.1
4850	10.1	36.4	20.0	24.7	8.8
4851	8.1	43.8	25.9	17.5	4.7
4852	8.1	29.8	28.0	27.2	6.9
4854	13.1	3.2	28.4	46.7	8.6
4855	13.6	2.9	22.8	52.6	8.0
4857	13.7	4.3	30.0	43.6	8.3
4858	13.0	4.2	20.6	54.1	8.1
4860	6.0	3.5	29.2	55.4	6.0
4861	5.8	3.8	28.6	56.0	5.8
4863	11.7	3.6	48.8	32.7	3.2
4864	11.2	3.3	46.3	36.4	2.7

Table 1 (cont'd). Fatty Acid Methyl Ester (FAME) - Area %

Sample code	16:0	18:0	18:1	18:2	18:3
2175	19.4	11.8	27.6	28.0	13.2
2176	12.3	8.4	25.8	43.6	10.0
2177	14.5	10.7	19.4	41.0	14.4
2179	15.9	4.9	22.1	41.8	15.3
2180	13.8	4.1	18.0	50.1	14.0
2182	19.0	6.2	23.7	37.4	13.7
2183	15.0	5.1	11.0	51.2	17.7
2185	12.6	6.1	19.4	47.8	14.2
2186	5.1	3.8	24.2	61.0	5.9
2188	16.6	6.2	25.4	41.3	10.4
2189	14.5	4.1	30.5	44.0	7.0
2745	14.9	22.1	20.5	31.9	10.6
2746	17.3	25.4	28.9	22.4	6.0
2747	13.4	26.8	28.7	24.5	6.5
2748	20.4	6.2	22.8	37.0	13.5
2749	15.0	4.8	21.4	47.6	11.2
2750	14.1	5.3	17.7	53.4	9.4
2751	23.5	7.6	24.3	33.7	10.9
2752	15.2	5.4	22.3	46.2	11.0
2753	12.1	6.0	13.6	54.4	13.9
2754	11.2	6.7	29.4	43.9	8.8
2755	7.5	4.7	24.9	56.0	6.9
2756	7.6	4.8	29.7	51.1	6.8
2757	17.5	6.0	31.7	38.1	6.6
2758	16.4	5.2	30.5	42.3	5.6
2759	16.8	5.4	26.3	42.4	9.0
3121	14.2	16.2	20.8	34.9	13.9
3122	10.7	18.4	19.3	37.7	13.9
3124	14.3	4.6	25.5	40.6	14.9
3125	12.4	4.0	18.3	53.6	11.7
3127	13.5	4.7	24.6	45.5	11.8
3128	12.3	5.3	14.7	53.9	13.8
3130	8.0	4.6	22.0	53.8	11.7
3131	5.5	4.0	20.5	62.7	7.3
3133	14.7	4.1	28.5	44.4	8.3
3134	12.7	3.9	33.1	45.1	5.2
3790	12.2	26.3	19.6	31.3	10.5
3791	8.9	35.2	22.2	26.2	7.5
3792	9.9	31.0	26.1	25.1	8.0
3794	12.7	3.5	30.2	45.5	8.1
3795	13.3	5.0	19.4	53.0	9.3
3797	12.2	4.3	24.1	51.7	7.7
3798	13.9	4.9	23.2	48.8	9.2
3800	5.3	3.2	41.8	45.2	4.5
3801	4.9	3.9	21.0	65.8	4.4
3803	11.1	3.0	48.5	34.6	2.7
3804	12.3	3.9	27.5	52.7	3.5