

FACILITATING BREEDING FOR RESISTANCE TO SOUTHERN STEM CANKER AND  
ELEVATED SEED OLEIC ACID CONTENT IN SOYBEAN

by

ZACHARY SHEARIN

(Under the Direction of H. Roger Boerma)

ABSTRACT

Disease resistance and seed oil composition are two economically important aspects of soybean (*Glycine max*) production in the southeastern USA. Soybean resistance to Southern stem canker caused by the fungus *Diaporthe phaseolorum* f. sp. *meridionalis* and methods of selection for elevated seed oleic acid content have been studied in these experiments. The stem canker resistance genes *Rdc2*, *Rdc3*, *Rdc4*, and *Rdc?(PI398469)* have been mapped using linkage analysis of phenotypic data from greenhouse resistance screens and genotypic data from simple sequence repeat (SSR) and single nucleotide polymorphism (SNP) DNA markers. Two genes, *Rdc2* from D85-10412 and *Rdc4* from Dowling both mapped to Gm02, but are located at different regions of the chromosome. The other two genes, *Rdc3* from Crockett and *Rdc?(PI398469)* from PI 398469 mapped to a similar location on Gm14 and appear to be clustered with several genes with known disease resistance motifs. All four resistance genes have mapped less than 3 cM from SSR or SNP markers that may be suitable for marker assisted selection. In addition to mapping disease resistance genes, three methods of selecting single plants with the elevated oleic acid trait from breeding line N00-3350 have been compared. They

include phenotypic selection (PS) using gas chromatography, stratified phenotypic selection (SPS) using gas chromatography while stratifying based on maturity, and marker assisted selection (MAS) using SNP markers associated with oleic acid quantitative trait loci. The three selection methods were tested in three different populations, BoggsRR x N00-3350, H7242RR x N00-3350, and PrichardRR x N00-3350. Stratified phenotypic selection and MAS were found to be more effective than PS when oleic acid QTL and maturity were segregating within a population. Plants selected using SPS and MAS gave rise to lines with higher average oleic acid and avoided a shift towards earlier maturity that was observed in some cases with PS. Maturity was significantly negatively correlated with oleic acid content in two of the three populations.

INDEX WORDS: Soybean, *Glycine max*, southern stem canker, *Diaporthe phaseolorum*, molecular mapping, SSR, SNP, bulked segregant analysis, BSA, oleic acid, selection methods, phenotypic selection, stratified phenotypic selection, marker assisted selection, MAS, QTL

FACILITATING BREEDING FOR RESISTANCE TO SOUTHERN STEM CANKER AND  
ELEVATED SEED OLEIC ACID CONTENT IN SOYBEAN

by

ZACHARY SHEARIN

BS, North Carolina State University, 2004

MS, The University of Georgia, 2007

A Dissertation Submitted to the Graduate Faculty of The University of Georgia in Partial  
Fulfillment of the Requirements for the Degree

DOCTOR OF PHILOSOPHY

ATHENS, GEORGIA

2011

© 2011

Zachary Shearin

All Rights Reserved

FACILITATING BREEDING FOR RESISTANCE TO SOUTHERN STEM CANKER AND  
ELEVATED SEED OLEIC ACID CONTENT IN SOYBEAN

by

ZACHARY SHEARIN

|                  |                 |
|------------------|-----------------|
| Major Professor: | H. Roger Boerma |
| Committee:       | Joe Bouton      |
|                  | Charles Brummer |
|                  | David Knauft    |
|                  | Shavannor Smith |

Electronic Version Approved:

Maureen Grasso  
Dean of the Graduate School  
The University of Georgia  
May 2011

## DEDICATION

This dissertation is dedicated to all those who provide the world with food and fiber and those who work to improve it through research. I would like to thank my family, friends, mentors, and colleagues for their unwavering support.

## TABLE OF CONTENTS

|  | Page |
|--|------|
| LIST OF TABLES .....                       | vii  |
| LIST OF FIGURES .....                      | viii |
| CHAPTER                                    |      |
| 1 INTRODUCTION .....                       | 1    |
| Breeding Goals.....                        | 3    |
| References.....                            | 6    |
| 2 STEM CANKER LITERATURE REVIEW .....      | 9    |
| Research Impetus .....                     | 9    |
| Taxonomy .....                             | 10   |
| Disease History .....                      | 12   |
| Disease Symptoms .....                     | 13   |
| Epidemiology .....                         | 14   |
| Factors Determining Disease Severity ..... | 17   |
| Disease Control.....                       | 22   |
| Sources of Resistance .....                | 23   |
| References.....                            | 25   |
| 3 OLEIC ACID LITERATURE REVIEW .....       | 29   |
| Benefits of Elevated Oleic Acid .....      | 29   |
| Breeding for Elevated Oleic Acid.....      | 33   |

|   |   |     |
|---|---|-----|
|   | Marker Assisted Selection .....   | 42  |
|   | References.....   | 48  |
| 4 | MAPPING RESISTANCE GENES TO SOUTHERN STEM CANKER.....                     | 54  |
|   | Abstract.....   | 55  |
|   | Introduction.....   | 56  |
|   | Materials and Methods.....  | 58  |
|   | Results and Discussion .....  | 61  |
|   | References.....   | 66  |
| 5 | COMPARING METHODS OF SELECTION FOR ELEVATED OLEIC ACID IN<br>SOYBEAN..... | 75  |
|   | Abstract.....   | 76  |
|   | Introduction.....   | 77  |
|   | Materials and Methods.....  | 81  |
|   | Results and Discussion .....  | 86  |
|   | References.....   | 91  |
| 6 | CONCLUSIONS.....  | 102 |



## LIST OF TABLES

|  | Page |
|--|------|
| Table 3.1: Summary of lines grown during the summer of 2008 at the Universty of Georgia Plant Sciences Farm .....  | 59   |
| Table 4.1: Phenotypic data for stem canker reaction in F <sub>2</sub> and F <sub>2:3</sub> populations .....   | 73   |
| Table 4.2: Cloned mRNA sequences of resistance genes with unknown functions in the vicinity of the map locations of stem canker resistance genes <i>Rdc2</i> , <i>Rdc3</i> , <i>Rdc4</i> , and <i>Rdc?</i> ( <i>PI 398469</i> ) .....            | 74   |
| Table 5.1: Number of experimental lines selected from each family, total number of lines grown per family, and the generation in which selection took place for three populations where N00-3350 was used as the elevated 18:1 donor parent..... | 95   |
| Table 5.2: Number of plants selected from each stratum for SPS .....   | 96   |
| Table 5.3: Oleic acid SNP markers segregating in each family, mean and range 18:1 concentration and maturity across three populations.....   | 97   |
| Table 5.4: Mean and range 18:1 concentration and maturity of lines selected by MAS, SPS, and PS .....  | 98   |
| Table 5.5: Mean oleic acid concentration of selected lines separated by SNP genotype at oleic acid QTL L2 and G1. ....   | 99   |

## LIST OF FIGURES

|  | Page |
|--|------|
| Figure 1.1: Soybean production area of the USA by state.....   | 8    |
| Figure 4.1: Linkage map of Gm14 based on the USDA consensus soybean genetic map (left) and<br>a linkage map of Gm14 including <i>Rdc?</i> ( <i>PI 398469</i> ) based on 94 F <sub>2</sub> plants of <i>PI 398469</i> x<br><i>J77-339</i> (right) ..... | 69   |
| Figure 4.2: Linkage map of Gm14 based on the USDA consensus soybean genetic map (left) and<br>a linkage map of Gm14 including <i>Rdc3</i> based on 94 F <sub>2</sub> plants of <i>Crockett</i> x <i>J77-339</i><br>(right) .....                       | 70   |
| Figure 4.3: Linkage map of Gm02 based on the USDA consensus soybean genetic map (left) and<br>a linkage map of Gm02 including <i>Rdc2</i> based on 92 F <sub>2:3</sub> plants of <i>D85-10412</i> x <i>J77-339</i><br>(right) 71                       |      |
| Figure 4.4: Linkage map of Gm02 based on the USDA consensus soybean genetic map (left) and<br>a linkage map of Gm02 including <i>Rdc4</i> based on 87 F <sub>2:3</sub> plants of <i>Dowling</i> x <i>J77-339</i><br>(right) 72                         |      |
| Figure 5.1: Correlations between maturity and oleic acid concentration of single plants grown in<br>2008 of POP1 (top panel) POP2 (middle panel) and POP3 (bottom panel).....  | 100  |
| Figure 5.2: Maximum and minimum daily temperature at The University of Georgia Plant<br>Sciences Farm from 15 August, 2008 to 31 October, 2008.....  | 101  |

## CHAPTER 1

### INTRODUCTION

Soybean (*Glycine max* L. Merr.) is a leguminous species native to eastern Asia.

Domestication of soybean most likely occurred in China during the Zhou Dynasty (1122 – 256 BCE) after it was introduced from the northeast, perhaps from the Manchurian Plain (Ho, 1977). Centuries later, Samuel Bowen introduced soybean to North America when he asked Henry Yonge, the Surveyor General of the Colony of Georgia, to plant soybean on Bowen's farm near Savannah, GA in 1765 (Hymowitz and Harlan, 1983). Bowen, a seaman employed by the East India Company, grew soybean on his plantation and made soy sauce and vermicelli from them for export to England. Almost 100 years later, in 1851 John H. Lea was the first person to plant soybean in the Midwest near Alton, IL where they were disseminated throughout the rest of the region in the following years (Hymowitz, 1986).

In 2009, soybean was planted on nearly 31.4 million hectares (77.5 million acres) in the USA. The 2009 soybean crop in the USA produced almost 91.5 million metric tons (3.4 billion bushels) worth an estimated \$31.8 billion at an average price of \$347 per metric ton (\$9.45 per bushel). While production and hectareage in the USA increased in 2009 over the previous year, the average price paid to farmers fell by \$0.65 per bushel. However, this is still over a dollar higher than the average price paid to farmers in 2006 when prices were already considered favorable by farmers. Almost 38% of the soybeans produced in 2009 in the USA were exported, with most exports sent to China, Mexico, Japan, the European Union, Taiwan, and Indonesia (Soy Stats 2009, <http://www.soystats.com>; verified 8 April, 2011).

Soybean has a wide variety of uses. The seeds are the most useful part of the plant. During harvest, the seeds are separated from the rest of the plant and soybean fodder is occasionally used as hay and fed directly to horses and livestock. Soybean seeds are usually processed and separated into two components, meal and oil. Soybean meal is by far the most important livestock protein feed, making up nearly 65% of the world protein feed supply (Economic Research Service, [http://usda.mannlib.cornell.edu/usda/ers/OCS//2000s/2006/OCS-04-04-2006\\_Special\\_Report.pdf](http://usda.mannlib.cornell.edu/usda/ers/OCS//2000s/2006/OCS-04-04-2006_Special_Report.pdf); verified 8 April, 2011). About half of all soybean meal produced in the USA is fed to poultry, while about a quarter is fed to swine, and the last quarter is fed to cattle, included in pet food, and put to other miscellaneous uses. Soybean oil is the other important component of soybean seeds. Soybean oil comprises over 70% of the world's edible fat and oil sources for direct human consumption. Three quarters of all soybean oil produced in the USA is used as food-grade vegetable oil for cooking and salad dressing. Another 5% of soybean oil is made into margarine. The remaining 20% of soybean oil is used in industrial products such as paint and lubricants. Soybeans are also used in other foods such as tofu (soy curd), natto (fermented soybeans), edamame (raw or steamed soybeans), and soy milk. An increasing amount of soybean is converted into diesel fuel, called biodiesel, which can be used in most diesel engines without making any mechanical modifications. In 2008, biodiesel consumption in the USA reached an all-time high of 2.6 billion liters (691 million gallons)(Soy Stats 2009, <http://www.soystats.com>; verified 8 April 2011).

Soybean is grown in 31 of the 48 states that make up the continental USA (Fig. 1.1). Florida, Texas, North Dakota, and New York make up the four corners of the soybean producing region of the USA, with every state within the region planting at least 8100 hectares of soybean

in 2007. Midwestern states such as Iowa, Missouri, Illinois, Indiana, and Ohio usually have the highest number of hectares planted to soybean (Fig. 1.1) and typically have the highest yields.

### Breeding Goals

Many traits are important in soybean. The most important and essential trait by far is seed yield. Yields of elite cultivars developed by public breeding programs increased by an average of about 1% per year over a 60-yr period from 1941 to 2001 (Wilcox, 2001). This rate of improvement was based on data from regional yield trials conducted prior to release of new cultivars. However, average seed yields in production environments across the USA have held fairly constant for the past 4 years with averages of 2.86, 2.67, 2.96, and 2.93 Mg ha<sup>-1</sup> in years 2007, 2008, 2009, and 2010, respectively (Soy Stats 2011, <http://www.soystats.com>; verified 10 April, 2011).

In addition to yield, there are several other agronomic traits that are essential to the success of a soybean cultivar. Cultivars must maintain an erect growth habit. Plants failing to grow correctly in favor of a prostrate growth habit are referred to as lodging plants. Lodging negatively affects yield by preventing plants from reaching their full yield potential and making harvest more difficult and less efficient (Woods and Swearingin, 1977). Resistance to shattering is another essential agronomic trait. Soybeans seeds are produced in pods that are broken open during the harvesting process. Shattering occurs when pods open prior to harvest and the seed are dropped to the ground. This seed cannot be collected from the field using any normal harvesting process, resulting in lowered yields. Shattering is usually caused by the pods either weathering or losing too much moisture prior to harvest, and can be genetically controlled (Philbrook and Oplinger, 1989). Maturity is another essential trait that controls the range of

latitude where a cultivar is adapted. In North America, soybean cultivars are classified into 13 maturity groups (MG 000-X) largely according to their latitude adaptation. A cultivar's maturity group dictates the region in which it is most adapted. The growth of a soybean plant is controlled by day length and temperature (Major et al., 1975). The way in which a soybean plant responds to day length and temperature dictates the maturity group in which it is most productive. Flowering is triggered when a plant's number of hours in darkness exceeds a genetically determined length. Temperature then dictates the number of days it takes for flowers to appear after that point. As a result, soybean cultivars adapted to the shorter day lengths and higher temperatures of the southern maturity groups will take too long to flower and produce seed when they are grown at higher latitudes. Soybean cultivars adapted to longer day lengths and lower temperatures of the northern maturity groups mature too quickly when grown at lower latitudes (Heatherly and Elmore, 2004).

With the seed being the most valuable part of a soybean plant, seed composition traits are also important breeding goals. As stated before, soybean seeds are usually separated into their two principle components, oil and meal (protein). Total oil and protein content in soybean seeds are negatively correlated (Burton, 1987). However, advancements have been made in altering the fatty acid profiles, or the various types and relative amounts of fatty acids and amino acids that make up the total oil or protein content of a seed. The typical fatty acid profile of soybean seed is 100 g kg<sup>-1</sup> palmitic acid, 40 g kg<sup>-1</sup> stearic acid, 220 g kg<sup>-1</sup> oleic acid, 540 g kg<sup>-1</sup> linoleic acid, and 100 g kg<sup>-1</sup> linolenic acid (Wilson, 2004). Ideally, the most usable form of soybean oil would have 70 to 150 g kg<sup>-1</sup> palmitic and stearic acid combined, more than 550 g kg<sup>-1</sup> oleic acid, and less than 30 mg g<sup>-1</sup> linolenic acid (Lee et al., 2008). Most breeding efforts for oil traits have focused on lowering saturated fat content (palmitic and stearic acids), maintaining flavor stability

without the need for chemical hydrogenation by reducing the amount of linolenic acid, and increasing the amount of oleic acid (Wilson, 2004).

Important traits related to seed protein content are the amounts of essential amino acids and reduced environmental impact of livestock waste after ingesting soybean meal. Significant breeding efforts have been made to increase the amounts of amino acids cysteine, lysine, and methionine in soybean meal. These three amino acids are referred to as essential amino acids for swine and poultry. Swine and poultry are not capable of producing these amino acids themselves, so they must be included in their diet (Wilson, 2004). To reduce the environmental impact of livestock waste as a result of ingesting soybean meal, breeders have tried to reduce the amount of phytic acid in soybean seeds. Soybean is very efficient in converting inorganic phosphorous from the soil to phytic acid, a metabolically useful form of phosphorous to both plants and animals. However, a significant amount of phytic acid is converted to phytate during the processing of soybean seed, which is nondigestible (Wilson, 2004). Phytate is excreted in livestock waste and has significant adverse impacts on the environment, especially water quality (Nahm, 2000). Low-phytate soybean lines have been created using chemical mutagenesis and breeders have been working to develop low phytate cultivars (Wilcox et al., 2000).

Disease and insect resistance are also important traits. One estimate attributed yield losses of more than 11 million Mg in 2005 to disease (Wrather and Koenning, 2006). By far, the most devastating soybean pathogen in terms of yield loss is soybean cyst nematode (*Heterodera glycines*), causing yield losses totaling almost 3.4 million Mg (Wrather and Koenning, 2006). Almost 30 different insect species are known to feed on soybean in the USA. Insects are particularly damaging in the southern most areas of the soybean growing region, especially along the Gulf of Mexico and Atlantic Ocean (Boethel, 2004).

The original research presented in this dissertation aims to improve both the seed composition and disease resistance of soybean. The objectives of this research are to enhance soybean resistance to southern soybean stem canker (caused by the fungus *Diaporthe phaseolorum* (Cooke & Ellis) Sacc. f. sp. *meridionalis* Morgan-Jones) and to enhance soybean seed fatty acid composition by; i) mapping the genomic location of soybean resistance genes to southern stem canker using SSR and SNP markers, and ii) comparing methods of selecting lines with elevated seed oleic acid content.

## References

- Boethel D.J. (2004) Integrated management of soybean insects, in: H. R. Boerma and J. E. Specht (Eds.), *Soybeans: Improvement, Production, and Uses*, ASA, CSSA, SSSA, Madison, WI. pp. 853-881.
- Burton J.W. (1987) Quantitative genetics: results relevant to soybean breeding, in: J. R. Wilcox (Ed.), *Soybeans: Improvement, Production, and Uses*, ASA, CSSA, SSSA, Madison, WI. pp. 211-248.
- Heatherly L.G., Elmore R.W. (2004) Managing inputs for peak production, in: H. R. Boerma and J. E. Specht (Eds.), *Soybeans: Improvement, Production, and Uses*, ASA, CSSA, SSSA, Madison, WI. pp. 451-536.
- Ho P.-t. (1977) The Indigenous Origins of Chinese Agriculture, in: C. A. Reed (Ed.), *The Origins of Agriculture*, Mouton, Chicago. pp. 413-484.
- Hymowitz T. (1986) Introduction of the soybean to Illinois. *Economic Botany* 41:28-32.
- Hymowitz T., Harlan J.R. (1983) The introduction of soybean to North America by Samuel Bowen in 1765. *Economic Botany* 37:371-379.
- Lee J.-D., Bilyeu K.D., Shannon J.G. (2008) Genetics and breeding for modified fatty acids in soybean seed oil. *J. Crop Sci. Biotech.* 10:201-210.
- Major D.J., Johnson D.R., Tanner J.W., Anderson I.C. (1975) Effects of day length and temperature on soybean development. *Crop Sci.* 15:174-179.
- Nahm K.H. (2000) A strategy to solve environmental concerns caused by poultry production. *World's Poult. Sci. J.* 56:379-388.



- Philbrook B.D., Oplinger E.S. (1989) Soybean field losses as influenced by harvest delays. *Agron. J.* 81:251-258.
- Wilcox J.R. (2001) Sixty years of improvement in publicly developed elite soybean lines. *Crop Sci.* 41:1711-1716.
- Wilcox J.R., Premachandra G.S., Young K.A., Raboy V. (2000) Isolation of high seed organic P, low phytate soybean mutants. *Crop Sci.* 40:1601-1605.
- Wilson R.F. (2004) Seed Composition, in: H. R. Boerma and J. E. Specht (Eds.), *Soybeans: Improvement, Production, and Uses*, ASA,CSSA,SSSA, Madison, WI. pp. 621-677.
- Woods S.J., Swearingin M.L. (1977) Influence of simulated early lodging upon soybean seed yield and its components 69:239-242.
- Wrather J.A., Koenning S.R. (2006) Estimates of disease effects on soybean yields in the United States 2003 to 2005. *Journal of Nematology* 38:173-180.



Figure 1.1. Soybean production area of the USA by state. The numbers within each state indicate the number of acres (top number, listed in thousands) and number of hectares (bottom number in parentheses, listed in thousands) planted within each state in 2009 (Soy Stats 2010, <http://www.soystats.com>; verified 8 April, 2011).

## CHAPTER 2

### STEM CANKER LITERATURE REVIEW

#### Research Impetus

Stem canker, caused by the fungus *Diaporthe phaseolorum* (Cooke & Ellis) Sacc. is a very economically and culturally important disease of soybean (*Glycine max* (L.) Merr.). Major revenue losses in the form of reduced yield potential have been observed in the USA and most other major soybean producing countries in the world. In 1994, seven of the top 10 soybean producing countries reported yield losses due to stem canker, including (in order of greatest to least estimated loss) Brazil, Paraguay, USA, Argentina, Bolivia, Italy, and Canada. Of the various diseases and pests of soybean, stem canker caused the second highest estimated yield loss at 1.9 million metric tons in these countries, with only soybean cyst nematode (*Heterodera glycines* Ichinohe) causing higher estimated losses (Wrather et al., 1997). Stem canker in North America has been found in the Midwestern and Southern regions of the USA and in Ontario, Canada. Stem canker became a serious problem in the southeastern USA in the early 1980's. In terms of percent of infected plants, infestation rates as high as 80% have been observed in some areas of the Southeast (Krausz and Fortnum, 1983). The economic impact of losses in the Southeast were estimated to cost \$37 million in 1983 (Backman et al., 1985). More recently, losses due to stem canker in the USA were estimated to cost \$67.1 million in 2003 (Wrather, 2004).

## Taxonomy

It is generally accepted that stem canker is caused by a fungal member of the *Diaporthe/Phomopsis* complex. The telomorphic, or sexually reproductive stage of this complex is identified as *Diaporthe phaseolorum*. The anamorphic, or asexually reproductive stage is identified as *Phomopsis phaseoli* (Morgan-Jones, 1989). Further classification of the fungal organism causing stem canker has been under continuous debate since it was first connected to the disease. Welch and Gilman (1948) were the first to distinguish stem canker from pod and stem blight, a disease of soybean and other crops caused by *Diaporthe phaseolorum* var. *sojae* (Lehman) Wehm. However, they incorrectly suggested that a strain of *Diaporthe phaseolorum* var. *batatatis* (Harter & Field) Wehm. was the causal organism of stem canker.

Athow and Caldwell (1954) were the first to correctly identify the fungus causing stem canker and gave it its own varietal name, *Diaporthe phaseolorum* (Cke. & Ell.) Sacc. var. *caulivora* Athow & Caldwell. They differentiated *D. phaseolorum* var. *caulivora* from *D. phaseolorum* var. *sojae* by its lack of a conidial (anamorphic) stage, the shape and grouping of its perithecia, the shape and length of its perithecial beaks, and the smaller size of its asci and ascospores.

However, in the late 1950's and 1960's evidence began accumulating that the causal organisms of stem canker and pod and stem blight were not in fact distinct varieties of *D. phaseolorum*.

Both the similar responses of the two proposed varieties to radiation-induced mutation *in vitro* and the identification of a conidial stage of *D. phaseolorum* var. *caulivora* (Threinen et al., 1959) provided the first evidence of a possible misclassification. Whitehead (1966) reported finding a strain of var. *sojae* that caused symptoms on soybean similar to stem canker, further invalidating this separation. However, strains associated with stem canker can consistently be distinguished from other strains of *D. phaseolorum*. As a result, Kulik (1984) suggested distinguishing strains

causing stem canker as different *formae speciales* from those lacking the pathogenic capability to induce the stem canker disease. This division recognized that the difference between strains was mostly in the host-pathogen relationship, with few morphological and *in vitro* behavioral differences otherwise distinguishing the strains. Backman et al. (1985) observed differences between strains of fungi that caused the stem canker disease. Differences in strains that were isolated from plants with stem canker symptoms in the northern USA and strains that were isolated from plants with stem canker symptoms in the southeastern USA were great enough to justify partitioning northern and southern biotypes into separate *formae speciales*.

In 1989, Morgan-Jones officially recognized three *formae speciales* of *D. phaseolorum* (Morgan-Jones, 1989). The organism associated with pod and stem blight was recognized as *Diaporthe phaseolorum* (Cooke & Ellis) Sacc. f. sp. *sojae* Morgan-Jones. The organism associated with stem canker in the northern USA and Canada was recognized as *Diaporthe phaseolorum* (Cooke & Ellis) Sacc. f. sp. *caulivora* Morgan-Jones and the organism associated with stem canker in the southeastern USA was recognized as *Diaporthe phaseolorum* (Cooke & Ellis) Sacc. f. sp. *meridionalis* Morgan-Jones. The latter was differentiated from f. sp. *caulivora* based on differences in colony appearance, stomatal size and occurrence, perithecium morphology production and overall arrangement, conidiomata formation, ascospore size and shape, growth response to different temperatures, and host symptom development.

However, the classification of these fungi as *formae speciales* has not been universally accepted, and they are still often referred to as varieties in the literature. From this point forward, *D. phaseolorum* f. sp. *caulivora* will be referred to as DPC, and *D. phaseolorum* f. sp. *meridionalis* will be referred to as DPM. Based on differential host cultivar reactions to certain isolates,

several races of DPC have been identified (Higley and Tachibana, 1987; Keeling, 1985).

Presently, no specific races of DPM have been identified.

### Disease History

Northern stem canker was first observed in the late 1940's. Although it probably existed before that time period, it was not differentiated from pod and stem blight. By the early 1950's stem canker incidence had increased dramatically due to the widespread use of two highly susceptible soybean cultivars, Blackhawk and Hawkeye. The repeated planting of these two cultivars year after year allowed the DPC population to increase over time to levels high enough to greatly reduce soybean yield (Hildebrand, 1952). Moving away from the use of Blackhawk and Hawkeye to other cultivars eliminated stem canker as a disease concern by the late 1950's (Weaver et al., 1984).

In contrast, the history of southern stem canker is more complex. Significant damage due to southern stem canker was first reported in Mississippi in 1973, followed by Alabama in 1977, Tennessee in 1981, and South Carolina and Georgia in 1982, Florida, Louisiana, and Arkansas in 1983, and Texas in 1984 (Backman et al., 1985). Unlike northern stem canker, outbreaks of southern stem canker could not be traced back to one or two highly susceptible cultivars because the cultivars grown in each state had a wide range of reactions to the pathogen. However, Bragg was growing on over 70% of the soybean hectareage in Georgia, South Carolina, Florida, and Alabama when stem canker was identified as a serious problem in the Southeast. Presently, stem canker still causes yield losses in some areas of the USA, but the development of resistant cultivars and altered cultural practices (both discussed in detail in later sections) have dramatically reduced the number of outbreaks in the USA.

## Disease Symptoms

Stem canker is named for the symptoms it causes in the plant. The first symptoms of both northern and southern stem canker appear during the plant's early reproductive stages as small reddish-brown superficial lesions on the stem, usually near a lower leaf node. The lesions expand longitudinally and form reddish-brown cankers that become slightly sunken as the growing season progresses. Older lesions may appear dark brown with a grayish-brown center and a reddish-brown margin (Fernandez et al., 1999). Tissue above and below the canker remains green (Grau et al., 2004). Lesions of northern stem canker range from 2 to 10 cm in length and usually girdle the stem. The dead stem tissue blocks the upward flow of water through the vascular tissue, and the seed-bearing portion of the plant becomes water-stressed and usually dies before the plant's full yield potential can be reached (Hildebrand, 1952). The leaves usually remain attached after plant death (Fernandez et al., 1999), but in some cases a top dieback can occur (Hobbs et al., 1981).

In southern stem canker the lesions expand along the length of the stem, but rarely girdle the stem (Fernandez et al., 1999). Leaves usually remain attached to the plant and show symptoms of interveinal chlorosis and necrosis. A phytotoxin plays a role in foliar symptoms and premature plant death (Lalitha et al., 1989). As with northern stem canker, plants with southern stem canker retain their leaves after death. Necrosis of the terminal meristem occasionally occurs, causing a characteristic shepherd's crook curling. Premature plant death causes a reduction in seed number and size (Fernandez et al., 1999). The reduction in seed size caused by the disease contributes to even greater yield loss during harvest as small seeds are often lost during the harvesting process (Hildebrand, 1952). Since the cankered stem tissue also

becomes dry and brittle any significant lateral force applied to the plant at or above this point can easily snap the stem, further contributing to yield loss (Hildebrand, 1956).

### Epidemiology

Most epidemiological research involving stem canker has involved DPM rather than DPC due to the greater threat and economic impact of DPM and southern stem canker. Plants are infected with DPM early in the growing season during the early vegetative growth stages from V3 through V10 (Fehr et al., 1971). Infection occurs primarily by rain splashing inoculum from infected soybean residue from the previous growing season onto the new crop during heavy rains (Ploetz and Shokes, 1985). Both conidia and ascospores are capable of causing infection (Ploetz and Shokes, 1985). Plants are usually infected through scars on stems, petioles, or petiole bases (Ploetz and Shokes, 1987a). Infection can also occur in the leaf tissue, but much less frequently than in other plant parts. Leaf infection does not lead to stem canker symptoms as often as does infection of stems, petioles, or petiole bases (Ploetz and Shokes, 1987b). Once a plant becomes infected, there is a latent period where no disease symptoms can be observed. Symptoms then appear during the early reproductive growth stages, usually during podfill. After stem canker symptoms have been observed, perithecia and pycnidia are occasionally produced in the cankers before the end of the growing season on highly susceptible cultivars. These perithecia and pycnidia can produce ascospores and conidia, respectively, which can serve as a secondary inoculum source during the growing season. The secondary inoculum may cause infection, but probably does not contribute to yield loss or disease development. Instead, it probably serves to increase the inoculum potential for the following growing season (Fernandez et al., 1999). In moderately susceptible cultivars, reproductive structures begin to develop after plant senescence



in late winter. The inoculum then overwinters on colonized plant debris left in the field after harvest (Grau et al., 2004). Ascospores and conidia are released in late April through June and infect the following season's crop during the V3 through V10 growth stages primarily by the previously mentioned mechanisms, at which point the cycle of disease is complete.

The pathogen is spread from one area to another by high winds during rainstorms, the transfer of infected debris, and to a lesser extent by infected seed. Splashing, blowing rain, and high winds during an early season storm can spread spores up to 2.0 m from a point source (Damicone et al., 1990). The movement of infested soybean debris contributes to the spread of the disease over greater distances (Backman et al., 1985; Grau et al., 2004). There has been much debate about the spread of stem canker via infested seed. Research has shown that seed infested with DPC has a greatly reduced germination rate (Hildebrand, 1952). Further research indicated that planting DPC infested seed does not increase the incidence of stem canker nor does it decrease the yield of the resulting plants in the absence of any other inoculum source (Hildebrand, 1956). Plöetz and Shokes (1987a) also conclude that DPM-infested seed does not play a substantial role in the spread of southern stem canker from one field to another. They also point out that no research has shown that seed infested with DPM results in infected plants when the seed is planted with no other exposure to the pathogen. Backman et al. (1985) however, argue that severe cases of stem canker have developed with seed as the only apparent inoculum source. Seed infestation rarely exceeds 1% in plants infected with DPM, but can range from 10% to 20% in plants infected with DPC (Fernandez et al., 1999). As shown by the inconsistent results in the literature, the exact role of infected seed in the spread of stem canker is unclear, and more research concerning the matter may prove beneficial.

Alternate hosts for DPC and DPM may play a role in the spread of stem canker.

Alternate hosts for DPC include alfalfa (*Medicago sativa* L.), barley (*Hordeum vulgare* L.), flax (*Linum usitatissimum* L.), lambsquarters (*Chenopodium album* L.), snapbean (*Phaseolus vulgaris* L.), and to lesser extents red clover (*Trifolium pratense* L.), white sweetclover (*Melilotus alba annua* Desr.), corn (*Zea mays* L.), rye (*Secale cereale* L.), oats (*Avena sativa* L.), barnyard grass (*Echinochloa crus-galli*), rough pigweed (*Amaranthus retroflexus* L.), witch grass (*Panicum capillare* L.), wheat (*Triticum aestivum* L.), and green foxtail (*Setaria viridis* L. Beauv.) (Frosheiser, 1957). These alternate hosts were found *in vitro* by evaluating the reproductive ability of a fungal strain on a putative host grown in culture media. While most of the alternate hosts were asymptomatic, stem canker symptoms were observed on alfalfa, red clover, and snapbean. At least 16 alternate hosts have been found for DPM. DPM that was isolated from cotton (*Gossypium hirsutum* L.) in Mississippi has been found to cause stem canker symptoms on soybean (Roy and Miller, 1983). Black et al. (1996) evaluated 17 weed species common to Louisiana soybean fields for their potential as alternate hosts. Of the 17 species they evaluated, 14 were identified as potential alternate hosts. They include black nightshade (*Solanum nigrum*), entireleaf morning glory (*Ipomoea hederacea* var. *integrifolia*), hairy indigo (*Indigofera hirsuta*), hemp sesbania (*Sesbania exaltata*), ivy-leaf morning glory (*Ipomoea hederacea*), northern joint-vetch (*Aeschynomene virginica*), pitted morning glory (*Ipomoea lacunosa*), prickly sida (*Sida spinosa*), redweed (*Melochia corchorifolia*), sicklepod (*Cassia obtusifolia*), smallflower morning glory (*Jacquemontia tamnifolia*), spiny amaranth (*Amaranthus spinosus*), tall morning glory (*Ipomoea purpurea*), and wild poinsettia (*Euphorbia heterophylla*). Only two of these species, hemp sesbania and hairy indigo, showed stem canker symptoms.

After isolating a phytotoxin from DPM that plays a role in the foliar symptoms of southern stem canker, Lalitha et al. (1989) tested 12 plant species for reaction to both the isolated toxin and the fungal strains from which the toxin was isolated. Only soybean and lima bean (*Phaseolus lunatus* L.) expressed symptoms of stem canker in the presence of the phytotoxin and the fungal strains. As a result, lima bean is considered to be an additional alternate host for DPM.

### Factors Determining Disease Severity

In order to provide protection for soybean against southern stem canker, a considerable amount of research has attempted to determine the factors that affect the severity of the disease. The growth stage at the time of infection, environmental conditions such as air temperature and moisture, cultural practices, and the presence of other pests have been shown to affect the extent to which soybean will react to DPM. The percent of infected soybean plants in a given field can increase from a negligible amount to epidemic levels in as little as one year. In one previously uninfested field, 1.6% of soybean plants had symptoms of stem canker in the first season stem canker was found at that location in 1983. During the 1984 growing season, 72.4% of the plants had symptoms of stem canker at the same location (Rothrock et al., 1985). It is clear that a combination of many external factors played a role in such a dramatic and rapid increase in the effect of stem canker on the crop grown in this field.

The growth stage of the host at the time of introduction to DPM has been shown to play a significant role in how stem canker affects the performance of the plant. It was first observed in northern stem canker that if soybean was attacked by DPC early in the growing season, both seed quality and quantity would be greatly reduced, but if the attack occurred later in the growing season, the effects were negligible (Hildebrand, 1952; Hildebrand, 1956). The findings that

early infection appeared to be related to final disease severity were also confirmed for southern stem canker. Rothrock observed that in 1985, 34% of plants of the moderately susceptible cultivar Hutton were infected by 21 DAP, and 37% of these sampled plants died prematurely, most likely from stem canker. The following year, 4% of the Hutton plants sampled 24 DAP were infected, and 6% of the sampled plants died prematurely with the most probable cause of death also being stem canker (Rothrock et al., 1988). DPM exposure at the V3 stage of development is associated with the highest disease severity. Severity is progressively reduced when exposure to the pathogen is delayed until stages V4 to V10 (Grau et al., 2004). In order to more fully understand the relationship, Smith and Backman (1989) conducted two different experiments to determine if planting date and infection at different soybean developmental stages influenced the occurrence or severity of the disease. In one experiment all plots were planted at the same time and inoculated at various growth stages. In the second experiment, the plots were planted at different times and all of the plots were inoculated at the same time. Similar results were obtained from both experiments despite the different experimental designs, cultivars, and environmental conditions. The data from both experiments were fitted to Cauchy distribution models that predicted an exponential increase in disease (measured at R6) for plants exposed to inoculum at stages V1 to V3 (12 to 22 DAP). Their models predicted that disease incidence and severity would then progressively decrease when plants were exposed to inoculum at V5 through R2. Their experiments suggest that disease severity largely depends on time of infection relative to plant growth stage. Additional studies that used the development of foliar symptoms as a measurement of disease incidence and seed weight and number as measurements of disease severity determined that stem canker symptoms can occur regardless of growth stage at the time of infection (Rupe et al., 1999). However, foliar symptoms, which are usually observed after the

presence of cankers, require a minimum incubation period of 34 to 41 days and a reproductive developmental stage. Foliar symptoms have never been observed earlier than R2 (the onset of flowering) even when inoculation occurs as early as V1. This corresponds to a trend that plants inoculated at V1 have the longest incubation period, while plants inoculated at R2 have the shortest incubation period for the disease.

Environmental conditions during vegetative growth play a role in the development of stem canker. Of these, air temperature has the greatest effect (Grau et al., 2004). A wide range of temperatures promote maximum recovery of DPM from artificially inoculated soybean plants, but a upper and lower temperature limits do appear to exist. Growth chamber experiments have determined that temperatures of 28 to 34°C promote the highest levels of DPM recovery after artificial inoculation, with optimal recovery at 28.5°C. Temperatures of 10 to 22°C are associated with less infection (Rupe et al., 1996). Infection at temperatures above 40°C is not known to occur (Ploetz and Shokes, 1987b). Moisture during vegetative growth of the plant also appears to play a role in stem canker infection. After spore deposition has occurred, either a prolonged and continuous wetting event such as rainfall or irrigation, or many discontinuous periods of wetting facilitate infection and stem canker development (Damicone et al., 1987). Stem canker epidemics are more likely to occur in an irrigated field than a nonirrigated field (Subbarao et al., 1992). Rupe et al. (1996) combined treatments of various temperatures (15 to 30°C) and moisture periods (8 to 96 h) on artificially inoculated soybean plants grown in the greenhouse and measured the recovery of DPM from surface-sterilized stem and petiole sections placed on potato dextrose agar (PDA) after the experiment was completed as a measure of infection. Maximum recovery of DPM was observed at wetness periods of 72 to 96 h, and

temperatures of 28 to 34°C. A regression model ( $R^2 = 0.847$ ) fitted to the data predicted high fungal recovery over a broad range of near-optimum temperatures and longer wetness periods.

Certain cultural practices have been found to affect disease severity. Tilling under infected debris and delayed planting have been shown to reduce disease severity (Grau et al., 2004; Hilty, 1991; Rothrock et al., 1985; Rothrock et al., 1988). Doublecropping soybean with wheat has been shown to increase disease severity (Rothrock et al., 1985; Rothrock et al., 1988). Crop rotations in general have not been shown to reduce stem canker infestations (Grau et al., 2004). Tilling that buries infested soybean residue reduces disease development by lowering or eliminating the number of spores near above ground plant parts that would otherwise be available to colonize crops early in the growing season (Hilty, 1991; Rothrock et al., 1985; Rothrock et al., 1988). Delayed planting can reduce stem canker incidence and severity by avoiding the critical time period when spores are released and deposited onto plants (Grau et al., 2004). However, the reduced yield potential usually associated with delayed planting is often greater than the reduced yield potential of crops with stem canker. Doublecropping soybean after wheat has been shown to promote the onset of stem canker symptoms (Rothrock et al., 1985). Stem canker incidence and severity is significantly higher during doublecropping with wheat than in monocropping with soybean alone, but there is no increase in DPM infection. This indicates that the relationship between cropping system and stem canker is related to symptom expression rather than rates of infection (Rothrock et al., 1988).

The severity of stem canker symptoms can change when a host plant is parasitized by other organisms. Three-cornered alfalfa hopper (*Spissistilus festinus* Say) (TCAH) feeding on soybean causes characteristic symptoms described as main stem feeding girdles. TCAH infestations reduce soybean yields by causing plant death, reducing seed weight, and causing

seed loss during harvest due to stem lodging and breakage. Stem canker symptom severity, especially canker length, is usually increased in plants with main stem girdles induced by TCAH when compared to plants parasitized with stem canker only (Padgett et al., 1994; Russin and Boethel, 1986). This relationship appears to be mostly additive. However, the authors qualify their findings by saying the relationship between these two pests could be due to a general reduction in plant vigor caused by TCAH rather than any actual physiological relationship within the plant (Russin and Boethel, 1986). Soybean looper (*Pseudoplusia includens* Walker), a defoliating insect of soybean, and soybean cyst nematode (*Heterodera glycines* Ichinohe) a parasitic nematode of soybean, have been found to reduce the severity of stem canker symptoms when either soybean looper, soybean cyst nematode, or both pests are found on plants with stem canker (Padgett et al., 1994; Russin et al., 1989).

DPM produces a phytotoxin after it infects its host that leads to the foliar symptoms associated with southern stem canker. Different strains of DPM have been found to produce different amounts of toxin. Disease severity has been shown to be related to the amount of phytotoxin to which a plant has been exposed (Lalitha et al., 1989). Lalitha et al. (1989) were the first to isolate this phytotoxin from soybean plants with stem canker symptoms that were infected with single-spore isolates of DPM. They reintroduced the isolated toxin in serial dilutions to healthy soybean plants and found a positive linear relationship ( $R^2 = 0.61$ ) between disease severity and toxin concentration. The single-spore isolates of DPM used in this experiment produced different amounts of toxin in culture. The amount of toxin produced in culture by a given DPM strain was associated with the length of cankers caused by inoculating plants with that DPM strain and with the length of cankers caused by exposing the plant to the isolated phytotoxin produced by the strain.

## Disease Control

When, where, and even if stem canker outbreaks will occur in a given growing season is difficult to predict and depends on many external factors. Therefore, most control strategies focus on preventing the spread of the disease, reducing inoculum levels in the field, avoiding conditions that promote infection, and using resistant cultivars rather than fungicides. To prevent the spread of the disease from infested fields to uninfested fields, Backman et al. (1985) recommended cleaning equipment before moving it from an infested field to a noninfested field. They also discouraged planting infested seed in a noninfested field even though the spread of the disease via seed is debatable. Infected soybean residue can also spread the disease if it is moved from one field to another (Grau et al., 2004). Inoculum levels in the field are reduced by tilling under infected soybean debris, as discussed earlier. To avoid conditions that promote infection, planting later in the season has also been recommended in some cases. Delaying planting until late June or July avoids the initial burst of inoculum released during May and early June. However, delayed planting is not a guarantee against infection because spores may still be released in late June and July, and plants may still be infected if temperature and rainfall conditions are within the parameters for infection. Chemical control with fungicides is certainly possible, but is not always economically feasible. Fungicides can be an economically effective means of controlling stem canker if they are part of a management strategy that also employs the use of cultivars that are moderately resistant to the disease (Weaver et al., 1984). Control of stem canker cannot be achieved with fungicides paired with highly susceptible cultivars because production costs would quickly exceed the crop returns. Using fungicides with highly resistant cultivars provides little or no benefit in terms of profits (Backman et al., 1985). If foliar fungicides are utilized, they must be applied well before any evidence of disease. Backman et al.



(1985) suggested that fungicides be applied during early vegetative growth periods when spores are actively being produced to prevent infection even though symptoms may not appear until much later in the growing season.

### Sources of Resistance

In most cases, stem canker can be controlled by removing or deep-tilling infested soybean debris combined with planting resistant cultivars. Cultivar resistance to stem canker is conditioned by at least five different dominant resistance genes. One resistance allele at any of these loci will condition resistance to DPM. The exact functions of these genes are not known, but in each case the resistant reaction is activated after infection has occurred, and thus does not prevent infection itself (Ploetz and Shokes, 1987b). In the late 1970's and early 1980's, soybean cultivars and breeding lines were observed to have different reactions to DPM under natural infection in the field (Keeling, 1982; Weaver et al., 1984). In most cases, the cultivar Tracy-M had significantly lower disease levels than other cultivars, and was considered highly resistant. The suspected source of resistance in Tracy-M is the cultivar CNS, which is a common ancestor of several highly resistant cultivars including 'Bay' (Keeling, 1982).

To determine the mode of resistance in Tracy-M, it was crossed to the highly susceptible cultivar J77-339 (Kilen et al., 1985). The parents, F<sub>1</sub> generation, F<sub>2</sub> generation, and F<sub>3</sub> lines were artificially inoculated and evaluated for resistance. All members of the F<sub>1</sub> generation were resistant, indicating that the resistant allele(s) in Tracy-M was dominant. The F<sub>2</sub> generation segregated in an approximate 15:1 ratio (resistant: susceptible). The F<sub>3</sub> generation segregated in an approximate 7:8:1 ratio (resistant: segregating: susceptible). The segregation ratios in the F<sub>2</sub>

and F<sub>3</sub> generations indicate the presence of two major dominant genes conditioning resistance to stem canker in Tracy-M.

Kilen and Hartwig (1987) used F<sub>4</sub> lines of individual F<sub>3</sub> plants from an earlier study (Kilen et al., 1985) that were uniformly resistant in order to separate the two resistance genes in Tracy-M into two separate lines. To do this, they chose two F<sub>4</sub> lines that were uniformly resistant, crossed them to each other and to J77-339 and evaluated them for resistance. The F<sub>2</sub> generation of the populations created from the F<sub>4</sub> lines crossed to J77-339 segregated in a 3:1 ratio (resistant: susceptible). When members of the two F<sub>4</sub> lines were crossed to each other they segregated 15:1 (resistant: susceptible). Their results indicated that the two F<sub>4</sub> lines both had single dominant resistance genes at two different loci. They designated these two resistance genes *Rdc1* and *Rdc2*. In 1994, two germplasm lines, D85-10404 and D85-10412, were released with resistance to stem canker (Kilen and Hartwig, 1994). The source of resistance in D85-10404 was *Rdc1* from Tracy-M and the source of resistance in D85-10412 was *Rdc2* from Tracy-M. Both germplasm lines were developed from the cross of Tracy-M x J77-339 reported in Kilen et al. (1985).

Additional resistance genes have been found in the cultivars Crockett and Dowling (Bowers et al., 1993). In that study, Crockett, Dowling, and Tracy-M were used as resistant parents and ‘Coker 338’ and ‘Johnston’ as susceptible parents. They crossed each resistant parent to each susceptible parent and to the other resistant cultivars. F<sub>1</sub> plants from each cross were backcrossed to the susceptible parents. F<sub>2</sub> plants from the original crosses were harvested individually and used to create F<sub>2:3</sub> lines. Members of all generations were then evaluated for resistance. Based on their segregation ratios, Crockett and Dowling were each found to contain single dominant genes conditioning resistance to stem canker. The resistance genes from

Crockett and Dowling were at different loci from each other and also from *Rdc1* and *Rdc2* found in Tracy-M. They assigned the symbols *Rdc3* and *Rdc4* to the resistance genes found in Crockett and Dowling respectively.

Additional sources of resistance to stem canker have been found in two soybean plant introductions (Tyler, 1995). PI 230976 and PI 398469 both have single dominant genes conditioning resistance to stem canker that are at different loci from *Rdc1-4*, but as of yet have not been assigned symbols. It is not known if the genes in these plant introductions are at different loci from each other.

## References

- Athow K.L., Caldwell R.M. (1954) A comparative study of *Diaporthe* stem canker and pod and stem blight of soybean. *Phytopathology* 44:319-325.
- Backman P.A., Weaver D.B., Morgan-Jones G. (1985) Soybean stem canker: an emerging disease problem. *Plant Disease* 69:641-647.
- Black B.D., Padgett G.B., Russin J.S., Griffin J.L., Snow J.P., Berggren G.T. (1996) Potential weed hosts for *Diaporthe phaseolorum* var. *caulivora*, causal agent for soybean stem canker. *Plant Disease* 80:763-765.
- Bowers G.R., Ngeleka K., Smith O.D. (1993) Inheritance of stem canker resistance in soybean cultivars Crockett and Dowling. *Crop Science* 33:67-70.
- Damicone J.P., Berggren G.T., Snow J.P. (1987) Effect of free moisture on soybean stem canker development. *Phytopathology* 77:1568-1572.
- Damicone J.P., Snow J.P., Berggren G.T. (1990) Spatial and temporal spread of soybean stem canker from an inoculum point source. *Phytopathology* 80:571-578.
- Fehr W.R., Caviness C.E., Burmood D.T., Pennington J.S. (1971) Stage of development descriptions for soybeans, *Glycine max* (L.) Merrill. *Crop Science* 11:929-931.
- Fernandez F.A., Phillips D.V., Russin J.S., Rupe J.C. (1999) Stem Canker, in: G. L. Hartman (Ed.), *Compendium of soybean diseases*, APS Press, St. Paul, MN. pp. 33-35.

- Frosheiser F.I. (1957) Studies on the etiology and epidemiology of *Diaporthe phaseolorum* var. *caulivora*, the cause of stem canker of soybeans. *Phytopathology* 47:87-94.
- Grau C.R., Dorrance A.E., Bond J., Russin J.S. (2004) Fungal Diseases, in: H. R. Boerma and J. Specht (Eds.), *Soybeans: Improvement, Production, and Uses*, American Society of Agronomy, Madison, WI. pp. 679-763.
- Higley P.M., Tachibana H. (1987) Physiologic specialization of *Diaporthe phaseolorum* var. *caulivora* in soybean *Plant Disease* 71:815-817.
- Hildebrand A.A. (1952) Stem canker: a disease of increasing importance on soybeans in Ontario. *Soybean Digest* 12:12-15.
- Hildebrand A.A. (1956) Observations on stem canker and pod and stem blight of soybeans in Ontario. *Canadian Journal of Botany* 34:577-599.
- Hilty J.W. (1991) The role of soybean debris in survival and inoculum production of *Diaporthe phaseolorum* var. *caulivora*, the cause of stem canker on soybean. *Journal of the Tennessee Academy of Science* 66:99-101.
- Hobbs T.W., Schmitthenner A.F., Ellett C.W. (1981) Top dieback of soybean caused by *Diaporthe phaseolorum* var. *caulivora*. *Plant Disease* 65:618-620.
- Keeling B.L. (1982) A seedling test for resistance to soybean stem canker caused by *Diaporthe phaseolorum* var. *caulivora*. *Phytopathology* 72:807-809.
- Keeling B.L. (1985) Soybean cultivar reactions to soybean stem canker caused by *Diaporthe phaseolorum* var. *caulivora* and pathogenic variation among isolates. *Plant Disease* 69:132-133.
- Kilen T.C., Hartwig E.E. (1987) Identification of single genes controlling resistance to stem canker in soybean. *Crop Science* 27:863-864.
- Kilen T.C., Hartwig E.E. (1994) Registration of soybean germplasm lines resistant to stem canker and *Phytophthora* rot: D85-10404 and D85-10412. *Crop Science* 34:292.
- Kilen T.C., Keeling B.L., Hartwig E.E. (1985) Inheritance of reaction to stem canker in soybean. *Crop Science* 25:50-51.
- Krausz J.P., Fortnum B.A. (1983) An epiphytotic of *Diaporthe* stem canker of soybean in South Carolina. *Plant Disease* 67:1128-1129.
- Kulik M.M. (1984) Symptomless infection, persistence, and production of pycnidia in host and non-host plants by *Phomopsis batatatae*, *Phomopsis phaseoli*, and *Phomopsis sojae*, and the taxonomic implications. *Mycologia* 76:274-291.

- Lalitha B., Snow J.P., Berggren G.T. (1989) Phytotoxin production by *Diaporthe phaseolorum* var. *caulivora*, the causal organism of stem canker of soybean. *Phytopathology* 79:499-504.
- Morgan-Jones G. (1989) The *Diaporthe/Phomopsis* complex: taxonomic considerations, World Soybean Research Conference IV, Buenos Aires, Argentina. pp. 1699-1706.
- Padgett G.B., Russin J.S., Snow J.P., Boethel D.J., Berggren G.T. (1994) Interactions among the soybean looper (*Lepidoptera: Noctuidae*), threecornered alfalfa hopper (*Homoptera Membracidae*), stem canker, and red crown rot in soybean. *Journal of Entomological Science* 29:110-119.
- Ploetz R.C., Shokes F.M. (1985) Soybean stem canker incited by ascospores and conidia of the fungus causing the disease in the southeastern United States. *Plant Disease* 69:990-992.
- Ploetz R.C., Shokes F.M. (1987a) Factors influencing infection of soybean seedlings by southern *Diaporthe phaseolorum*. *Phytopathology* 77:786-790.
- Ploetz R.C., Shokes F.M. (1987b) Infection of different plant parts of soybean seedlings by southern *Diaporthe phaseolorum* and its role in the development of stem canker symptoms. *Canadian Journal of Botany* 65:2104-2108.
- Rothrock C.S., Hobbs T.W., Phillips D.V. (1985) Effects of tillage and cropping system on incidence and severity of southern stem canker of soybean. *Phytopathology* 75:1156-1159.
- Rothrock C.S., Phillips D.V., Hobbs T.W. (1988) Effects of cultivar, tillage, and cropping system on infection of soybean by *Diaporthe phaseolorum* var. *caulivora* and southern stem canker symptom development. *Phytopathology* 78:266-270.
- Roy K.W., Miller W.A. (1983) Soybean stem canker incited by isolates of *Diaporthe* and *Phomopsis* spp. from cotton in Mississippi. *Plant Disease* 67:135-137.
- Rupe J.C., Sutton E.A., Becton C.M., Gbur E.E. (1996) Effect of temperature and wetness period on recovery of the southern biotype of *Diaporthe phaseolorum* var. *caulivora* from soybean. *Plant Disease* 80:255-257.
- Rupe J.C., Sutton E.A., Becton C.M., Gbur E.E. (1999) Effect of soybean growth stage at the time of inoculation with *Diaporthe phaseolorum* var. *meridionales* on stem canker development and yield. *Plant Disease* 83:582-586.
- Russin J.S., Boethel D.J. (1986) Effects of girdling by the threecornered alfalfa hopper on symptom expression of soybean stem canker and associated soybean yields. *Plant Disease* 70:759-761.
- Russin J.S., Layton M.B., Boethel D.J., McGawley E.C., Snow J.P., Berggren G.T. (1989) Development of *Heterodera glycines* on soybean damaged by soybean looper and stem canker. *Journal of Nematology* 21:108-114.

- Smith E.F., Backman P.A. (1989) Epidemiology of soybean stem canker in the southeastern United States: Relationship between time of exposure to inoculum and disease severity. *Plant Disease* 73:464-468.
- Subbarao K.V., Snow J.P., Berggren G.T., Damicone J.P., Padgett G.B. (1992) Analysis of stem canker epidemics in irrigated and nonirrigated conditions on differentially susceptible soybean cultivars. *Phytopathology* 82:1251-1256.
- Threinen J.T., Kommedahl T., Klug R.J. (1959) Hybridization between radiation-induced mutants of two varieties of *Diaporthe phaseolorum*. *Phytopathology* 49:797-801.
- Tyler J.M. (1995) Additional sources of stem canker resistance in soybean plant introductions. *Crop Science* 35:376-377.
- Weaver D.B., Coper B.H., Backman P.A., Crawford M.A. (1984) Cultivar resistance to field infestations of soybean stem canker. *Plant Disease* 68:877-879.
- Welch A.W., Gilman J.C. (1948) Hetero- and homo-thallic types of *Diaporthe* on soybeans. *Phytopathology* 38:628-637.
- Whitehead M.D. (1966) Stem canker and blight of birdsfoot-trefoil and soybeans incited by *Diaporthe phaseolorum* var. *sojae*. *Phytopathology* 56:396-400.
- Wrather J.A. (2004) Soybean disease loss estimates for the United States in 2003. United Soybean Board domestic report, final report for project # 4215.
- Wrather J.A., Anderson T.R., Arsyad D.M., Gai J., Ploper L.D., Porta-Puglia A., Ram H.H., Yorinori J.T. (1997) Soybean disease loss estimates for the top 10 soybean producing countries in 1994. *Plant Disease* 81:107-110.

## CHAPTER 3

### OLEIC ACID LITERATURE REVIEW

#### Benefits of Elevated Oleic Acid

Breeding mid-oleic soybean, or soybean that produces oleic acid at a concentration of greater than 500 g kg<sup>-1</sup> of total oil content, would increase the overall value of soybean oil and the price paid to growers for their crop. The typical fatty acid profile of soybean seeds is 100 g kg<sup>-1</sup> palmitic acid (16:0), 40 g kg<sup>-1</sup> stearic acid (18:0), 220 g kg<sup>-1</sup> oleic acid (18:1), 540 g kg<sup>-1</sup> linoleic acid (18:2), and 100 g kg<sup>-1</sup> linolenic acid (18:3) (Wilson, 2004). The first number in the parentheses refers to the number of carbon atoms in the molecule, and the second number refers to the number of double bonds between carbon atoms present in the molecule. Fatty acid molecules with zero double bonds such as palmitic acid and stearic acid are referred to as “saturated fats.” Molecules with a single double bond such as oleic acid are referred to as “monounsaturated fats,” and molecules with multiple double bonds such as linoleic and linolenic acids are referred to as “polyunsaturated fats.”

Breeding for increased oleic acid in soybean seeds could potentially have positive effects on the marketing of soybean oil due to increased flavor stability and health benefits. Developing soybean cultivars with altered fatty acid compositions allows breeders to tailor soybean oil to specific markets or end users. About 44% of soybean oil in the USA is consumed as salad dressings and cooking oil. Wilson (2004) suggests that the target oil composition for oil consumed in this manner should have 7% 16:0 and 18:0 combined, 60% 18:1, 31% 18:2, and 2%

18:3. Wilson (2004) suggests developing soybean cultivars specifically for this fatty acid profile for this end use.

Soybean oil higher in 18:1 would also enhance its value for soy biodiesel. Biodiesel blenders are forced to alter the chemical composition of diesel fuel in colder climates due to poor cetane and cold flow properties. The cetane index is a measurement of a diesel fuel's quality of combustion. Specifically, cetane number is the relative amount of time between injection of the fuel into the engine and the beginning of combustion (ignition). A higher cetane number, or shorter time period between injection and ignition, allow diesel engines to operate at higher speeds more effectively. Increasing the 18:1 level of soybean oil increases the cetane index of biodiesel created from soybean oil. Cold flow is a measurement of the lowest operating temperature of a diesel fuel. At low temperatures, biodiesel becomes more viscous and difficult to pump into the combustion chamber of the engine. Lowering the 16:0 level of soybean oil improves the cold flow of biodiesel created from soybean oil. In combination, higher 18:1 and lower 16:0 would improve ignition and reduce performance problems associated with the use of soy biodiesel fuel in colder climates (Dunn et al., 1996).

Lowering the relative amounts of 18:2 and 18:3 could improve the viability of soybean oil as a high temperature frying oil for restaurants and food manufacturers. There is a well-established negative correlation between the amount of 18:1 in soybean seeds and the amount of polyunsaturated fatty acids. Breeding for increased 18:1 also indirectly decreases the polyunsaturated fats (Wilson, 2004). The double bonds in unsaturated fatty acids found in soybean oil are susceptible to oxidation, especially during frying at high temperatures and during storage at room temperature for long periods of time. Oxidation of unsaturated fats produces flavorless but unstable compounds called hydroperoxides. Hydroperoxides are broken down into



to secondary products such as aldehydes, alcohols, ketones, acids, hydrocarbons, esters, and lactones that cause undesired off-flavors in the oil (Frankel, 1991). The more unsaturated the fatty acid, the less stable it is and the faster it is oxidized. The reaction rate with oxygen and the hydroperoxide decomposition rate for 18:3 is faster than for 18:2, which is faster than 18:1 (Gunstone and Hilditch, 1945). These rates are increased under high temperature frying conditions. Decreasing the amount of 18:2 and 18:3 in soybean oil through hydrogenation or breeding improves the oil's oxidative stability during frying and storage at room temperature (Liu and White, 1992b). Soybean lines A6, A16, A17, A87 were developed at Iowa State Univ. with lower 18:3 than commercial cultivars. Chemical and taste panel tests determined these cultivars to be more stable to oxidation than cultivars with normal linolenic acid content (Liu and White, 1992b). Oils from these cultivars were also more suitable for frying, most likely due to their improved oxidative stability at higher temperatures (Liu and White, 1992a). Warner and Gupta (2005) determined that soybean oil high in 18:1 is stable under storage and frying conditions. However, taste panels that tested potato chips fried in high oleic soybean oil reported easily detectable fishy and rancid flavors. The authors postulated that the off flavors were easier to detect in high oleic soybean oil because oleic acid is relatively flavorless and lacks the desirable intense deep fried flavor of other frying oils (Warner and Gupta, 2005). If this is the case, high oleic soybean oil would need to be blended with other oils for a favorable flavor while maintaining the improvement in shelf life and frying stability.

Soybean oil high in oleic acid and low in polyunsaturated fatty acids is also healthier than soybean oil with the traditional fatty acid profile. Chang and Huang (1998) found that monounsaturated fats are more effective at preventing arteriosclerosis than polyunsaturated fats in rats. A chemical hydrogenation process is often used to improve the shelf life and stability of

standard soybean oil. Catalytic hydrogenation is a method of increasing the oxidative stability of soybean oil by adding hydrogen atoms to olefinic double bonds and converting 18:3 to 18:2, to 18:1, and eventually to 18:0. However, hydrogenation produces unsaturated fatty acids with *trans*-isomers as opposed to the naturally occurring *cis*-isomers (Puri, 1978). *Trans* fatty acids have been linked to adverse health effects (Hayakawa et al., 2000). Unsaturated fatty acids in the *cis* configuration have a natural bend in the molecule. Fatty acids in the *trans* configuration have a straighter molecular structure that may emulate saturated fatty acids during human or animal consumption (Wilson, 2004). Ingesting large quantities of *trans* fatty acids increases total blood serum cholesterol levels (Zock and Katan, 1992). *Trans* fatty acids are also associated with increased blood serum levels of low density lipoprotein (LDL, “bad cholesterol”) and reduced levels of high density lipoprotein (HDL, “good cholesterol”). This results in an elevated LDL to HDL ratio which may increase the risk of heart attack by as much as 27% (Zock and Katan, 1992). *Trans* fatty acids could be considered to have a more negative effect on serum cholesterol levels than saturated fatty acids since both saturated and *trans* fatty acids increase LDL levels, but only *trans* fatty acids lower HDL (Hayakawa et al., 2000). Consuming unhydrogenated, unsaturated fatty acids such as 18:1 in the naturally occurring *cis* configuration in place of saturated and unsaturated fatty acids in the *trans* configuration is more effective in preventing coronary heart disease in women than simply reducing overall fat intake (Zock and Katan, 1992). The consumption of nonhydrogenated oils was also found to be associated with a reduced risk of breast cancer (Dai et al., 2002). Increasing the relative amount of oleic acid in soybean seeds has been suggested as a method of reducing the need for hydrogenation (Wilson, 2004).

## Breeding for Elevated Oleic Acid

Research suggests that oleic acid content in soybean seeds is a quantitative trait controlled by both major fatty acid biosynthesis genes and modifier genes with smaller effects (Rebetzke et al., 1998). As a result, recurrent selection has been used successfully to increase 18:1 content in soybean seeds. The germplasm line N78-2245 was most likely the first line developed with higher 18:1 (Burton et al., 1983). The recurrent selection population used to develop N78-2245 was created by crossing six lines with elevated 18:1 to the male sterile maintainer line N69-2774. After four cycles of recurrent selection, Burton et al. (1983) had increased oleic acid from 24.8 to 33.0%. While phenotypically selecting only for increased 18:1, 18:2 decreased from 533 to 470 g kg<sup>-1</sup>, and 18:3 decreased from 78 to 63 g kg<sup>-1</sup>. The average rate of gain for 18:1 was 1.6 +/- 0.2% per cycle. Carver et al. (1986) intermated two plant introductions with elevated 18:1, PI 90406 and PI 92567 to produce a recurrent selection population segregating for 18:1 content to evaluate cumulative response to eight cycles of recurrent selection. Six F<sub>4</sub> lines from this population were crossed to the male sterile maintainer line N69-2774. Cycles 1 to 3 involved individual or mass selection, while mass plus within half-sib family selection was used for cycles 4 to 7, and S1 progeny selection was employed in cycle 8. In cycle 6, reverse selection for low oleic acid was initiated to ensure that oleic acid biosynthesis genes were still segregating within the population. Selected lines from each cycle were composited and tested in eight environments. Oleic acid increased from 227 g kg<sup>-1</sup> in cycle 0 to 416 g kg<sup>-1</sup> in cycle 8. Average rate of gain in 18:1 percentage ranged from 11.5 g kg<sup>-1</sup> +/- .1.7 for mass selection to 26.4 g kg<sup>-1</sup> +/- 2.4 for mass plus within half-sib family selection. Linoleic acid decreased from 540 to 387 g kg<sup>-1</sup> and 18:3 decreased from 87 to 59 g kg<sup>-1</sup> over the

same period while selecting only based on 18:1 content. In cycle 6, changes in oleic acid percent after reverse selection indicated that oleic acid biosynthesis genes had not been fixed after six cycles of selection.

Mutation breeding has also been an effective means of developing soybean lines with increased 18:1. Wilcox et al. (1984) treated seeds of 'Century' soybean with EMS and evaluated M2 progeny for seed fatty acid composition. They found greater variation in relative contents of each fatty acid in mutated seeds compared to the control. Rahman et al. (1994) irradiated seeds of the cultivar Bay using x-rays to create M23, a soybean mutant line with high oleic acid. Takagi and Rahman (1996) performed inheritance studies to determine the inheritance of the high oleic trait in M23 by making the cross M23 x Bay and its reciprocal, Bay x M23. Oleic acid concentration of F<sub>1</sub> seeds of both crosses was significantly different than both parents and the midparent value, indicating partial dominance for high oleic acid trait. The F<sub>2</sub> generation segregated in an approximate 1:2:1 ratio of normal (20-26%), intermediate (27-36%), and high (39-55%) relative oleic acid content. A population of F<sub>1</sub> plants backcrossed to M23 segregated in a 1:1 ratio of intermediate and high oleic acid plants. These results indicate that high oleic acid in the mutant line M23 is conditioned by a single recessive gene. This gene was given the designation *Ol*, such that homozygous recessive plants with the genotype *olol* have the highest oleic acid content. Linoleic acid content was inversely related to oleic acid content in each generation, whereas linolenic acid remained unchanged. Inheritance studies determined that the *Ol* gene segregated independently from *fap1* and *sop1* controlling reduced palmitic acid, and also from *fan* and *fanx<sup>a</sup>* conditioning reduced 18:3 (Rahman et al., 2004).

Transformation has also been used to create soybean lines with elevated 18:1. The main transgenic approach is to downregulate the *FAD2-1* gene that encodes a microsomal omega-6

desaturase responsible for converting the monounsaturated 18:1 to the polyunsaturated 18:2 (Bilyeu et al., 2003). Buhr et al. (2002) reduced expression of *FAD2-1* using two different methods. One method involved transforming soybean with a cassette that replaced the standard 3' untranslated region with a 3' self-cleaving ribozyme transcript that retains *FAD2-1* gene transcripts in the nucleus. This cassette also included a copy of the *FAD2-1* gene. Their second method was to transform plants with an antisense copy of the *FAD2-1* gene that activated post-transcriptional gene silencing mechanisms. Transformants with ribozyme transcripts had oleic acid contents as high as 62.3%. Transformants with antisense transcripts had oleic acid contents as high as 77.8%. Buhr et al. (2002) also produced transgenic plants that downregulated both the *FAD2-1* gene and a palmitoyl-thioesterase gene, *FatB*, involved in the synthesis of palmitic acid. Transformants with both genes downregulated had oleic acid levels that exceeded 85% and less than 6% saturated fatty acids. Pioneer Hi-Bred International, Inc. plans to market high 18:1 soybeans under the Plenish<sup>®</sup> tradename. Plenish soybeans have been transformed with seven abbreviated or partial copies of the *FAD2-1* gene that activate the plant's gene silencing mechanisms and reduce the expression of the native intact *FAD2-1* gene (Kinney et al., 2008).

In addition to *FAD2-1*, many alleles exist at other loci that alter unsaturated fat content in soybean seeds. Alt et al. (2005a) created populations segregating for unsaturated fatty acid alleles by making all possible crosses of FA22, N98-4445A, and M23. FA22 is a soybean line developed at Iowa State Univ. with elevated oleic acid and N98-4445A is a germplasm line developed by the USDA-ARS and North Carolina State Univ. with elevated 18:1 (Burton et al., 2006; Wilson, 2004). Transgressive segregates were found in F<sub>2</sub> and F<sub>3</sub> generations of all crosses, with 18:1 concentrations as high as 73% found in the N98-4445A x M23 population. Their data indicates that many alleles in these populations affect 18:1 content and each of the

three high oleic parents differed from the others in at least some of the alleles that condition their oleic acid content (Alt et al., 2005a).

With a few exceptions, such as *ol*, alleles that condition altered unsaturated fatty acids have primarily been given *fad* and *fan* designations. The allele in N78-2245, the first elevated 18:1 germplasm line, was given the original *fad* designation in soybean. Wilcox and Cavins (1985) exposed Century soybean to chemical mutagenesis and selected C1640, a M<sub>2</sub> line that had the lowest 18:3, about 3.5%. This low 18:3 phenotype was determined by inheritance studies to be conditioned by a single recessive allele designated *fan*. It has been hypothesized that mutations in *fad* alleles affect  $\omega$ -6 desaturase activity and mutations in *fan* alleles affect  $\omega$ -3 desaturase activity. Omega-6 desaturase catalyzes a double bond to 18:1, converting it to 18:2. Omega-3 desaturase catalyzes another double bond to 18:2, converting it to 18:3 (Wilson, 2004). This hypothesis was tested in a cross of N78-2245 (*fadfad FanFan*) x PI 123440 (*FadFad fanfan*). The segregation ratio of the F<sub>3</sub> generation fit a model for two independently segregating genes (Wilson and Burton, 1986). The F<sub>4</sub> generation of a cross of N83-375 (*FadFad FanFan*) x N85-2176 (*fadfad fanfan*) was screened for appropriate phenotypic segregation of  $\omega$ -6 desaturase and  $\omega$ -3 desaturase activity to confirm their conclusion (Wilson et al., 1990). Later, this was confirmed at the molecular level when cDNA probes from the *FAD2-1* and *FAD3* genes were used to probe mRNA and DNA of inbred lines of the major phenotypic and predicted genotypic classes of the same cross. The *fan1* allele is located at the *FAD3* gene locus (Byrum et al., 1995). The *ol* allele in the mutant line M23 should have been given a *fad* designation since it conditions elevated 18:1 through a deletion in the *FAD2-1* gene (Kinoshita et al., 1998). Hawkins et al. (1983) mutagenized the line FA9525 and selected a line with 3.5% 18:3, designating it as A5. The recessive allele *fan1* conditioned the low 18:3 phenotype in A5. Two

additional mutations, *fan2* and *fan3* were also described in this population. Fehr et al. (1992) created a line A29 that was homozygous for all 3 mutations, *fan1 fan2* and *fan3*. This line had 18:3 concentrations as low as 1.1%.

There is a very wide range in reported heritability estimates for the oleic acid trait. Realized heritability for 18:1 in the recurrent selection population from which N78-2245 was developed was 0.21 +/- 0.06 after four cycles of recurrent selection (Burton et al., 1983). Hawkins et al. (1983) planted 19 lines from the 1979 Northern States Uniform Soybean Tests in seven different environments (Hawkins et al., 1983). Broad-sense heritability estimates on a seed sample, plant, plot, and entry-mean basis for oleic acid were 0.50, 0.52, 0.58, and 0.92 respectively. Alt et al. (2005b) estimated the narrow-sense heritability of 18:1 content in M23 based on F<sub>2</sub> plants and F<sub>2:3</sub> lines by crossing M23 x Archer, a cultivar with normal oleic acid content. Narrow-sense heritability was estimated at 0.33 based on F<sub>3</sub> seed and 0.44 based on F<sub>2</sub> plants. Broad-sense heritability for 88 F<sub>2:3</sub> lines was estimated at 0.37 to 0.46 on a plot basis and 0.82 on an entry-mean basis. Bachlava et al. (2008a) created three F<sub>5</sub> RIL populations to study oleic acid inheritance. The FAE population was a cross of N98-4445A x 'Satelite' and is segregating for oleic acid genes and the *fap<sub>nc</sub>* allele conditioning lower palmitic acid. Satelite is a cultivar with low palmitic and linolenic acids (Cardinal et al., 2007). The FAF population was a cross of N97-3363-3 x PI 423893. N97-3363-3 is a high oleic sister line of N98-4445A and PI 423893 is a mid-oleic plant introduction of unknown ancestry (Burton et al., 2006). FAF was segregating for oleic acid genes and the *fan(PI 123440)* allele conditioning lower linolenic acid (Bachlava et al., 2008a). FAS was from the cross of N98-4445A x PI 423893. FAS is segregating for oleic acid genes and the *fan(PI 123440)* allele conditioning lower linolenic acid (Bachlava et al., 2008a). Narrow-sense heritability for oleic acid content in FAS was estimated

at 0.76 and 0.88 on a plot-mean and entry-mean basis, respectively, based on 231 F<sub>3</sub>-derived lines grown in one environment in 1 yr (Bachlava et al., 2008b). Heritability in this population may be overestimated as a consequence of possible confounding with genotype x environment interaction due to only one environment being included in the estimate. Narrow-sense heritability for oleic acid content in FAF was estimated at 0.25, 0.8 and 0.96 on a parent-offspring, plot-mean, and entry-mean basis, respectively, based on 118 F<sub>5</sub>-derived lines grown in six environment over 2 yr. In the FAE population, narrow-sense heritability was estimated at 0.22, 0.69 and 0.95 on a parent-offspring, plot-mean, and entry-mean basis, respectively, based on 721 F<sub>5</sub>-derived lines grown in six environments over 2 yr. Parent-offspring heritabilities estimated in FAF and FAE populations by regressing F<sub>5:6</sub> progeny on F<sub>2:3</sub> progenitors suggested that early generation selection for oleic acid based on unreplicated lines grown in a single environment should not be done any earlier than the F<sub>3</sub> generation (Bachlava et al., 2008a).

Similarly to heritability, soybean lines developed with elevated 18:1 have a wide range in stability. Lines developed through mutation breeding or transformation are generally more stable than lines developed through recurrent selection. Oliva et al. (2006) grew 17 soybean lines with different fatty acid profiles in 10 different environments to evaluate the stability of each fatty acid. High oleic lines N97-3363-4 and N98-4445A bred through recurrent selection were the least stable for oleic acid, with b-values of 2.53 and 3.28 respectively. They found that much of the variation in oleic acid content in these lines could be attributed to temperature changes. The mutant line M23 and Holl, a cultivar derived from M23, were significantly more stable, with b-values of 0.13 and 0.73 respectively (Oliva et al., 2006). RG9 and AN145-66 were included in a study with 12 other soybean lines with altered fatty acid profiles and three cultivars that were grown in four locations in Southern Ontario, Canada over 3 yr (Primomo et al., 2002).



RG9 is a soybean line with elevated oleic acid levels that was developed from mutating seeds of cultivar Elgin 87 using EMS (Primomo et al., 2002). AN145-66 is a line developed from the cross A5 x N78-2245 after several generations of recurrent selection for elevated oleic acid levels (Primomo et al., 2002). Using a regression stability analysis, RG9 had a b-value of 1.15, indicating it was stable across environments. This was expected since the altered oleic acid levels in this line is most likely due to the mutation of a single gene with large phenotypic effects. AN145-66 had a b-value of 1.63, indicating that oleic acid levels in this line were not stable across different environments. This was attributed to the likelihood that elevated oleic acid in this line was conditioned by multiple genes with smaller effects due to this line being the product of recurrent selection rather than mutagenesis (Primomo et al., 2002). In their study, oleic and linoleic acids were more sensitive overall to environmental interactions than the other fatty acids found in soybean seed oil. In an ANOVA analysis, all main effects of genotype, location, and year, and all of their two- and three-order interactions were significant for differences in oleic acid. Other studies have also reported significant genotype x environment interactions for fatty acid traits. In one recurrent selection breeding population for altered 18:1 content, the main effect of environment and genotype x environment interaction were both significant for 18:1, 18:2, and 18:3 (Carver et al., 1986).

Research has well established that lines with altered 18:1 have different phenotypic expressions of the trait based on the environment in which they are grown. Environmental temperature may account for some of these differences. As environmental temperature increases, the percent total oil deposition into seeds increases (Wolf et al., 1982). Greenhouse and growth chamber studies have found that total oil concentration in seeds increased as mean daily temperature increased, with maximum oil deposition between 25 and 28°C (Dornbos and

Mullen, 1992; Gibson and Mullen, 1996). Above 28°C oil concentration declined. Piper and Boote (1999) found similar results using field and weather data from the USDA Uniform Regional Soybean Tests with a significant positive correlation between temperature and oil concentration, and optimum oil deposition occurring at 27.7°C (Piper and Boote, 1999). Even though there is a general trend of increased oil with higher temperatures, each fatty acid in soybean oil reacts differently to temperature changes. Relative percent of 18:1 increases, relative percents of 18:2 and 18:3 decrease, and relative percents of 16:0 and 18:0 remain fairly constant with increasing temperature (Wilson, 2004). Membrane fluidity at lower temperatures is maintained by desaturation of fatty acids. Higher temperatures reduce the requirement for membrane bound unsaturated fatty acids. The temperature dependence of fatty acid desaturase enzymes may account for this trend (Schlueter et al., 2007). Using greenhouse experiments with controlled temperatures, Dornbos et al. (1989) determined that the effects of temperature during podfill have the greatest impact on oleic acid concentration. During podfill, or the period when nutrients are being deposited into soybean seeds, higher temperatures were associated with increased oleic acid concentrations.

Maturity may directly or indirectly have an affect on oleic acid concentration. In general, earlier maturing plants in populations segregating for 18:1 concentration have higher amounts of 18:1 (Wilson, 2004). This may be due to the fact that earlier maturing lines are exposed to higher temperatures during podfill because their podfill period takes place during the earlier, warmer part of the growing season. When selecting lines for elevated 18:1 in populations with wide ranges in maturity, researchers must take great care not to select lines that appear to have higher 18:1 because of genetic control of the trait when in reality their higher 18:1 phenotype is conditioned by the confounding factor of earlier maturity. If a range in maturity is not

intentionally maintained during selection in a population for increased 18:1, population shifts toward earlier maturity usually occur. In one recurrent selection population, flowering date and maturity date were correlated responses to selection for both high and low oleic acid percentage, with number of days to flowering and maturity significantly decreasing in response to selection for high oleic acid percent, and significantly increasing in response to reverse selection for lower oleic acid percentage (Carver et al., 1986). Burton et al. (1983) also reported a 6-d shift towards earlier maturity over the selection period in their recurrent selection population while selecting only for increased 18:1. However, the relationship between 18:1 concentration, temperature, maturity, and day length isn't completely clear. In the FAE, FAF, and FAS populations, 18:1 was positively correlated with average daily temperature during stages R2 to R8 for the FAE population, but negatively correlated for the latter two populations (Bachlava and Cardinal, 2009). The authors propose that the earlier maturities of the FAF and FAS populations may account for their unexpected results. These three populations also have a very wide range in maturity within each population. Linoleic and linolenic acids were negatively correlated with average daily temperature in FAE, but positively correlated in FAF and FAS. They propose that the unexpected direction of correlation in FAF and FAS could be due to the effect of photoperiod rather than average daily temperature. Stratifying the lines by maturity prior to selection for altered 18:1 may assist in phenotypically selecting lines with the most desirable genotype conducive to expression of the desired level of 18:1. The expression of elevated 18:1 in lines with low stability is enhanced by warmer environments (Oliva et al., 2006). Monteros et al. (2008) used increased day length and temperature to compact the range in maturity in mapping populations while successfully mapping and confirming six QTL for increased 18:1.

Other traits are also correlated with changes in soybean seed unsaturated fatty acid composition. Tocopherols inhibit lipid oxidation and are a natural antioxidant. Tocopherol amount and relative amount of seed linolenic acid are highly positively correlated (Dolde et al., 1999). Oleic acid is negatively correlated with linolenic acid. Recurrent selection for high oleic acid usually also results in lower linolenic acid content (Burton et al., 1983). The strong inverse relationship between oleic and linoleic acids, supports the hypothesis of sequential desaturation, or the conversion of 18:0 first to 18:1, then to 18:2 and then to 18:3, in the biosynthesis of unsaturated fatty acids in soybean seeds (Wilcox et al., 1984).

### Marker Assisted Selection

Marker assisted selection (MAS) of genes conditioning elevated 18:1 concentrations would be helpful to soybean breeders due to the difficulty associated with phenotypic selection for this trait. The significant genotype x environment interactions, poor stability of some elevated 18:1 breeding lines, and the low heritability of the trait in early generations makes it difficult for breeders to accurately select desirable genotypes based on phenotypic information. Phenotypic selection for elevated oleic acid is complicated by maternal effects in some populations. In these populations, neither phenotypic fatty acid analysis of F<sub>2</sub> seeds nor means of individual F<sub>3</sub> plants provide accurate information on the genotypes of F<sub>2</sub> plants (Primomo et al., 2002). With molecular markers linked to loci that condition favorable 18:1 concentrations, plants lacking desirable alleles can be eliminated from the breeding process in earlier generations. Marker-assisted selection would increase the heritability of elevated 18:1 by providing a reliable method of selecting single plants that eliminates confounding environmental and maternal effects. As a result, breeders that employ MAS can save time and

resources by avoiding the selection of plants or lines in early generations that appear to be phenotypically desirable but ultimately lack the desirable combination of alleles. Additionally, it is impossible to determine seed fatty acid composition phenotypically for a single plant prior to reproductive maturity (Pantalone et al., 2004). As a result, breeders are forced to spend time and resources making more crosses than would be necessary with the use of MAS. Using MAS, individual plants from segregating populations that do not possess desirable alleles could be eliminated prior to crossing. This reduces the number of crosses needed in order to ensure the desired combination of alleles is captured in the next generation.

The utility of molecular markers linked to oleic acid alleles in soybean has been recognized since the beginning of marker development in the species. Diers and Shoemaker (1992) tested 243 RFLPs on 60 F<sub>2</sub>-derived lines of a *G. max* x *G. soja* cross A81-356022 x PI 468916. Three RFLPs that mapped to current Lg-A1 (Gm05) were significantly associated with higher 18:1 and lower 18:2 in *G. soja* relative to *G. max*. Two RFLPs that mapped to current Lg-E (Gm15) were significantly associated with lower 18:1 and higher 18:2 levels in *G. soja*. One RFLP that mapped to current Lg-B2 (Gm14) was significantly associated with higher 18:1 levels in *G. soja*, but was not associated with altered levels of 18:2. Lee et al. (1996) conducted the first study on fatty acid QTL that analyzed the consistency of QTL across multiple environments. This study mapped QTL for total oil content rather than specific fatty acids, but is indicative of the environment-specific and population-specific nature of many QTL associated with soybean oil traits. They used single-factor ANOVA across locations to detect QTL that were significant across all environments in a populations of 120 F<sub>4</sub>-derived lines of 'Young' x PI 416937 grown in four environments. Young is a cultivar with competitive yields for its time of release (Burton et al., 1987). PI 416937 has been shown to be tolerant to drought in the field (Sloane et al.,

1990). A second population of 111 F<sub>2</sub>-derived lines of PI 97100 x 'Coker 237' grown in two environments was also analyzed. Progeny of this cross segregated for growth habit, with PI 97100 being indeterminate and Coker 237 being determinate (Lee et al., 1996). In the Young x PI 416937 population, they found six RFLP markers at three independent QTL that were consistent across all environments. They also found 5 RFLP markers at three independent QTL in PI 97100 x Coker 237 population. One of the three QTL wasn't significant at one location, but was still significant in the single-factor ANOVA across all environments, most likely because of its very large effect in a single environment. None of the QTL identified were consistent across the two populations, but this could be attributed to the low marker density of early soybean RFLP maps, or that the effects of total oil QTL vary depending on genetic background. In the PI 97100 x Coker 237 population, two of the three previously identified QTL were confirmed in an independent population of the same cross (Fasoula et al., 2004). Only one of the three previously identified QTL in the Young x PI416937 population was confirmed in an independent population of the same cross (Fasoula et al., 2004).

Brummer et al. (1997) found similar results where many QTL were unstable across multiple years or populations. They evaluated eight soybean populations developed in the Midwestern USA for total seed oil QTL that were significant across multiple years and multiple populations. Their definition of a stable marker-QTL association was one that had a probability of  $P \leq 0.05$  in two or more years and  $P \leq 0.05$  for the 3-yr average using single factor ANOVA. Marker-QTL associations that didn't meet both criteria were considered unstable and less beneficial to breeding programs. Markers on seven different linkage groups were stable across at least 2 of the 3 yr tested and the 3-yr average. Six of the eight populations had at least one stable QTL across multiple years. Ten additional significant associations were found but were unstable

across years. Several QTL were stable across multiple populations, however, no QTL were stable across all eight populations.

Research suggests that modifier genes affect oil composition phenotype in addition to major fatty acid genes, leading to the quantitative nature of oil composition traits (Rebetzke et al., 1998). The greater abundance of SSR markers over RFLPs in soybean provided the technology and map resolution needed to elucidate this theory. Hyten et al. (2004) created 131  $F_6$ -derived recombinant inbred lines (RILs) of the cross 'Essex' x 'Williams' and grew them in five environments over a period of 2 yr. One hundred SSR markers were used to map fatty acid QTL this population. They found QTL for all five fatty acids, including two for 18:1, a minor QTL ( $R^2 = 7.2$ ) on Lg-D1b (Gm02), and a major QTL ( $R^2 = 35.3$ ) on Lg-L (Gm19). The QTL on Gm19 was located in the same interval as the gene, *Dt1*, which conditions plant growth habit or stem termination (determinance or indeterminance). The range in 18:1 content in the mapping population was larger than the range for the other fatty acids. The authors postulated that modifier genes or maturity differences could have contributed to the larger range in 18:1 content. The RILs in the mapping population ranged in maturity from MG III to MG V. A minor maturity QTL was also mapped on Gm19 near the fatty acid QTL and could explain the large effect of the Gm19 QTL for oleic acid. The authors suggested verifying the QTL found in their study in populations where maturity effects are less prominent (Hyten et al., 2004).

As previously discussed, maturity is a major confounding factor of 18:1 concentration.

Monteros et al. (2008) reduced the range in maturity among lines by increasing day length in greenhouse and growing plants in a winter nursery environment to map 18:1 QTL in a population of 'Boggs-RR' x N00-3350. N00-3350 is a single plant selection from N98-4445A with elevated 18:1. Boggs-RR is a well adapted cultivar with resistance to the herbicide

glyphosate (Boerma et al., 2000). Six QTL, one on Gm05, one on Lg-D2 (Gm17), two on LG-G (Gm18), and two on Gm19, were identified with the alleles for increased 18:1 inherited from N00-3350. The most highly significant simple sequence repeat (SSR) markers for these QTL were Satt211 ( $R^2 = 4\%$ ) on Gm05, Satt389 ( $R^2 = 6\%$ ) on Gm17, Satt394 ( $R^2 = 13\%$ ) and Satt191 ( $R^2 = 7\%$ ) on Gm18, and Satt418 ( $R^2 = 9\%$ ) and Satt561 ( $R^2 = 25\%$ ) on Gm19. Together, these six markers accounted for 30% of the variation in oleic acid in the Boggs-RR x N00-3350 population. These markers were also confirmed in an independent population of 'H7242RR' x N00-3350. Due to the narrow range of maturity in these mapping populations, it is likely that these QTL are less affected by the environment.

Bachlava et al. (2009b) identified six QTL each in FAF and FAS populations that were significant across all environments and explained 54.9 and 57.4% of the variation in oleic acid respectively. QTL were mapped to Lg-N (Gm03), Lg-A2 (Gm08), Lg-F (Gm13), Lg-I (Gm20), Gm05, and Lg-E (Gm15) in the FAF population, and to Lg-M (Gm07), Lg-G (Gm18), Gm17, Lg-O (Gm10), Lg-I (Gm20), and Lg-F (Gm13) in the FAS population. The QTL allele with largest effect was contributed by PI 423893 in both populations and mapped to Gm13. It explained 16.5 and 18.3% of the phenotypic variation for oleic acid in both populations, respectively. The alleles for elevated 18:1 at the other five QTL in each population were inherited from N97-3363-3. Only three of the oleic acid QTL were inherited from N98-4445A in the FAS population (Bachlava et al., 2009b). QTL identified on linkage groups Gm19 and Gm05 were not significant across all environments, but did confirm QTL found in other studies (Monteros et al., 2008).

More recently, the soybean genome has been sequenced by the US Department of Energy, Joint Genome Institute (<http://www.phytozome.net/soybean.php>; verified 10 March



2010). Sequencing the soybean genome has facilitated the isolation and mapping of genes involved in fatty acid biosynthesis. Research has shown that *FAD2* genes encode omega-6 fatty acid desaturases responsible for converting 18:1 to 18:2, and that *FAD3* genes encode omega-3 fatty acid desaturases responsible for converting 18:2 to 18:3 (Bilyeu et al., 2003; Schlueter et al., 2007). The *FAD2* gene family in soybean consists of at least five members in four regions of the soybean genome. The *FAD2-1A*, *FAD2-1B*, and *FAD2-2A* genes mapped to Gm10, Gm20, and Gm19, respectively. Previously, *FAD2-2B* and *FAD2-2C* could not be mapped due to a lack of marker polymorphisms. A high degree of sequence homeology was observed between *FAD2-1A* and *FAD2-1B*. RT-PCR determined that *FAD2-2B* and *FAD2-2C* are candidates for the temperature-dependent expression of omega-6 desaturase in soybean seeds during podfill. Quantitative RT-PCR showed an eightfold increase in expression of *FAD2-2C* in pods developing in cooler conditions compared to pods developing in warmer conditions (Schlueter et al., 2007).

The *FAD3* gene family also consists of multiple genes with varying levels of expression in different plant tissues including *GmFAD3A*, *GmFAD3B*, and *GmFAD3C*. A deletion within the *GmFAD3A* gene sequence was associated with the *Fan* locus and was found to be the source of reduced linolenic acid in the soybean line A5 (Bilyeu et al., 2003). This gene was also found to be actively expressed in seed tissue. The *GmFAD3B* and *GmFAD3C* genes do not have seed-specific expression and are candidate genes for the *fan2*, *fan3*, and *fanx* mutations that have minor effects on linolenic acid concentration (Bilyeu et al., 2003).

Since the discovery of these two gene families in soybean, much work has been done to establish associations between fatty acid QTL and the genes within these families. Bachlava et al. (2008a) designed SNP primers to amplify *FAD2-1A*, *FAD2-1B*, *FAD2-2A*, and *FAD2-2B* in

the previously mentioned FAF, FAS, and FAE soybean populations. The previously unmapped *FAD2-2B* mapped closely to *FAD2-2A* on Gm19. Oleic acid QTL with smaller than expected effects were found near *FAD2-1B* and *FAD2-2B*. Maturity QTL also mapped closely to the *FAD2* genes. The *FAD2* genes mapped in this study were not considered to be the major genes that are significant contributors to elevated oleic acid levels in sister lines N98-4445A and N97-3363-3. Bachlava et al. (2009a) found and mapped a fourth isoform of *FAD2*, *FAD2-2D* and two isoforms of an aminoalcoholphosphotransferase gene, *AAPT1a* and *AAPT1*. The previously unmapped *FAD2-2C* gene and the newly discovered *FAD2-2D* gene mapped to Gm15 and Gm03, respectively, while *AAPT1a* mapped to Gm02. Another gene involved in fatty acid biosynthesis, *FAD6*, also mapped to Gm02 in this study. A QTL for 18:3 mapped near the position of *FAD6* in the FAS population that explained 4.6% of the data (Bachlava et al., 2009a). This QTL was associated with higher 18:3 in N98-4445A and expresses dominant gene action. QTL were also found near *AAPT1a* in the FAF and FAS populations that increased 18:1, decreased 18:2 and 18:3, and acted additively. This QTL was not confirmed for linolenic acid, but accounted for 3.4 and 1.5% of the variation in oleic and linoleic acids, respectively (Bachlava et al., 2009a). Multiple interval mapping revealed a minor QTL near *FAD2-2C* that explained 4.5 and 3.6% of the oleic and linoleic acid variation, respectively, in the FAF population, but not in the FAS population. However, single factor analysis indicated that the *FAD2-2C* marker is significantly associated with increased oleic acid and decreased linoleic acid from N98-4445A in the FAS population (Bachlava et al., 2009a).

## References

Alt J.L., Fehr W.R., Welke G.A., Sandhu D. (2005a) Phenotypic and molecular analysis of oleate content in the mutant soybean line M23. *Crop Science* 45:1997-2000.

- Alt J.L., Fehr W.R., Welke G.A., Shannon J.G. (2005b) Transgressive segregation for oleate content in three soybean populations. *Crop Science* 45:2005-2007.
- Bachlava E., Cardinal A.J. (2009) Correlation between temperature and oleic acid seed content in three segregating soybean populations. *Crop Science* 49:1328-1335.
- Bachlava E., Dewey R.E., Burton J.W., Cardinal A.J. (2009a) Mapping candidate genes for oleate biosynthesis and their association with unsaturated fatty acid seed content in soybean. *Molecular breeding* 23:337-347.
- Bachlava E., Dewey R.E., Burton J.W., Cardinal A.J. (2009b) Mapping and comparison of quantitative trait loci for oleic acid seed content in two segregating soybean populations. *Crop Science* 49:433-442.
- Bachlava E., Burton J.W., Brownie C., Wang S., Auclair J., Cardinal A.J. (2008a) Heritability of oleic acid content in soybean seed oil and its genetic correlation with fatty acid and agronomic traits. *Crop Science* 48:1764-1772.
- Bachlava E., Dewey R.E., Auclair J., Wang S., Burton J.W., Cardinal A.J. (2008b) Mapping genes encoding microsomal w-6 desaturase enzymes and their cosegregation with QTL affecting oleate content in soybean. *Crop Science* 48:640-650.
- Bilyeu K.D., Palavalli L., Sleper D.A., Beuselinck P.R. (2003) Three microsomal omega-3 fatty acid desaturase genes contribute to soybean linolenic acid levels. *Crop Science* 43:1833-1838.
- Boerma H.R., Hussey R.S., Phillips D.V., Wood E.D., Rowan G.B., Finnerty S.L. (2000) Registration of 'Boggs' soybean. *Crop sci.* 40:294-295.
- Brummer E.C., Graef G.L., Orf J., Wilcox J.R., Shoemaker R.C. (1997) Mapping QTL for seed protein and oil content in eight soybean populations. *Crop Science* 37:370-378.
- Buhr T., Sato S., Ebrahim F., Xing A., Zhou Y., Mathiesen M., Schweiger B., Kinney A., Staswick P. (2002) Ribozyme termination of RNA transcripts down-regulate seed fatty acid genes in transgenic soybean. *Plant Journal* 30:155-163.
- Burton J.W., Wilson R.F., Brim C.A. (1983) Recurrent selection in soybeans. IV. selection for increased oleic acid percentage in seed oil. *Crop Science* 23:744-747.
- Burton J.W., Brim C.A., Young M.F. (1987) Registration of Young soybean. *Crop Science* 27:1093.
- Burton J.W., Wilson R.F., Rebetzke G.J., Pantalone V.R. (2006) Registration of N98-4445A mid-oleic soybean germplasm line. *Crop Science* 46:1010-1012.
- Byrum J.R., Kinney A.J., Shoemaker R.C., Diers B.W. (1995) Mapping of the microsomal and plastid omega-3 fatty acid desaturases in soybean. *Soybean genetics newsletter* 22:121-124.

- Cardinal A.J., Burton J.W., Camacho-Roger A.M., Yang J.H., Wilson R.F., Dewey R.E. (2007) Molecular analysis of soybean lines with low palmitic acid content in the seed oil. *Crop Science* 47:304-310.
- Carver B.F., Burton J.W., Wilson R.F., Carter T.E., Jr. (1986) Cumulative response to various recurrent selection schemes in soybean: oil quality and correlated agronomic traits. *Crop Science* 26:853-858.
- Chang N.W., Huang P.C. (1998) Effects of the ratio of polyunsaturated and monounsaturated fatty acid on rat plasma and liver lipid concentration. *Lipids* 33:481-487.
- Dai Q., Shu X.O., Jin F., Gao Y.T., Ruan Z.X., Zheng W. (2002) Consumption of animal foods, cooking methods, and risk of breast cancer. *Cancer epidemiology biomarkers and prevention* 11:801-808.
- Diers B.W., Shoemaker R.C. (1992) Restriction fragment length polymorphism analysis of soybean fatty acid content. *Journal of the american oil chemists society* 69:1242-1244.
- Dolde D., Vlahakis C., Hazebroek J. (1999) Tocopherols in breeding lines and effects of planting location, fatty acid composition, and temperature development. *Journal of the american oil chemists society* 76:349-355.
- Dornbos D.L., Mullen R.E. (1992) Soybean seed protein and oil contents and fatty acid composition adjustments by drought and temperature. *Journal of the american oil chemists society* 69:228-231.
- Dornbos D.L., Mullen R.E., Hammond E.G. (1989) Phospholipids of environmentally stressed soybean seeds. *Journal of the american oil chemists society* 66:1371-1373.
- Dunn R.O., Shockley M.W., Bagby M.O. (1996) Improving the low-temperature properties of alternative fuels: Vegetable oil-derived methyl esters. *Journal of the american oil chemists society* 73:1719-1728.
- Fasoula V.A., Harris D.K., Boerma H.R. (2004) Validation and designation of quantitative trait loci for seed protein, seed oil, and seed weight from two soybean populations. *Crop Science* 44:1218-1225.
- Frankel E.N. (1991) Recent advances in lipid oxidation. *Journal of the Science of Food and Agriculture* 54:495-511.
- Gibson L.R., Mullen R.E. (1996) Soybean seed composition under high day and night growth temperatures. *Journal of the american oil chemists society* 73:733-737.
- Gunstone F.D., Hilditch T.P. (1945) The union of gaseous oxygen with methyl oleate, linoleate, and linolenate. *Journal of the chemical society* 1945:836.
- Ha B.-K., Monteros M., Boerma H.R. (2010) Development of SNP assays associated with oleic acid QTLs in N00-3350 soybean *Euphytica* 176:403-415.

- Hawkins S.E., Fehr W.R., Hammond E.G. (1983) Resource allocation in breeding for fatty acid composition of soybean oil. *Crop Sci* 23:900-904.
- Hayakawa K., Linko Y.-Y., Linko P. (2000) The role of *trans* fatty acids in human nutrition. *Starch* 52:229-235.
- Hyten D.L., Pantalone V.R., Saxton A.M., Schmidt M.E., Sams C.E. (2004) Molecular mapping and identification of soybean fatty acid modifier quantitative trait loci. *Journal of the American Oil Chemists Society* 81:1115-1118.
- Kinney A.J., Stecca K.L., Meyer K. (2008) Soybean event DP-305423-1 and compositions and methods for the identification and/or detection thereof, in: U. S. P. Office (Ed.), USA. pp. 1-118.
- Kinoshita T., Rahman S.M., Anai T., Takagi Y. (1998) Genetic analysis of restriction fragment length polymorphism on the fatty acid synthesis in soybean mutants and their progenies: II. High oleic acid mutants with two microsomal omega-6 fatty acid desaturase cDNAs as probes. *Bulletin of the Agricultural Saga University* 83:37-42.
- Lee S.H., Bailey M.A., Mian M.A.R., Carter T.E., Jr., Shipe E.R., Ashley D.A., Parrott W.A., Hussey R.S., Boerma H.R. (1996) RFLP loci associated with soybean seed protein and oil content across populations and locations. *Theoretical and Applied Genetics* 93:649-657.
- Liu H.R., White P.J. (1992a) High-temperature stability of soybean oils with altered fatty acid compositions. *Journal of the American Oil Chemists Society* 69:533-537.
- Liu H.R., White P.J. (1992b) Oxidative stability of soybean oils with altered fatty acid compositions. *Journal of the American Oil Chemists Society* 69:528-532.
- Monteros M.J., Burton J.W., Boerma H.R. (2008) Molecular mapping and confirmation of QTLs associated with oleic acid content in N00-3350 soybean. *Crop Science* 48:2223-2234.
- Oliva M.L., Shannon J.G., Sleper D.A., Eilersieck M.R., Cardinal A.J., Paris R.L., Lee J.D. (2006) Stability of fatty acid profile in soybean genotypes with modified seed oil composition. *Crop Science* 46:2069-2075.
- Pantalone V.R., Walker D.R., Dewer R.E., Rajcan I. (2004) DNA marker-assisted selection for improvement of soybean oil concentration and quality, in: R. F. Wilson, et al. (Eds.), *Legume crop genomics*, AOCS Press, Champaign, IL. pp. 283-311.
- Piper E.L., Boote K.J. (1999) Temperature and cultivar effects on soybean seed oil and protein concentrations. *Journal of the American Oil Chemists Society* 76:1233-1241.
- Primomo V.S., Falk D.E., Ablett G.R., Tanner J.W., Rajcan I. (2002) Genotype x environment interactions, stability, and agronomic performance of soybean with altered fatty acid profiles. *Crop Science* 42:37-44.

- Puri P. (1978) Correlations for trans-isomer formation during partial hydrogenation of oils and fats. *Journal of the American Oil Chemists' Society* 55:865-869.
- Rahman S.M., Takagi Y., Kubota K., Miyamoto K., Kawakita T. (1994) High oleic acid mutant in soybean produced by X-ray irradiation. *Bioscience Biotechnology Biochemistry* 58:1070-1072.
- Rahman S.M., Anai T., Kinoshita T., Arima S., Takagi Y. (2004) Three novel soybean germplasms with unique fatty acid composition using multiple mutant alleles. *Breeding Science* 54:225-229.
- Rebetzke G.J., Burton J.W., Carter T.E., Jr., Wilson R.F. (1998) Genetic variation for modifiers controlling reduced saturated fatty acid content in soybean. *Crop Science* 38:303-308.
- Schlueter J.A., Vasylenko-Sanders I.F., Deshpande S., Yi J., Siegfried M., Roe B.A., Schlueter S.D., Scheffler B.E., Shoemaker R.C. (2007) The FAD2 Gene Family of Soybean:: Insights into the Structural and Functional Divergence of a Paleopolyploid Genome. *Crop Sci* 47:S-14-26. DOI: 10.2135/cropsci2006.06.0382tpg.
- Sloane R.J., Patteson R.P., Carter T.E., Jr. (1990) Field drought tolerance of a soybean plant introduction. *Crop Science* 30:118-123.
- Stacey G., Shoemaker R.C., Jackson S., Schmutz J., Rokhsar D. (2010) Soybean genome project, DoE Joint Genome Institute.
- Takagi Y., Rahman S.M. (1996) Inheritance of high oleic acid content in the seed oil of soybean mutant M23. *Theoretical and Applied Genetics* 92:179-182.
- Warner K., Gupta M. (2005) Potato chip quality and frying oil stability of high oleic acid soybean oil. *Journal of food science* 70:S395-S400.
- Wilcox J.R., Cavins J.F. (1985) Inheritance of low linolenic acid content of the seed oil of a mutant in *Glycine max*. *Theoretical and Applied Genetics* 71:74-78.
- Wilcox J.R., Cavins J.F., Nielsen N.C. (1984) Genetic alteration of soybean oil composition by a chemical mutagen. *Journal of the American Oil Chemists Society* 61:97-100.
- Wilson R.F. (2004) Seed Composition, in: H. R. Boerma and J. E. Specht (Eds.), *Soybeans: Improvement, Production, and Uses*, ASA, CSSA, SSSA, Madison, WI. pp. 621-677.
- Wilson R.F., Burton J.W. (1986) Regulation of linolenic acid in soybeans and gene transfer to high yielding, high protein germplasm, in: A. R. Baldwin (Ed.), *Proceedings on the World Conference on Emerging Technology in the Fats and Oils industry*, Cannes, France 3-8 Nov. 1985, AOCS Press, Champaign, IL. pp. 386-391.
- Wilson R.F., Burton J.W., Kwanyuen P. (1990) Effect of genetic modification of fatty acid composition of soybeans on oil quality, in: D. R. Erickson (Ed.), *Edible fats and oils*

processing: Basic principles and modern practices, AOCS Press, Champaign, IL. pp. 355-359.

Wolf R.B., Cavins J.F., Kleiman R., Black L.T. (1982) Effect of temperature on soybean seed constituents: Oil, protein, moisture, fatty acids, amino acids, and sugars. *Journal of the American Oil Chemists Society* 59:230-232.

Zock P.L., Katan M.B. (1992) Hydrogenation alternatives: effects of trans fatty acids and stearic acid versus linoleic acid on serum lipids and lipoproteins in humans. *J. Lipid Res.* 33:399-410.

## CHAPTER 4

### MAPPING RESISTANCE GENES TO SOUTHERN STEM CANKER<sup>1</sup>

---

<sup>1</sup>Shearin, Z.P., Yates, J.L., Bowers, G.R., and H.R. Boerma. To be submitted to *Crop Science*.



## Abstract

Southern stem canker, caused by the fungus *Diaporthe phaseolorum* (Cooke & Ellis) Sacc., is a very devastating disease of soybean (*Glycine max* (L.) Merr.) worldwide, and particularly in the southern USA. The most successful method for controlling stem canker involves the use of resistant soybean cultivars. The resistance genes *Rdc1*, *Rdc2*, *Rdc3*, *Rdc4*, and *Rdc?*(*PI398469*) have been identified using traditional phenotypic segregation ratios. The objective of this study was to locate stem canker resistance genes on the USDA consensus soybean genetic linkage map. Our approach to mapping the *Rdc* loci involved crossing a source of a single *Rdc* gene with the highly susceptible soybean breeding line J77-339 to create F<sub>2</sub> or F<sub>2:3</sub> soybean mapping populations. The mapping populations used in this study included D85-10412 x J77-339, ‘Crockett’ x J77-339, ‘Dowling’ x J77-339, and PI 398469 x J77-339 for mapping *Rdc2*, *Rdc3*, *Rdc4*, and *Rdc?*(*PI 398469*), respectively. Greenhouse and field screens for stem canker reaction (resistant vs. susceptible) produced the expected 3:1 (resistant:susceptible) segregation ratio for all four mapping populations. Two genes, *Rdc2* from D85-10412 and *Rdc4* from Dowling both mapped to linkage group (Lg)-D1b (Gm02), but are located at different regions on the chromosome. The other two genes, *Rdc3* from Crockett and *Rdc?*(*PI398469*) from PI 398469 mapped to a similar location on Lg-B2 (Gm14) and appear to be clustered with several genes with known disease resistance motifs. Several other genes with known disease resistance motifs are also located near *Rdc2* and *Rdc4*. Markers less than 3 cM from each resistance gene have been identified and may be suitable for marker assisted selection for stem canker resistance.

## Introduction

Southern stem canker, caused by the fungus *Diaporthe phaseolorum* (Cooke & Ellis) Sacc., is an economically important disease of soybean, *Glycine max* (L.) Merr. Yield losses due to stem canker in the USA were estimated to cost growers \$67.1 million in 2003 (Wrather, 2004). The disease has especially been a problem in the southern USA in recent years. After previously being considered as separate varieties, the northern and southern biotypes of stem canker-causing fungi have been classified as separate *formae speciales*, with the organism associated with stem canker in the northern USA and Canada classified as *D. phaseolorum* (Cooke & Ellis) Sacc. f. sp. *caulivora* Morgan-Jones (DPC), and the organism associated with stem canker in the southern USA classified as *D. phaseolorum* (Cooke & Ellis) Sacc. f. sp. *meridionalis* Morgan-Jones (DPM; (Morgan-Jones, 1989). However, the classification of these fungi as *formae speciales* has not been universally accepted, and they are still often referred to as varieties in the literature.

Fungal infection by DPM occurs primarily by rain splashing inoculum from infested soybean residue from the previous growing season onto the new crop during heavy rains (Ploetz and Shokes, 1985). Plants are usually infected through scars on stems, petioles, or petiole bases during early vegetative growth stages (Ploetz and Shokes, 1987). Once a plant becomes infected, there is a latent period where no disease symptoms can be observed. The first symptoms of southern stem canker appear during the plant's early reproductive growth stages as small reddish-brown superficial lesions on the stem, usually near a lower leaf node. The lesions expand longitudinally and form reddish-brown cankers that become slightly sunken as the growing season progresses. Older lesions may appear dark brown with a grayish-brown center and a reddish-brown margin, rarely girdling the stem (Fernandez et al., 1999). Tissue above and

below the canker remains green (Grau et al., 2004). The dead stem tissue blocks the upward flow of water through the vascular tissue, and the seed-bearing portion of the plant becomes water-stressed and usually dies before the plant's full yield potential is reached (Hildebrand, 1952). Leaves usually remain attached to the plant and show symptoms of interveinal chlorosis and necrosis. A phytotoxin plays a role in foliar symptoms and premature plant death (Lalitha et al., 1989). Premature plant death causes a reduction in seed number and size (Fernandez et al., 1999). Since symptoms usually don't appear until the reproductive growth stages are reached, growers often spend valuable resources earlier in the growing season on infected plants that show no signs of infection but will most likely fall short of their full yield potential.

The stem canker disease can usually be managed by removing or deep-tilling infested soybean debris combined with planting resistant cultivars. Removing or deep-tilling infested soybean debris is not possible for growers that use no-till or reduced-tillage management practices. Cultivar resistance to stem canker is conditioned by at least five different dominant resistance genes. In 1994, two germplasm lines, D85-10404 and D85-10412, were released with resistance to stem canker (Kilen and Hartwig, 1994). 'Tracy-M' was the source of resistance for D85-10404 (*Rdc1*) and D85-10412 (*Rdc2*). Both germplasm lines were developed from the cross of Tracy-M x J77-339 (Kilen et al., (1985). Additional resistance genes have been found in the cultivars Crockett (*Rdc3*) and Dowling (*Rdc4*) (Bowers et al., 1993). Both PI 230976 and PI 398469 have single dominant genes conditioning resistance to stem canker that are at different loci than *Rdc1*, *Rdc2*, *Rdc3*, and *Rdc4*, but as of yet have not been assigned gene symbols because it is not known if the genes in these plant introductions are unique (Tyler, 1995).

Mapping stem canker resistance genes will allow soybean breeders to more efficiently select for stem canker resistance in their cultivar development programs. The USDA consensus

soybean genetic linkage map and the complete genome sequence have been used to identify many marker-trait associations in soybean (Schmutz et al., 2010; Song et al., 2004). An additional 60,000 SSR markers have been identified using the soybean genome sequence (Song et al., 2010). The objective of this study was to identify molecular markers associated with genes that condition resistance to stem canker.

### Materials and Methods

In the summer of 2004, the stem canker resistant D85-10404, D85-10412, Crockett, Dowling, and PI 398469 were each crossed to the highly susceptible breeding line J77-339 at the Univ. of Georgia Plant Sciences Farm near Watkinsville, GA to create the *Rdc1*, *Rdc2*, *Rdc3*, *Rdc4*, and *Rdc?*(PI 398469) mapping populations, respectively. The F<sub>1</sub> generation of each cross was grown in a greenhouse on the Univ. of Georgia campus in Athens, GA during the winter of 2005. Seed of the F<sub>1:2</sub> generation was harvested in bulk from single F<sub>1</sub> plants of the Crockett x J77-339 and PI 398469 x J77-339 populations and gave rise to the F<sub>2</sub> plants used for mapping. Three F<sub>1:2</sub> rows each from the crosses D85-10404 x J77-339, D85-10412 x J77-339, and Dowling x J77-339 were grown in the summer of 2005 at the Univ. of Georgia Plant Sciences Farm near Watkinsville, GA. Individual plants from each of the F<sub>1:2</sub> rows were harvested and single-plant threshed to create F<sub>2:3</sub> lines. Mapping populations consisted of 94 individually labeled F<sub>2</sub> plants of Crockett x J77-339, 94 individually labeled F<sub>2</sub> plants of PI 398469 x J77-339, 92 F<sub>2:3</sub> families of D85-10412 x J77-339, and 87 F<sub>2:3</sub> families of Dowling x J77-339. The D85-10404 x J77-339 mapping population was eliminated due to seed contamination or crossing complications and mapping *Rdc1* was not pursued.

Screening for stem canker reaction was done in the greenhouse (D85-10412 x J77-339 and Dowling x J77-339 populations) and in the field (Crockett x J77-339 and PI 398469 x J77-339 populations) using methods similar to those presented by Keeling (1982). The DPM inoculum was isolated in August of 2006 from the stems of infected soybean plants grown in a field in Mississippi. The DPM isolate was subcultured in a potato dextrose agar (PDA) solution with toothpicks. Infested toothpicks were inserted into the hypocotyls of individually-labeled F<sub>2</sub> soybean seedlings from the Crockett x J77-339 and PI 398469 x J77-339 populations planted in the field and 13 to 16 F<sub>3</sub> soybean seedlings of the D85-10404 x J77-339 population labeled according to F<sub>2,3</sub> family planted in clay pots. Plants were considered susceptible if they were dead after 10 d, moderately resistant if they were still alive but had stem lesions, and resistant if they showed no symptoms of stem canker.

For the Crockett x J77-339 and PI 398469 x J77-339 F<sub>2</sub> mapping populations, F<sub>2</sub> plants were individually labeled and leaves were harvested from each plant for genomic DNA extraction prior to DPM inoculation. For the D85-10412 x J77-339 and Dowling x J77-339 F<sub>2,3</sub> mapping populations, leaves from each F<sub>3</sub> plant were harvested and combined with leaves of other plants from the same F<sub>2</sub> family prior to DNA extraction in order to resynthesize the genome of the F<sub>2</sub> plant that gave rise to that particular F<sub>2,3</sub> family. The collected leaf tissue was lyophilized and ground to a powder. DNA was extracted using the CTAB method (Keim et al., 1988) and resuspended in TE buffer.

The PCR conditions for each SSR marker were similar to those used by Li et al. (2001). Each reaction had a total volume of 10 µL and consisted of 2 µL of 50 ng/µL template DNA, 1.0X PCR buffer, 2.5 mM MgCl<sub>2</sub>, 200 µM of each dNTP, 0.2 µM each of fluorescently-labeled forward and reverse SSR primers, and 5 U/µl of Promega Taq DNA polymerase. PCR products

were combined with a mixture of 2  $\mu$ L formamide, 0.75  $\mu$ L loading buffer, and 0.2  $\mu$ L ROX-500 size standard in a 2:3 ratio (PCR product: loading buffer). The mixture was denatured at 95°C for 5 minutes and loaded into a 96-lane, 12-cm 19:1 (acrylamide: bisacrylamide) gel and electrophoresced at 750 V for 1.5 to 2.0 hours using an ABI Prism 377 DNA sequencer [Applied Biosystems Incorporated (ABI), Foster City, CA] in order to assign a parental genotype to each PCR product. The computer program GeneScan Version 3.0 [Applied Biosystems Incorporated (ABI), Foster City, CA] was used to collect marker data.

Single nucleotide polymorphism (SNP) markers were developed using methods similar to Ha et al. (2010). In summary, potential SNP-containing sequence tagged sites (STS) were selected based on their proximity to the location of the stem canker resistance gene. The STS primers were used to sequence the parents of the mapping populations of interest using an Applied Biosystems Incorporated (ABI; Carlsbad, California, USA) 3730 capillary sequencer. The original primers used to amplify each STS can be found on the NCBI website (<http://www.ncbi.nlm.nih.gov>; verified 10 January, 2011). The STS sequences of the parents were aligned and compared in order to find SNPs that could be used during mapping. SNP markers were amplified in the mapping populations using high resolution melting. Primers for SNPs were developed using LightCycler Probe Design Software (Roche Diagnostics; Indianapolis, IN, USA). Primers were tested on the mapping population using a Roche LightCycler 480 and detected using a saturating dye.

SSR marker data on the F<sub>2</sub> plants was collected by using bulk segregant analysis (BSA) similar to the methods of Michelmore et al. (1991), with one bulk consisting of F<sub>2</sub> plants with the resistant phenotype, and one bulk consisting of F<sub>2</sub> plants with the susceptible phenotype. Each bulk was made up of no more than 12 plants of a mapping population with each plant

contributing approximately equal DNA to the bulk. Polymorphic SSR markers spaced approximately every 20 cM along the soybean genetic map on each of the 20 chromosomes were tested on the bulks and the parents of each cross. Using BSA, a putative location of the resistance locus was considered for further analysis when the band for the susceptible bulk matched the susceptible parent and bands for the resistant bulk matched both the resistant and the susceptible parents. Once a putative map location was found using BSA, these marker(s) were tested on the individual members of each bulk to verify the initial BSA results. After the confirmation of these markers for each cross, additional polymorphic SSR markers available in the region were tested on each member of the mapping population in order to resolve the most probable location of the resistance gene.

The genotypic and phenotypic data were analyzed using MAPMAKER/EXP V. 3.0 (Lander et al., 1987) to construct genetic linkage maps using the Kosambi mapping function (Kosambi, 1944). The stem canker rating (resistant or susceptible) was treated as a qualitative trait and mapped by treating the resistant rating as a dominant marker.

## Results and Discussion

Stem canker reaction of plants in the two F<sub>2</sub> mapping populations, Crockett x J77-339 for mapping *Rdc3* and PI 398469 x J77-339 for mapping *Rdc?(PI398469)*, fit a 3:1 (resistant : susceptible) segregation ratio (Table 4.1). This indicates a single dominant gene for stem canker resistance is present in each of these two F<sub>2</sub> mapping populations. For the F<sub>2:3</sub> mapping populations D85-10412 x J77-339 and Dowling x J77-339, plants of the F<sub>2</sub> generation that gave rise to at least 85% susceptible F<sub>3</sub> plants were classified as susceptible. All other F<sub>2</sub> plants were classified as resistant. Of course, these F<sub>2</sub> plants were either homozygous resistant or

segregating for resistance. Families of both F<sub>2:3</sub> mapping populations, D85-10412 x J77-339 for mapping *Rdc2* and Dowling x J77-339 for mapping *Rdc4*, fit a 3:1 (resistant : susceptible) F<sub>2</sub> plant segregation ratio (Table 4.1). This also indicated that single dominant genes for stem canker resistance are present in each of the two F<sub>2:3</sub> mapping populations.

During BSA, SSR marker Satt577 located on linkage group (Lg)-B2 (chromosome 14, Gm14) was tested on the PI 398469 x J77-339 F<sub>2</sub> mapping population. For this marker, bulks of stem canker resistant plants had SSR bands of two different lengths representative of both resistant parent and the susceptible parent. Bulks of stem canker susceptible plants had a single band matching the length of the susceptible parent. Similar results were found when SSR marker Sat\_264, also on Gm14, was tested using BSA of plants from the Crockett x J77-339 F<sub>2</sub> mapping population. All of the other 130 SSR markers tested on the PI 398469 x J77-339 population and the 102 SSR markers tested on the Crockett x J77-339 population bulks had both parental bands in both the resistant and susceptible bulks.

The identification of the putative linkage between *Rdc?(PI398469)* and Satt577 using BSA was verified by testing DNA of each plant used to make the bulks in individual PCR reactions with SSR marker Satt577. Every plant in the resistant bulk was either homozygous for the band corresponding to the resistant parent or heterozygous for both parental bands. Every plant in the susceptible bulk was homozygous for the band corresponding to the susceptible parent. Similar results were found when the putative linkage between *Rdc3* and Sat\_264 was tested in a similar manner on individuals used to create the resistant and susceptible bulks from the Crockett x J77-339 mapping population. The BSA provided strong evidence that both *Rdc?(PI398469)* and *Rdc3* are located on Gm14.



Based on results from BSA, additional SSR and SNP markers from Gm14 were tested on the 94 F<sub>2</sub> plants of the PI 398469 x J77-339 population and on the 94 F<sub>2</sub> plants of the Crockett x J77-339 population. The *Rdc?*(PI398469) gene mapped to the segment of Gm14 between SSR markers Sat\_177 and 14\_0115 (Fig. 4.1). The SSR marker 14\_0097 cosegregated perfectly with stem canker reaction in all 94 individuals. It is unknown whether 14\_0097 resides within *Rdc?*(PI 398469) or is outside the gene but tightly linked. Testing 14\_0097 on a greater number of individuals from this mapping population could possibly resolve this question. Tight linkage, rather than an intragenic SSR marker, would be indicated if a stem canker susceptible plant was found to have an allele from PI 398469. Satt577, the marker identified as being associated with *Rdc?*(PI398469) using BSA, was 6.7 cM from *Rdc?*(PI398469).

The *Rdc3* gene from Crockett mapped to the segment of Gm14 between SSR markers Sat\_264 and Satt416, 2.9 cM from Sat\_264 (Fig. 4.2). Sat\_264 was also the marker identified as being associated with *Rdc3* using BSA.

To initially narrow down the locations of *Rdc2* and *Rdc4* to single linkage groups for each gene, approximately 40 SNP markers were tested on the F<sub>2:3</sub> populations of D85-10412 x J77-339 and Dowling x J77-339. The Monsanto Company (St. Louis, MO) used proprietary SNP markers to putatively locate *Rdc2* on Lg-D1b (Gm02). Twelve SSR markers from Gm02 were tested on 92 F<sub>2:3</sub> lines from the D85-10412 x J77-339 population. The *Rdc2* gene from D85-10412 mapped to the segment of Gm02 between Satt141 and 02\_1635 (Fig. 4.3). No recombination was found between *Rdc2* and Satt189 in this mapping population. It is unknown whether Satt189 resides within *Rdc2* or is outside the gene but tightly linked.

The Monsanto Company used proprietary SNP markers to also putatively locate *Rdc4* on Gm02. Fourteen SSR and SNP markers from Gm02 were tested on 87 F<sub>2:3</sub> lines from the

Dowling x J77-339 population. The *Rdc4* gene mapped to the interval between SSR markers BE021153 and Satt157 (Fig. 4.4). The closest marker to *Rdc4* was Satt157, 2.4 cM away from the gene. Linkage analysis of the SSR markers used to map *Rdc2*, *Rdc3*, *Rdc4*, and *Rdc?(PI398469)* are in reasonable agreement with the USDA consensus soybean genetic linkage map (Song et al., 2004).

Both *Rdc3* and *Rdc?(PI398469)* mapped to similar regions on Gm14. SSR marker Sat\_177 flanked both *Rdc?(PI398469)* and *Rdc3* and mapped 4.9 cM and 3.8 cM away from Sat\_177, respectively. SSR marker 14\_0097 is also common to both maps, with zero recombination between this marker and *Rdc?(PI398469)* and 2.7 cM between this marker and *Rdc3*. Based on these recombinational distances, *Rdc3* should reside in the interval between *Rdc?(PI398469)* and Sat\_177, however whether these genes are allelic or at different, yet closely linked loci is beyond the scope of our research. Tyler (1995) reported that a Crockett x PI 398469 F<sub>2</sub> population segregated approximately 15:1 (119 resistant plants: 5 susceptible plants) indicating that the *Rdc3* and *Rdc?(PI398469)* resistance genes were located at independently segregating loci. However, there was no F<sub>3</sub> progeny confirmation of the F<sub>2</sub> resistant or susceptible plants. The general agreement of the genetic distance between the SSR markers on the USDA consensus soybean map and our linkage maps of Gm14 for PI 398469 x J77-339 and Crockett x J77-339 and the lack of map expansion after including the *Rdc?(PI398469)* and *Rdc3* genes with the DNA markers provides evidence of the validity of our data. Given the precision of our mapping data, it is possible that *Rdc3* and *Rdc?(PI398469)* are located at the same locus. Although our data cannot resolve this issue, it is clear that the stem canker resistance gene(s) in Crockett and PI398469 are not at independent loci.

Both *Rdc2* and *Rdc4* mapped to Gm02. Common to both maps, SSR markers Satt157 and Satt558 reside in the interval between *Rdc2* and *Rdc4*. Using the distance between *Rdc4* and Satt157 from the Dowling x J77-339 map, the distance between Satt157 and Satt558 from the USDA Consensus Soybean Genetic Map, and the distance between Satt558 and *Rdc2* from the D85-10412 x J77-339 map, the interval between *Rdc4* and *Rdc2* is approximately 41.6 cM (2.4 cM, 6.8 cM, and 32.4 cM for each of the three intervals, respectively). The centromere may also separate *Rdc2* and *Rdc4* on Gm02 (data not shown; Soybase, <http://soybeanbreederstoolbox.org>). This recombinational distance supports Tyler's (1995) hypothesis that these two genes segregate independently.

Two of the stem canker resistance genes, *Rdc3* and *Rdc?*(*PI398469*) have mapped to a putative disease resistance gene cluster in soybean (Soybase, <http://soybeanbreederstoolbox.org>). Four mRNA sequences encoding putative disease resistance genes have been cloned and are located within 15 cM of the *Rdc3* and *Rdc?*(*PI398469*) locations on Gm14 (Table 4.2; Soybase, <http://soybeanbreederstoolbox.org>). The other two resistance genes are also located near putative disease resistance genes. The *Rdc2* gene has mapped to nearly the same location as the resistance gene analog RGA1f (Arahana et al., 2001; Hayes et al., 2000; Kanazin et al., 1996). Four mRNA sequences encoding putative disease resistance genes have been cloned and located to the region near or between *Rdc2* and *Rdc4* chromosome 2 (Table 4.2; Soybase, <http://soybeanbreederstoolbox.org>). The genes listed in Table 4.2 may condition stem canker resistance. However, since their function is unknown they may also play roles in resistance to other soybean diseases.

The markers flanking these four stem canker resistance genes may be sufficient for marker-assisted selection. Markers less than 3 cM away from each gene have been identified.

These markers should be validated in an independent set of meiotic events before using them to select for stem canker resistance among segregating soybean lines in a breeding program. Once validated, these markers could be used in lieu of phenotyping for disease resistance using the more traditional greenhouse and field methods which require maintenance and culturing of the stem canker-causing fungus. These or other validated markers in the same regions that cosegregate with stem canker resistance will empower breeders to select homozygous resistant plants and lines in early breeding generations and practice selection for other traits such as seed yield in later generations of inbreeding among stem canker resistant lines.

#### References

- Arahana V.S., Graef G.L., Specht J., Steadman J.R., Eskridge K.M. (2001) Identifications of QTLs for resistance to *Sclerotinia sclerotiorum* in soybean. *Crop Science* 41:180-188.
- Bowers G.R., Ngeleka K., Smith O.D. (1993) Inheritance of stem canker resistance in soybean cultivars Crockett and Dowling. *Crop Science* 33:67-70.
- Fernandez F.A., Phillips D.V., Russin J.S., Rupe J.C. (1999) Stem Canker, in: G. L. Hartman (Ed.), *Compendium of soybean diseases*, APS Press, St. Paul, MN. pp. 33-35.
- Grau C.R., Dorrance A.E., Bond J., Russin J.S. (2004) Fungal Diseases, in: H. R. Boerma and J. Specht (Eds.), *Soybeans: Improvement, Production, and Uses*, American Society of Agronomy, Madison, WI. pp. 679-763.
- Ha B.-K., Monteros M., Boerma H.R. (2010) Development of SNP assays associated with oleic acid QTLs in N00-3350 soybean *Euphytica* 176:403-415.
- Hayes A.J., Ma G., Buss G.R., Maroof M.A.S. (2000) Molecular marker mapping of *Rsv4*, a gene conferring resistance to all known strains of soybean mosaic virus. *Crop Science* 40:1434-1437.
- Hildebrand A.A. (1952) Stem canker: a disease of increasing importance on soybeans in Ontario. *Soybean Digest* 12:12-15.
- Kanazin V., Marek L.F., Shoemaker R.C. (1996) Resistance gene analogs are conserved and clustered in soybean. *Genetics* 93:11746-11750.

- Keeling B.L. (1982) A seedling test for resistance to soybean stem canker caused by *Diaporthe phaseolorum* var. *caulivora*. *Phytopathology* 72:807-809.
- Keim P., Olson T.C., Shoemaker R.C. (1988) A rapid protocol for isolating soybean DNA. *Soybean Genetics Newsletter* 15:150-152.
- Kilen T.C., Hartwig E.E. (1994) Registration of soybean germplasm lines resistant to stem canker and *Phytophthora* rot: D85-10404 and D85-10412. *Crop Science* 34:292.
- Kilen T.C., Keeling B.L., Hartwig E.E. (1985) Inheritance of reaction to stem canker in soybean. *Crop Science* 25:50-51.
- Kosambi D.D. (1944) The estimation of map distances using recombination values. *Annals of Human Genetics* 12:172-175.
- Lalitha B., Snow J.P., Berggren G.T. (1989) Phytotoxin production by *Diaporthe phaseolorum* var. *caulivora*, the causal organism of stem canker of soybean. *Phytopathology* 79:499-504.
- Lander E.S., Green P., Abrahamson J., Barlow A., Daly M.J., Lincoln S.E., Newburg L. (1987) MAPMAKER: an interactive computer package for constructing primary genetic linkage maps of experimental and natural populations. *Genomics* 1:174-181.
- Li Z., Jakkula L., Hussey R.S., Tamulonis J.P., Boerma H.R. (2001) SSR mapping and confirmation of the QTL from PI96354 conditioning resistance to southern root-knot nematode. *Theoretical and Applied Genetics* 103:1167-1173.
- Michelmore R.W., Paran I., Kesseli R.V. (1991) Identification of markers linked to disease-resistance genes by bulked segregant analysis: A rapid method to detect markers in specific genomic regions by using segregating populations. *Proceedings of the National Academy of Sciences, USA* 88:9828-9832.
- Morgan-Jones G. (1989) The *Diaporthe/Phomopsis* complex: taxonomic considerations, World Soybean Research Conference IV, Buenos Aires, Argentina. pp. 1699-1706.
- Ploetz R.C., Shokes F.M. (1985) Soybean stem canker incited by ascospores and conidia of the fungus causing the disease in the southeastern United States. *Plant Disease* 69:990-992.
- Ploetz R.C., Shokes F.M. (1987) Infection of different plant parts of soybean seedlings by southern *Diaporthe phaseolorum* and its role in the development of stem canker symptoms. *Canadian Journal of Botany* 65:2104-2108.
- Schmutz J., Cannon S.B., Schlueter J., Ma J., Mitros T., Nelson W., Hyten D.L., Song Q., Thelen J., Cheng J., Xu D., Hellsten U., May G.D., Yu Y., Sakurai T., Umezawa T., Bhattacharyya M.K., Sandhu D., Valliyodan B., Lindquist E., Peto M., Grant D., Shu S., Goodstein D., Barry K., Futrell-Griggs M., B. A., J. D., X. T., Zhu L., Gill N., Joshi T.,

- Libault M., Sethuraman A., Zhang X.-C., Shinozaki K., Nguyen H.T., Wing R.A., Cregan P.B., Specht J.E., Grimwood J., Rokhsar D., Stacey G., Shoemaker R.C., Jackson S.A. (2010) Genome sequence of the palaeopolyploid soybean. *Nature* 463:178-183.
- Song Q., Jia G., Zhu Y., Grant D., Nelson R.T., Hwang E.-Y., Hyten D.L., Cregan P.B. (2010) Abundance of SSR Motifs and Development of Candidate Polymorphic SSR Markers (BARCSOYSSR\_1.0) in Soybean. *Crop Sci.* 50:1950-1960.
- Song Q.J., Marek L.F., Shoemaker R.C., Lark K.G., Concibido V.C., Delannay X., Specht J., Cregan P.B. (2004) A new integrated genetic linkage map of the soybean. *Theoretical and Applied Genetics* 109:121-128.
- Tyler J.M. (1995) Additional sources of stem canker resistance in soybean plant introductions. *Crop Science* 35:376-377.
- Wrather J.A. (2004) Soybean disease loss estimates for the United States in 2003. United Soybean Board domestic report, final report for project # 4215.
- Zhu Y.L., Song Q.J., Hyten D.L., Van Tassell C.P., Matukumalli L.K., Grimm D.R., Hyatt S.M., Fikus E.W., Young N.D., Cregan P.B. (2003) Single-nucleotide polymorphisms in soybean. *Genetics* 163:1123-1134.

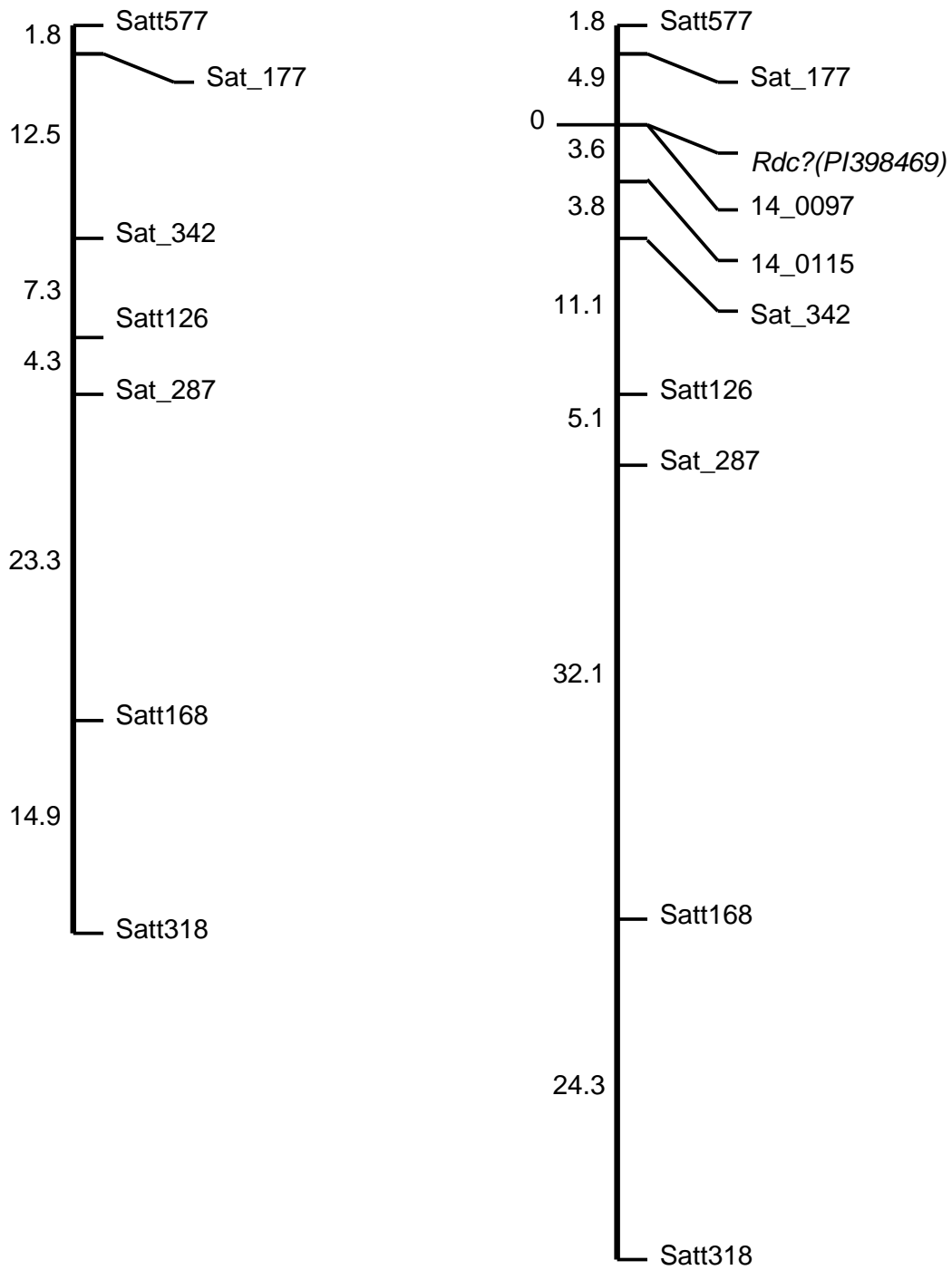


Figure 4.1. Linkage map of Gm14 based on the USDA consensus soybean genetic map (left) and a linkage map of Gm14 including *Rdc?(PI 398469)* based on 94 F<sub>2</sub> plants of PI 398469 x J77-339 (right).

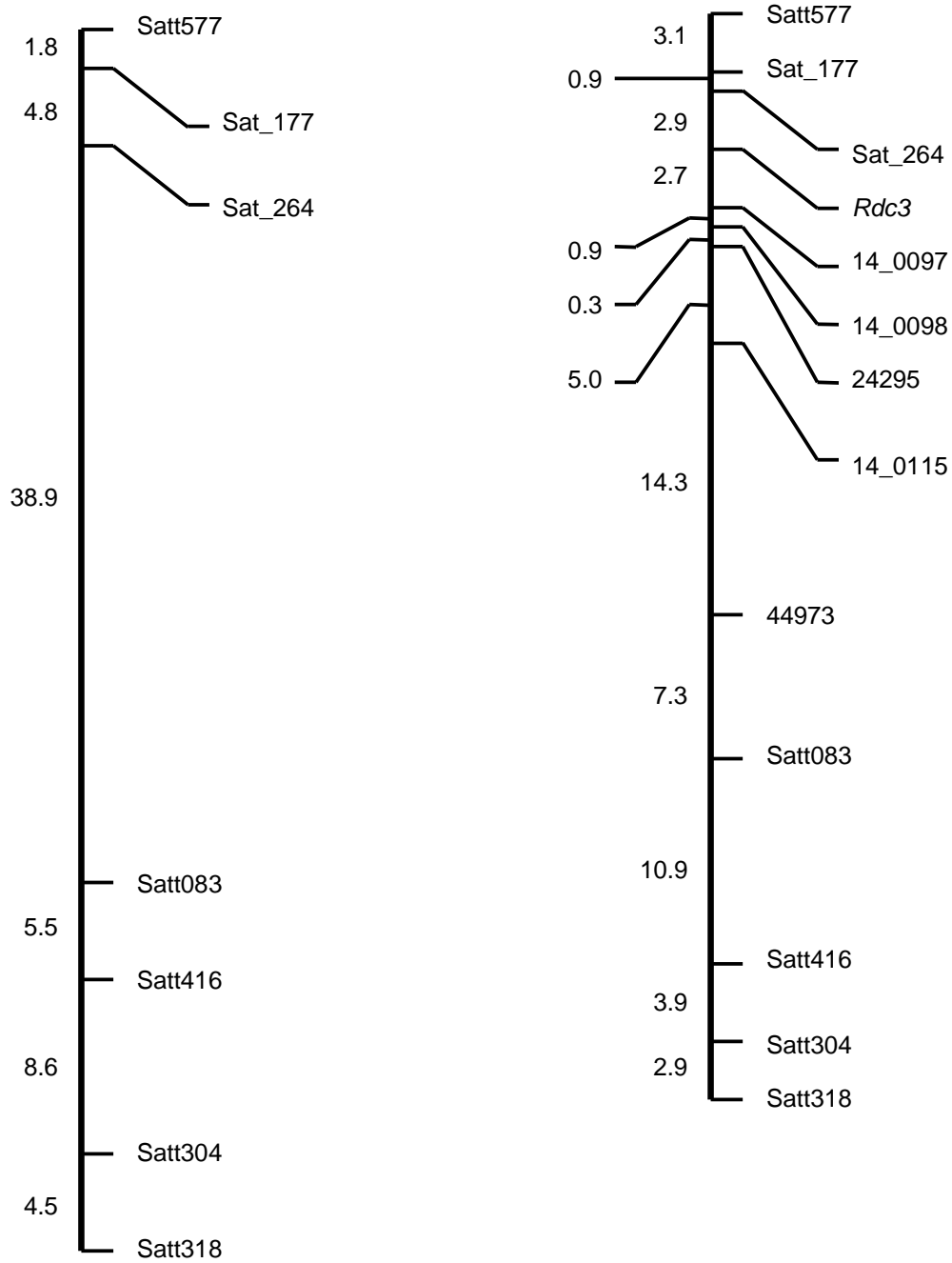


Figure 4.2. Linkage map of Gm14 based on the USDA consensus soybean genetic map (left) and a linkage map of Gm14 including *Rdc3* based on 94 F<sub>2</sub> plants of Crockett x J77-339 (right).



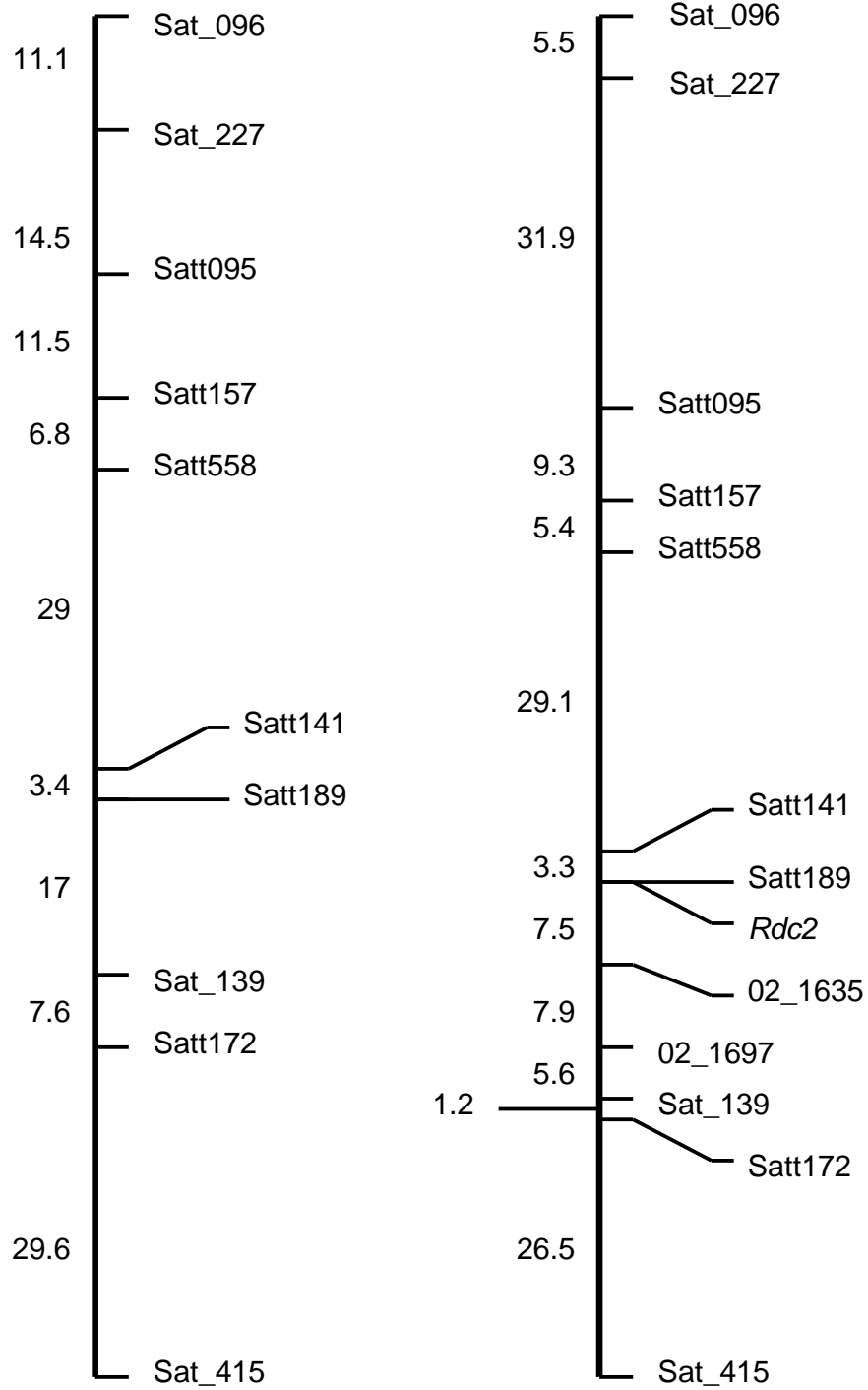


Figure 4.3. Linkage map of Gm02 based on the USDA consensus soybean genetic map (left) and a linkage map of Gm02 including *Rdc2* based on 92 F<sub>2:3</sub> plants of D85-10412 x J77-339 (right).

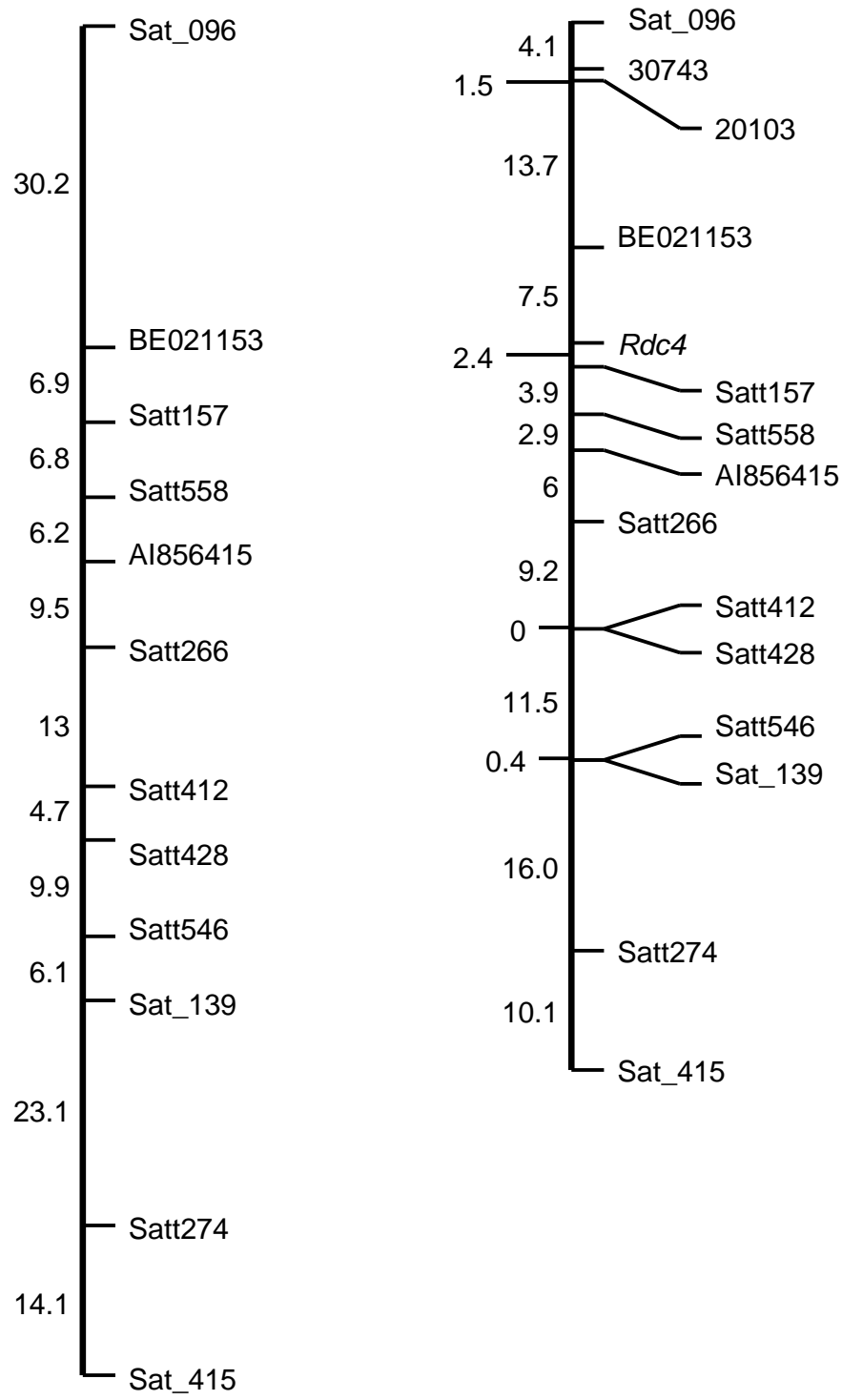


Figure 4.4. Linkage map of Gm02 based on the USDA consensus soybean genetic map (left) and a linkage map of LG-D1b including *Rdc4* based on 87 F<sub>2:3</sub> plants of Dowling x J77-339 (right).

Table 4.1. Phenotypic data for stem canker reaction in F<sub>2</sub> and F<sub>2:3</sub> populations. †Chi square values are for the expected 3:1 (resistant: susceptible) segregation ratios.  $X^2_{(0.10)} = 2.71$   $X^2_{(0.05)} = 3.84$  for 1 degree of freedom.

| Cross               | Population | Resistance gene | Resistant             | Susceptible              | Total                    | $\chi^2$ † |
|---------------------|------------|-----------------|-----------------------|--------------------------|--------------------------|------------|
|                     |            |                 | (no. plants or lines) | (no. of plants or lines) | (no. of plants or lines) |            |
| D85-10412 x J77-339 | F2:3       | <i>Rdc2</i>     | 68                    | 24                       | 92                       | 0.5        |
| Crockett x J77-339  | F2         | <i>Rdc3</i>     | 71                    | 23                       | 94                       | 0.01       |
| Dowling x J77-339   | F2:3       | <i>Rdc4</i>     | 63                    | 24                       | 87                       | 0.3        |
| PI 398469 x J77-339 | F2         | <i>Rdc?</i>     | 71                    | 23                       | 94                       | 0.01       |

Table 4.2. Cloned mRNA sequences of resistance genes with unknown functions in the vicinity of the map locations of stem canker resistance genes *Rdc2*, *Rdc3*, *Rdc4*, and *Rdc?*(*PI 398469*). †SSR marker used for mapping *Rdc2*, *Rdc3*, *Rdc4*, or *Rdc?*(*PI 398469*)

| Genbank Accession Number | Clone    | Resistance gene motif | Chromosome | Position start | Position end |
|--------------------------|----------|-----------------------|------------|----------------|--------------|
| FJ014875.1               | cw14     | TIR                   | 14         | 1745797        | 1748126      |
| EU888316                 | 8070607  | RLK                   | 14         | 1895959        | 1902475      |
| N/A                      | †Sat_342 | N/A                   | 14         | 2954822        | 2954895      |
| FJ014836.1               | cw156    | LRR                   | 14         | 3102336        | 3108378      |
| FJ014848.1               | cw171    | not specified         | 14         | 3259732        | 3262016      |
| EU888319.1               | 8070610  | PK-LRR-TM             | 14         | 3269402        | 3270403      |
| FJ014849.1               | cw172    | PK                    | 14         | 3663167        | 3666230      |
| N/A                      | †Satt126 | N/A                   | 14         | 4935860        | 4935913      |
| N/A                      | †Satt227 | N/A                   | 2          | 3074234        | 3074479      |
| FJ014788.1               | cw105    | LRR-RLK               | 2          | 3334689        | 3338374      |
| N/A                      | †Satt095 | N/A                   | 2          | 6307407        | 6307574      |
| N/A                      | †Satt157 | N/A                   | 2          | 9239908        | 9240161      |
| EU669879.1               | SIK1     | stress-induced<br>RLK | 2          | 9352870        | 9357365      |
| N/A                      | †Satt141 | N/A                   | 2          | 35083059       | 35083258     |
| N/A                      | †Sat_139 | N/A                   | 2          | 44878908       | 44879093     |
| FJ014836.1               | cw156    | LRR                   | 2          | 48864551       | 48868364     |
| FJ014865.1               | cw4      | TIR                   | 2          | 50084223       | 50086285     |

## CHAPTER 5

### COMPARING METHODS OF SELECTION FOR ELEVATED OLEIC ACID IN SOYBEAN<sup>1</sup>

---

<sup>1</sup>Shearin, Z.P., and H.R. Boerma. To be submitted to *Crop Science*.

## Abstract

Breeding mid-oleic soybean (*Glycine max* (L.) Merr.), or soybean that produces oleic acid (18:1) at a concentration of greater than 500 g kg<sup>-1</sup> of total seed oil content, would increase the overall value of soybean oil and the price paid to growers for their crop. Breeding for increased 18:1 is difficult due to its highly quantitative nature, significant genotype x environment interactions, and poor stability of some elevated 18:1 breeding lines. This study evaluated three selection methods of incorporating elevated 18:1 from the mid-oleic breeding line N00-3350 into three different glyphosate-tolerant cultivars, Boggs RR, H7242 RR, and Prichard RR. The selection methods included phenotypic selection (PS) based on gas chromatography analysis (GCA) of ground seed, stratified phenotypic selection (SPS) based on GCA with stratification based on maturity, and marker-assisted selection (MAS) based on SNP markers near six 18:1 QTL. Selected lines were tested in a randomized complete block experimental design in two environments with two replications per environment. Mean 18:1 concentration was numerically higher for MAS than PS in five of nine families by 70 – 810 g kg<sup>-1</sup>. Mean 18:1 concentration was numerically higher for SPS than PS in all nine families by 60 – 720 g kg<sup>-1</sup>. PS was numerically superior to MAS in four of nine families by 40 – 260 g kg<sup>-1</sup>, but in no family did PS have the highest mean overall. However, not all differences were statistically significant. Given the results of our study, MAS or SPS would be expected to be more effective than PS in identifying lines with elevated 18:1 levels across a range of elite breeding populations.

## Introduction

Breeding mid-oleic soybean, or soybean that produces oleic acid at a concentration of greater than 50% of total seed oil content, would increase the overall value of soybean oil and the price paid to growers for their crop. The typical fatty acid profile of soybean seeds from current commodity soybean cultivars is 100 g kg<sup>-1</sup> palmitic acid (16:0), 40 g kg<sup>-1</sup> stearic acid (18:0), 220 g kg<sup>-1</sup> oleic acid (18:1), 540 g kg<sup>-1</sup> linoleic acid (18:2), and 100 g kg<sup>-1</sup> linolenic acid (18:3) (Wilson, 2004). Breeding for increased oleic acid in soybean seeds could potentially have positive effects on the marketing of soybean oil due to increased flavor stability and health benefits.

Research suggests that oleic acid content in soybean seeds is a quantitative trait controlled by both major fatty acid biosynthesis genes and modifier genes with smaller effects (Rebetzke et al., 1998). As a result, recurrent selection has been used successfully to increase 18:1 content in soybean seeds (Burton et al., 1983).

Many alleles exist at numerous loci that alter 18:1 content in soybean seeds. Alt et al. (2005a) created populations segregating for unsaturated fatty acid alleles by making all possible crosses of FA22, N98-4445A, and M23. FA22 is a soybean line developed at Iowa State University with elevated oleic acid and N98-4445A is a germplasm line developed by the USDA-ARS and North Carolina State University with elevated 18:1 (Burton et al., 2006; Wilson, 2004). The M23 line is a high oleic acid mutant line that was created by irradiating seeds of the cultivar Bay (Rahman et al., 1994). Transgressive segregates were found in F<sub>2</sub> and F<sub>3</sub> generations of all crosses, with 18:1 concentrations as high as 73% found in the N98-4445A x M23 population. Their data indicated that many alleles in these populations affect 18:1 content and each of the three high oleic parents differed from the others in at least some of the alleles that condition their oleic acid content (Alt et al., 2005a).

Soybean lines developed with elevated 18:1 have a wide range in stability. Lines developed through mutation breeding or transformation are generally more stable than lines developed through recurrent selection. Oliva et al. (2006) grew 17 soybean lines with different fatty acid profiles in 10 different environments to evaluate the stability of each fatty acid. High oleic lines N97-3363-4 and N98-4445A were both developed via recurrent selection and were the most unstable for oleic acid. They found that much of the variation in oleic acid content in these lines could be attributed to temperature changes. The mutant line M23 and 'Holl,' a cultivar derived from M23, were significantly more stable (Oliva et al., 2006).

Research has established that lines with altered 18:1 have different phenotypic expressions of the trait based on the environment in which they are grown. Environmental temperature during seed development may account for some of these differences. With increasing temperature the relative percent of 18:1 increases, relative percents of 18:2 and 18:3 decrease, and relative percents of 16:0 and 18:0 remain fairly constant (Wilson, 2004). Membrane fluidity at lower temperatures is maintained by desaturation of fatty acids. Higher temperatures reduce the requirement for membrane bound unsaturated fatty acids. The temperature dependence of fatty acid desaturase enzymes may account for this trend (Schlueter et al., 2007). Using greenhouse experiments with controlled temperatures, Dornbos et al. (1989) determined that the effects of temperature during the podfill growth stage have the greatest impact on oleic acid concentration. During podfill, or the period when nutrients are being deposited into soybean seeds, higher temperatures were associated with increased oleic acid concentrations.

Maturity may directly or indirectly have an effect on oleic acid concentration. In general, earlier maturing plants in populations segregating for 18:1 concentration have higher amounts of



18:1 (Wilson, 2004). This may be due to the fact that earlier maturing lines are exposed to higher temperatures during podfill because their podfill period takes place during the earlier, warmer part of the growing season.

When selecting lines for elevated 18:1 in populations with wide ranges in maturity, researchers must take great care not to select lines that appear to have higher 18:1 because of genetic control of the trait when in reality their higher 18:1 phenotype is conditioned by the confounding factor of earlier maturity. If a narrow range in maturity is not intentionally maintained during selection in a population for increased 18:1, population shifts toward earlier maturity usually occur. In one recurrent selection population, flowering date and maturity date were correlated responses to selection for both high and low oleic acid percentage, with number of days to flowering and maturity significantly decreasing in response to selection for high oleic acid percent, and significantly increasing in response to reverse selection for lower oleic acid percent (Carver et al., 1986). Burton et al. (1983) also reported a 6 d shift towards earlier maturity over the selection period in their recurrent selection population while selecting only for increased 18:1. Stratifying the lines by maturity prior to selection for altered 18:1 may assist in phenotypically selecting lines with the most desirable genotype conducive to expression of the desired level of 18:1. The expression of elevated 18:1 in lines with low stability is enhanced by warmer environments (Oliva et al., 2006).

Marker assisted selection (MAS) of genes conditioning elevated 18:1 concentrations would be helpful to soybean breeders due to the difficulty associated with phenotypic selection for this trait. The significant genotype x environment interactions, poor stability of some elevated 18:1 breeding lines, and the low heritability of the trait in early generations makes it difficult for breeders to accurately select desirable genotypes based on phenotypic information,

especially during early generations. Phenotypic selection for elevated oleic acid is complicated by maternal effects in some populations. In these populations, neither phenotypic fatty acid analysis of F<sub>2</sub> seeds nor means of individual F<sub>3</sub> plants provide accurate information on the genotypes of F<sub>2</sub> plants (Primomo et al., 2002).

Molecular markers have been linked to multiple QTL with effects on seed 18:1 content. Monteros et al. (2008) used supplemental lighting in a greenhouse and a Puerto Rican environment to control day length and compact the range in maturity while successfully mapping and confirming six QTL for increased 18:1 in a population of Boggs-RR x N00-3350. N00-3350 is a single plant selection from N98-4445A with elevated 18:1. Boggs-RR is a well adapted cultivar with resistance to the herbicide glyphosate (Boerma et al., 2000). Six oleic QTL, one on Gm05 (Lg-A1, 18:1 QTL A1), one on Gm17 (Lg-D2, 18:1 QTL D2), two on Gm18 (Lg-G, 18:1 QTL G1 and 18:1 QTL G2), and two on Gm19 (Lg-L, 18:1 QTL L1 and 18:1 QTL L2), were identified with the alleles for increased 18:1 at all six QTL inherited from N00-3350. The simple sequence repeat (SSR) markers identifying these QTL were Satt211 (18:1 QTL A1; R<sup>2</sup> = 4%) on Gm05, Satt389 (18:1 QTL D2; R<sup>2</sup> = 6%) on Gm17, Satt394 (18:1 QTL G1; R<sup>2</sup> = 13%) and Satt191 (18:1 QTL G2; R<sup>2</sup> = 7%) on Gm18, and Satt418 (18:1 QTL L1; R<sup>2</sup> = 9%) and Satt561 (18:1 QTL L2; R<sup>2</sup> = 25%) on Gm19. Together, these six markers accounted for 30% of the variation in oleic acid in the Boggs-RR x N00-3350 population.

Due to the narrow range of maturity expressed among the lines in this mapping population in both environments, it is likely that they experienced similar temperatures during their reproductive growth thus reducing the confounding effect of environment on the expression of these QTL. These markers were also confirmed in an independent population of G99-G3438 x N00-3350. Breeder-friendly, cost effective SNP markers were developed to aid in selection of

all six QTL, and candidate genes associated with fatty acid biosynthesis were identified for four of the six QTL (Ha et al., 2010).

Additional knowledge of the relationship between 18:1, maturity, and QTL associated with the trait would be beneficial to soybean breeders by allowing them to more efficiently and reliably develop soybean cultivars with elevated 18:1. The objective of this research project was to compare three methods of selecting for elevated 18:1 from N00-3350 in three different genetic backgrounds. The three methods of selection include:

- i. phenotypic selection (PS) based on gas chromatography (GC) analysis of seed from single plants,
- ii. stratified phenotypic selection (SPS) based on GC analysis of seed from single plants, with stratification based on the maturity date of the single plants, and
- iii. marker-assisted selection (MAS) of single plants based on SNP markers near the six oleic acid QTL developed by Ha et al. (2010).

## Materials and Methods

### **Population Development**

Due to the population-specific nature of oleic acid QTL, three different crosses were evaluated to compare the selection methods in different genetic backgrounds. The crosses consisted of Boggs RR x N00-3350 (POP1), H7242 RR x N00-3350 (POP2), and Prichard RR x N00-3350 (POP3).

In the summer of 2000, the glyphosate tolerant cultivar Boggs RR was crossed to N00-3350 to create F<sub>1</sub> seeds of POP1. The POP1 F<sub>1</sub> plants were grown in the greenhouse during the winter of 2001 and the resulting F<sub>2</sub> seed was harvested in bulk. In the summer of 2001, F<sub>2</sub> plants

were grown in the greenhouse and harvested individually to create two  $F_{2:3}$  lines hereto and after referred to as 375-1 and 375-2 (Table 5.1). During the summer of 2004, some of the  $F_{2:3}$  seeds from 375-2 were planted in the greenhouse and harvested individually to create one  $F_{3:4}$  line hereto and after referred to as 480-3. The remaining  $F_{2:3}$  seeds from 375-2 were saved.

In the summer of 2004, the glyphosate tolerant cultivar H7242 RR was crossed to N00-3350 to create  $F_1$  seeds of POP2. During the winter of 2005,  $F_1$  plants of POP2 were grown and harvested individually to create two  $F_{1:2}$  lines, hereto and after referred to as 318-1 and 318-2.

In the summer of 2000, the well adapted, glyphosate tolerant cultivar Prichard RR was crossed to N00-3350 to create  $F_1$  seeds of POP3. The POP3  $F_1$  plants were grown in the greenhouse during the winter of 2001 and harvested in bulk to produce  $F_2$  seed. In the summer of 2004,  $F_2$  plants of POP3 were grown in the greenhouse and harvested individually to create four  $F_{2:3}$  lines, referred to as 380-1, 380-2, 380-3, and 380-4.

### **Selection Procedure**

During the summer of 2008, the  $F_{1:2}$  families 318-1 and 318-2, the  $F_{2:3}$  families 375-1, 375-2, 380-1, 380-2, 380-3, and 380-4, and the  $F_{3:4}$  family 480-3 were each grown in separate rows at the Univ. of Georgia Plant Sciences Farm near Watkinsville, GA on Cecil coarse sandy loam (clayey, kaolinitic, thermic Typic Kanhapludults). Each plant within each row was individually labeled using a small plastic tag. A total of 213 plants from POP1 (375-1, 375-2, and 480-3), 351 plants from POP2 (318-1 and 318-2), and 352 plants from POP3 (380-1, 380-2, 380-3, and 380-4) were grown in three, two, and four rows, respectively (Table 5.1). The parents, Boggs RR, H7242 RR, Prichard RR, and N00-3350 were also grown in 1-row plots.

Immature trifoliolate leaves were collected from each plant for DNA extraction. SNP markers developed by Ha et al. (2010) near each of the six confirmed oleic acid QTL reported by Monteros et al. (2008) were tested on the DNA extracted from immature trifoliolate leaves of each individually-labeled plant. During the fall of 2008, bi-weekly maturity notes were taken for each plant.

At reproductive maturity, each plant was single-plant threshed to produce F<sub>2:3</sub> lines of families 318-1 and 318-2, F<sub>3:4</sub> lines of families 375-1, 375-2, 380-1, 380-2, 380-3, and 380-4, and F<sub>4:5</sub> lines of family 480-3. The single-plant-derived lines within each family served as the experimental lines for this experiment. A 20-seed sample of each experimental line was analyzed for fatty acid profile using gas chromatography.

For PS, the experimental lines in the top 15% of each family were selected based on the 18:1 content of a 20-seed sample determined by GC. For SPS, experimental lines within each family were separated into three strata based on the maturity of the individual plant grown in the summer of 2008 that gave rise to the corresponding experimental line (Table 5.2). Plants were separated into an early, middle, and late stratum within each family. Attempts were made to ensure that the number of plants in each stratum was similar. However, all plants that matured the same week were always assigned to the same strata. From each stratum, the top 15% of lines were selected based on the 18:1 content of a 20-seed sample determined by GC. For MAS, a selection index,  $I$ , was calculated for each experimental line such that  $I = \sum (b_i X_i)$  where  $b$  is the R<sup>2</sup> value of the  $i$ th QTL and  $X$  is the number of alleles from N00-3350 at a SNP marker linked to the  $i$ th QTL. The experimental lines with the highest 10% of index scores were selected.

### **Fatty Acid Analysis**

Fatty acid composition was determined using gas chromatography as described by Rebetzke et al. (1998). In general, seed samples were pulverized and extracted for 12 h in a solvent of chloroform/hexane/methanol (8:5:2 v/v/v) in stoppered glass test tubes. Fatty acid methyl esters of the lipid extracts were prepared by transesterification using sodium methoxide. Each sample was analyzed using an HP 6890 gas chromatograph (Agilent Technologies, Inc., Wilmington, DE). Chromatograms were analyzed to identify peaks and integrate unknowns relative to authentic standards using HP ChemStation software (Agilent Technologies).

### **SNP Genotyping**

For MAS, SimpleProbe primers were used to genotype each line at six confirmed 18:1 QTL as described by Ha et al. (2010). PCR reactions were performed in 384-well plates with a total volume of 3 uL per well in a LightCycler 480 (Roche Applied Science, Indianapolis, IN, USA) using a general protocol for asymmetrical PCR. The fluorescence signal (F) was graphed against temperature (T) to produce melting curves for each sample. LightCycler Data Analysis software (Roche Diagnostics, Indianapolis, IN) then converted the melting curves into negative derivative curves of fluorescence with respect to temperature ( $-dF/dT$ ). The software automatically separated similar melting curves into groups and assigned genotypic scores based on parental genotype melting standards included in the experiment.

### **Evaluation of Selected Lines**

The selected experimental lines were planted at the USDA Winter Nursery in Isabella, Puerto Rico during the winter of 2009 and grown under supplemental light to extend the natural

photoperiod to 20 hours of light for 45 d after planting. Each line was harvested in bulk to produce F<sub>2:4</sub> lines within 318-1 and 318-2, F<sub>3:5</sub> lines within 375-1, 375-2, 380-1, 380-2, 380-3, and 380-4, and F<sub>4:6</sub> lines within 480-3. These lines served as the seed source for each line the next season.

During the summer of 2009, the experimental lines were grown as single-row plots in two environments in Georgia (a 1 June planting date in a Wedowee coarse sandy loam (fine, kaolinitic, thermic Typic Kanhapludults) and 23 June planting date in Cecil coarse sandy loam (clayey, kaolinitic, thermic Typic Kanhapludults), both at The Univ. of Georgia Plant Sciences Farm) with two replications per environment in a randomized complete block experimental design. Maturity dates (date when 95% of the pods in a plot reach their mature pod color) were recorded for each line. Upon maturity, each line was harvested in bulk to produce F<sub>2:5</sub> lines of 318-1 and 318-2, F<sub>3:6</sub> lines of 375-1, 375-2, 380-1, 380-2, 380-3, and 380-4, and F<sub>4:7</sub> lines of 480-3. A 20-seed sample of each of these lines was analyzed for fatty acid profile using GC.

The 18:1 data were analyzed (assuming blocks and environments as random effects and lines as fixed effects) by ANOVA using Agrobase software (Agronomix Software Inc., Winnipeg, Canada). T-tests were used to make all possible pairwise comparisons between the mean 18:1 concentration of all lines selected using each of the three selection methods. Instead of combining data from each family to compare means over the entire population, comparisons were made within each family due to the fact that families within each population had been derived in different generations, and some families had already been fixed for various 18:1 QTL (Table 5.3). Welch's (1947) T-test for samples with unequal sample sizes and variances was used for all comparisons. Additional T-tests were used to compare maturities in a similar manner. The heritability estimate for maturity was calculated using Proc REG in SAS 9.1 (Cary,

NC USA) by regressing mean maturity of the lines grown in summer 2009 on their single plant maturity during the summer of 2008.

### Results and Discussion

As many as four and as few as two 18:1 QTL were fixed for either the male or female parental alleles in each of the single-plant-derived lines grown in 2008 from the Boggs RR x N00-3350 and Prichard RR x N00-3350 populations (Table 5.3). The single-plant-derived lines 318-1 and 318-2 from POP2 were segregating for all six 18:1 QTL.

Either MAS, SPS, or both selection methods were numerically superior to PS for selecting lines with elevated 18:1 in all nine families (Table 5.4). However, not all of these differences were statistically significant. In the two families of POP2 where all six 18:1 QTL were segregating, MAS was numerically superior to SPS and PS and statistically ( $P = 0.05$ ) superior to SPS and PS in the 318-2 family. Marker-assisted selection was statistically ( $P = 0.05$ ) superior to PS in two (380-1 and 480-3) of the seven remaining families and inferior to PS in two other families (375-1 and 380-4). Stratifying for maturity prior to phenotypic selection for 18:1 (SPS) was numerically superior to PS for selecting lines with increased 18:1 concentrations in all nine families, and statistically significant ( $P = 0.05$ ) in four of nine families (480-3, 318-1, 318-2, and 380-3). SPS was also significantly ( $P = 0.05$ ) superior to MAS in three of nine families (375-1, 380-3, and 380-4). By regressing the mean maturity of the derived experimental lines on their single-plant progenitors, the heritability estimate for maturity was 0.62 across all the families and populations (data not shown). This relatively high heritability on a single plant basis and the success of SPS across all populations indicates that SPS is a viable method of selecting for increased 18:1 concentration in these populations. Across the three populations,



SPS and MAS selected lines averaged 464 and 457 g kg<sup>-1</sup> compared to 439 g kg<sup>-1</sup> for PS selected lines (Table 5.4). In our study where N00-3350 was the elevated 18:1 donor parent and the lines were segregating for maturity, both SPS and MAS resulted in more lines with superior levels of 18:1 than PS.

In POP1 and POP2, seed 18:1 concentration of all 2008 plants (both selected and unselected) single plants was negatively correlated with plant maturity, where  $r = 0.14^*$  and  $r = 0.51^{**}$ , respectively (Fig. 5.1). This correlation between 18:1 concentration and maturity was not significant in POP3. In POP1, a 1 wk delay in maturity was associated with an 11 g kg<sup>-1</sup> decrease in seed 18:1 concentration. In POP2, a 1 wk maturity delay was associated with a more pronounced 37 g kg<sup>-1</sup> decrease in seed 18:1 concentration. These results generally support prior research that warmer temperatures (i.e., earlier maturity) during podfill are associated with higher seed 18:1 concentration (Dornbos et al, 1989). It follows that fewer lines in the later maturing POP3 are exposed to warmer temperatures during podfill, and thus selection for 18:1 concentration in this population is less affected by maturity (Fig 5.2).

In POP1, lines selected using PS had an average maturity 2 to 4 d earlier than lines selected by MAS and 1 to 3 d earlier than lines selected by SPS (Table 5.4). Lines selected by MAS in family 480-3 averaged 25 g kg<sup>-1</sup> higher in 18:1 and 1 d later in maturity than lines selected using SPS in this family. This indicated MAS was effective in POP1 to select lines with increased 18:1 concentration without a shift towards earlier maturity.

No significant ( $P > 0.05$ ) differences in maturity were observed among the three selection methods in POP2. Experimental lines in POP3 generally matured much later than lines in POP 1 or POP2 (Table 5.4). This was expected in this population given the late maturity of the MG VIII female parent, Prichard RR (58 d after 31 August). The low number of lines exposed to

warmer podfill temperatures in this population may account for the similarity in maturity of the high 18:1 lines selected by PS, SPS, and MAS (Table 5.4).

The line with the maximum 18:1 concentration from each of the nine families all approached or exceeded 18:1 levels of N00-3350 (Table 5.4). In POP1, the line with the highest 18:1 concentration in each family ranged from 97 to 114% of N00-3350. In POP2, the experimental line with the highest 18:1 concentration was 103% and 105% of N00-3350 for families 318-1 and 318-2, respectively. In POP3, the experimental line with the highest 18:1 concentration ranged from 79 to 103% of N00-3350, depending on the family. The line within each family with the highest 18:1 was selected by MAS or SPS in all nine families. Phenotypic selection identified the same highest 18:1 line in only two families, 380-1 and 380-4 of POP3. The SPS method also successfully identified the lines with highest 18:1 concentration in these two families. Interestingly, POP3 was the only population where PS was successful in selecting the highest 18:1 lines. This population was also the latest maturing population with the fewest lines exposed to warmer temperatures during podfill (Fig. 5.2). As a result, the segregation for maturity may have been less of a factor during the selection of lines with increased 18:1 in POP3.

The 18:1 concentration of the line within each family that had the lowest 18:1 concentration among all selected lines is also listed in Table 5.4. Phenotypic selection identified this line in eight of nine families, compared to once for MAS. The line in family 375-1 with the lowest 18:1 concentration was selected using MAS only. All lines selected using MAS in 375-1 also averaged 260 g kg<sup>-1</sup> less 18:1 than all lines selected in the same family using PS (Table 5.4). The lack of segregation of 18:1 QTL may partially account for the superiority of PS in this family. The two QTL with the highest reported R<sup>2</sup> values, L2 (25%) and G1 (13%), are both

fixed for the BoggsRR alleles, the low 18:1 parent in family 375-1 (Table 5.3). Of the seven families with two or more 18:1 QTL fixed in the population prior to selection, 375-1 is the only family where all the nonsegregating QTL are fixed for the parent with lower 18:1 concentration.

Significant ( $P = 0.05$ ) differences in 18:1 concentration were observed among marker classes at the six 18:1 QTL in many families. Generally, experimental lines homozygous for N00-3350 alleles at 18:1 QTL had a higher average 18:1 concentration than lines homozygous for the lower 18:1 parent (data not shown). As expected, differences between marker classes were the largest for the 18:1 QTL L2 and 18:1 QTL G1 (Table 5.5). Of the six 18:1 QTL, QTL L2 and QTL G1 conditioned the greatest amount of the phenotypic variation in 18:1 levels ( $R^2$  values of 25 and 13%, respectively) in the original mapping population (Monteros et al., 2008). Neither of these QTL were segregating in POP1 (Table 5.3). The largest differences between marker classes were observed in family 380-1 of POP3, where lines homozygous for N00-3350 alleles at 18:1 QTL L2 and 18:1 QTL G1 had 840 and 540 g kg<sup>-1</sup> higher 18:1 concentrations than lines homozygous for the female parent, respectively (Table 5.5). In POP2, where all six 18:1 QTL were segregating, experimental lines homozygous for N00-3350 alleles had a higher average 18:1 concentration than lines homozygous for the female parent at all QTL except for L1 (data not shown). Similar results were found in POP1 and POP3 at all segregating QTL, however some differences were not consistent across all families segregating for a particular QTL.

Based on results in this study, MAS and SPS were superior to PS for selecting lines with elevated 18:1 especially when considering maturity of the selected lines. The shift towards earlier maturity when using PS for selecting for elevated 18:1 was avoided in most cases by using MAS and SPS. Stratification for maturity, such as with SPS, reduces the confounding

effect of maturity when selecting for increased 18:1 by ensuring that later maturing lines with elevated 18:1 are selected even though they may not express the highest 18:1 concentrations in the selection environment. The MAS selection method also removes the confounding of maturity unless 18:1 and maturity QTL are linked and their alleles for high 18:1 and early maturity are in coupling phase. Lines selected using MAS and SPS had higher average 18:1 concentrations than lines selected using PS across all populations. The MAS and SPS methods also selected the individual lines from each family with the highest 18:1 concentration more often than PS, while PS selected the individual line from each family with the lowest 18:1 concentration more often than MAS and SPS.

However, MAS and SPS are not without limitations. The markers associated with the 18:1 QTL are linked markers rather than “perfect” markers. Thus, recombination between the marker and the actual 18:1 gene are possible. It is also possible that there are no existing polymorphic markers in the region of the QTL in the parents of the breeding population. The effectiveness of SPS may be limited if selection is conducted in environments that produce a low heritability for maturity on a single-plant basis. This results in the misclassification of the maturity of the resultant lines when they are evaluated in multiple environments.

Given the results of our study, MAS or SPS would be expected to be more effective than PS in identifying lines with elevated 18:1 levels across a range of elite breeding populations. The use of SPS, MAS, or a combination of both methods would be especially important in selecting for elevated 18:1 in a population of plants with a wide range of maturity. Given the effectiveness of MAS for oleic acid and the ability to extract sufficient DNA from the non-embryonic portion of a single F<sub>2</sub> seed (1/4-seed sample) for assaying the six oleic QTL SNPs, MAS allows the breeder to conduct selection for mid-oleic acid while maintaining the remaining

3/4-seed portion for planting of selected F<sub>2</sub> seeds. This eliminates the need to plant the entire F<sub>2</sub> population in the field, stratify the population for maturity, and harvest seed from each F<sub>2</sub> plant for oleic acid determination. The inability to phenotypically select for either maturity or oleic acid content (via GC) on a 1/4-seed sample provides MAS a significant advantage compared to SPS in terms of time, supply costs, and labor. Thus, the use of MAS in early generations to fix the mid-oleic phenotype followed by yield testing, agronomic evaluation and confirmation of the mid-oleic phenotype in superior inbred lines by GC analysis seems a highly efficient and effective breeding strategy.

#### References

- Alt J.L., Fehr W.R., Welke G.A., Shannon J.G. (2005a) Transgressive segregation for oleate content in three soybean populations. *Crop Science* 45:2005-2007.
- Alt J.L., Fehr W.R., Welke G.A., Sandhu D. (2005b) Phenotypic and molecular analysis of oleate content in the mutant soybean line M23. *Crop Science* 45:1997-2000.
- Bachlava E., Cardinal A.J. (2009) Correlation between temperature and oleic acid seed content in three segregating soybean populations. *Crop Science* 49:1328-1335.
- Bachlava E., Dewey R.E., Burton J.W., Cardinal A.J. (2009a) Mapping and comparison of quantitative trait loci for oleic acid seed content in two segregating soybean populations. *Crop Science* 49:433-442.
- Bachlava E., Dewey R.E., Burton J.W., Cardinal A.J. (2009b) Mapping candidate genes for oleate biosynthesis and their association with unsaturated fatty acid seed content in soybean. *Molecular breeding* 23:337-347.
- Bachlava E., Burton J.W., Brownie C., Wang S., Auclair J., Cardinal A.J. (2008a) Heritability of oleic acid content in soybean seed oil and its genetic correlation with fatty acid and agronomic traits. *Crop Science* 48:1764-1772.
- Bachlava E., Dewey R.E., Auclair J., Wang S., Burton J.W., Cardinal A.J. (2008b) Mapping genes encoding microsomal w-6 desaturase enzymes and their cosegregation with QTL affecting oleate content in soybean. *Crop Science* 48:640-650.

- Bilyeu K.D., Palavalli L., Slepner D.A., Beuselinck P.R. (2003) Three microsomal omega-3 fatty acid desaturase genes contribute to soybean linolenic acid levels. *Crop Science* 43:1833-1838.
- Boerma H.R., Hussey R.S., Phillips D.V., Wood E.D., Rowan G.B., Finnerty S.L. (2000) Registration of 'Boggs' soybean. *Crop sci.* 40:294-295.
- Burton J.W., Wilson R.F., Brim C.A. (1983) Recurrent selection in soybeans. IV. selection for increased oleic acid percentage in seed oil. *Crop Science* 23:744-747.
- Burton J.W., Wilson R.F., Rebetzke G.J., Pantalone V.R. (2006) Registration of N98-4445A mid-oleic soybean germplasm line. *Crop Science* 46:1010-1012.
- Byrum J.R., Kinney A.J., Shoemaker R.C., Diers B.W. (1995) Mapping of the microsomal and plastid omega-3 fatty acid desaturases in soybean. *Soybean genetics newsletter* 22:121-124.
- Cardinal A.J., Burton J.W., Camacho-Roger A.M., Yang J.H., Wilson R.F., Dewey R.E. (2007) Molecular analysis of soybean lines with low palmitic acid content in the seed oil. *Crop Science* 47:304-310.
- Carver B.F., Burton J.W., Wilson R.F., Carter T.E., Jr. (1986) Cumulative response to various recurrent selection schemes in soybean: oil quality and correlated agronomic traits. *Crop Science* 26:853-858.
- Chang N.W., Huang P.C. (1998) Effects of the ratio of polyunsaturated and monounsaturated fatty acid on rat plasma and liver lipid concentration. *Lipids* 33:481-487.
- Dai Q., Shu X.O., Jin F., Gao Y.T., Ruan Z.X., Zheng W. (2002) Consumption of animal foods, cooking methods, and risk of breast cancer. *Cancer epidemiology biomarkers and prevention* 11:801-808.
- Diers B.W., Shoemaker R.C. (1992) Restriction fragment length polymorphism analysis of soybean fatty acid content. *Journal of the american oil chemists society* 69:1242-1244.
- Dornbos D.L., Mullen R.E., Hammond E.G. (1989) Phospholipids of environmentally stressed soybean seeds. *Journal of the american oil chemists society* 66:1371-1373.
- Frankel E.N. (1991) Recent advances in lipid oxidation. *Journal of the Science of Food and Agriculture* 54:495-511.
- Gunstone F.D., Hilditch T.P. (1945) The union of gaseous oxygen with methyl oleate, linoleate, and linolenate. *Journal of the chemical society* 1945:836.
- Ha B.-K., Monteros M., Boerma H.R. (2010) Development of SNP assays associated with oleic acid QTLs in N00-3350 soybean *Euphytica* 176:403-415.

- Hawkins S.E., Fehr W.R., Hammond E.G. (1983) Resource allocation in breeding for fatty acid composition of soybean oil. *Crop Sci* 23:900-904.
- Hayakawa K., Linko Y.-Y., Linko P. (2000) The role of *trans* fatty acids in human nutrition. *Starch* 52:229-235.
- Hyten D.L., Pantalone V.R., Saxton A.M., Schmidt M.E., Sams C.E. (2004) Molecular mapping and identification of soybean fatty acid modifier quantitative trait loci. *Journal of the American Oil Chemists Society* 81:1115-1118.
- Kinney A.J., Stecca K.L., Meyer K. (2008) Soybean event DP-305423-1 and compositions and methods for the identification and/or detection thereof, in: U. S. P. Office (Ed.), USA. pp. 1-118.
- Kinoshita T., Rahman S.M., Anai T., Takagi Y. (1998) Genetic analysis of restriction fragment length polymorphism on the fatty acid synthesis in soybean mutants and their progenies: II. High oleic acid mutants with two microsomal omega-6 fatty acid desaturase cDNAs as probes. *Bulletin of the Agricultural Saga University* 83:37-42.
- Liu H.R., White P.J. (1992) High-temperature stability of soybean oils with altered fatty acid compositions. *Journal of the American Oil Chemists Society* 69:533-537.
- Monteros M.J., Burton J.W., Boerma H.R. (2008) Molecular mapping and confirmation of QTLs associated with oleic acid content in N00-3350 soybean. *Crop Science* 48:2223-2234.
- Oliva M.L., Shannon J.G., Sleper D.A., Ellersieck M.R., Cardinal A.J., Paris R.L., Lee J.D. (2006) Stability of fatty acid profile in soybean genotypes with modified seed oil composition. *Crop Science* 46:2069-2075.
- Pantalone V.R., Walker D.R., Dewar R.E., Rajcan I. (2004) DNA marker-assisted selection for improvement of soybean oil concentration and quality, in: R. F. Wilson, et al. (Eds.), *Legume crop genomics*, AOCS Press, Champaign, IL. pp. 283-311.
- Primomo V.S., Falk D.E., Ablett G.R., Tanner J.W., Rajcan I. (2002) Genotype x environment interactions, stability, and agronomic performance of soybean with altered fatty acid profiles. *Crop Science* 42:37-44.
- Puri P. (1978) Correlations for trans-isomer formation during partial hydrogenation of oils and fats. *Journal of the American Oil Chemists' Society* 55:865-869.
- Rahman S.M., Takagi Y., Kubota K., Miyamoto K., Kawakita T. (1994) High oleic acid mutant in soybean produced by X-ray irradiation. *Bioscience Biotechnology Biochemistry* 58:1070-1072.

- Rebetzke G.J., Burton J.W., Carter T.E., Jr., Wilson R.F. (1998) Genetic variation for modifiers controlling reduced saturated fatty acid content in soybean. *Crop Science* 38:303-308.
- Schlueter J.A., Vasylenko-Sanders I.F., Deshpande S., Yi J., Siegfried M., Roe B.A., Schlueter S.D., Scheffler B.E., Shoemaker R.C. (2007) The FAD2 Gene Family of Soybean:: Insights into the Structural and Functional Divergence of a Paleopolyploid Genome. *Crop Sci* 47:S-14-26. DOI: 10.2135/cropsci2006.06.0382tpg.
- Stacey G., Shoemaker R.C., Jackson S., Schmutz J., Rokhsar D. (2010) Soybean genome project, DoE Joint Genome Institute.
- Welch B.L. (1947) The generalization of "Student's" problem when several different population variances are involved. *Biometrika* 34:28-35.
- Wilcox J.R., Cavins J.F. (1985) Inheritance of low linolenic acid content of the seed oil of a mutant in *Glycine max*. *Theoretical and Applied Genetics* 71:74-78.
- Wilson R.F. (2004) Seed Composition, in: H. R. Boerma and J. E. Specht (Eds.), *Soybeans: Improvement, Production, and Uses*, ASA, CSSA, SSSA, Madison, WI. pp. 621-677.
- Wilson R.F., Burton J.W. (1986) Regulation of linolenic acid in soybeans and gene transfer to high yielding, high protein germplasm, in: A. R. Baldwin (Ed.), *Proceedings on the World Conference on Emerging Technology in the Fats and Oils industry*, Cannes, France 3-8 Nov. 1985, AOCS Press, Champaign, IL. pp. 386-391.
- Wilson R.F., Burton J.W., Kwanyuen P. (1990) Effect of genetic modification of fatty acid composition of soybeans on oil quality, in: D. R. Erickson (Ed.), *Edible fats and oils processing: Basic principles and modern practices*, AOCS Press, Champaign, IL. pp. 355-359.
- Zock P.L., Katan M.B. (1992) Hydrogenation alternatives: effects of trans fatty acids and stearic acid versus linoleic acid on serum lipids and lipoproteins in humans. *J. Lipid Res.* 33:399-410.



Table 5.1. Number of experimental lines selected from each family, total number of lines grown per family, and the generation in which selection took place for three populations where N00-3350 was used as the elevated 18:1 donor parent.

| Cross                 | Family | Generation | Plants per<br>family | Lines selected |     |    |
|-----------------------|--------|------------|----------------------|----------------|-----|----|
|                       |        |            |                      | MAS            | SPS | PS |
| BoggsRR x N00-3350    | 375-1  | F2:3       | 47                   | 5              | 7   | 7  |
| BoggsRR x N00-3350    | 375-2  | F2:3       | 61                   | 6              | 9   | 9  |
| BoggsRR x N00-3350    | 480-3  | F3:4       | 105                  | 11             | 16  | 16 |
| H7242RR x N00-3350    | 318-1  | F1:2       | 105                  | 11             | 16  | 16 |
| H7242RR x N00-3350    | 318-2  | F1:2       | 100                  | 10             | 15  | 15 |
| PrichardRR x N00-3350 | 380-1  | F2:3       | 120                  | 12             | 18  | 18 |
| PrichardRR x N00-3350 | 380-2  | F2:3       | 48                   | 5              | 7   | 7  |
| PrichardRR x N00-3350 | 380-3  | F2:3       | 56                   | 6              | 8   | 8  |
| PrichardRR x N00-3350 | 380-4  | F2:3       | 128                  | 13             | 19  | 19 |

Table 5.2. Number of plants selected from each stratum for SPS.

| Cross                           | Total<br>selected/total<br>plants | Selections/total plants |           |           |
|---------------------------------|-----------------------------------|-------------------------|-----------|-----------|
|                                 |                                   | Stratum 1               | Stratum 2 | Stratum 3 |
| BoggsRR x N00-3350<br>(POP1)    | no.                               | no.                     | no        | no        |
| 375-1                           | 7/47                              | 2/12                    | 2/13      | 3/22      |
| 375-2                           | 9/61                              | 2/13                    | 4/28      | 3/20      |
| 480-3                           | 16/105                            | 7/46                    | 5/33      | 4/26      |
| H7242RR x N00-3350<br>(POP2)    |                                   |                         |           |           |
| 318-1                           | 16/105                            | 5/32                    | 5/35      | 6/38      |
| 318-2                           | 15/100                            | 4/26                    | 5/34      | 6/40      |
| PrichardRR x N00-3350<br>(POP3) |                                   |                         |           |           |
| 380-1                           | 18/120                            | 8/53                    | 6/39      | 4/28      |
| 380-2                           | 7/48                              | 3/20                    | 3/21      | 1/7       |
| 380-3                           | 8/56                              | 2/14                    | 3/19      | 3/23      |
| 380-4                           | 19/128                            | 5/34                    | 7/45      | 7/49      |

Table 5.3. Oleic acid SNP markers segregating in each family, mean and range 18:1 concentration and maturity across three populations. <sup>†</sup> SNP marker segregating at the oleic acid QTL. <sup>‡</sup> SNP marker fixed for the female parent at the oleic acid QTL. <sup>§</sup> SNP marker fixed for the male parent at the oleic acid QTL. <sup>Ω</sup> Weeks after 31 August

| Cross                               | Oleic acid QTL (R <sup>2</sup> ) |         |                |         |         |          | No. segregating QTL | Combined R <sup>2</sup> of segregating QTL | Mean 18:1 concentration | Mean maturity   |
|-------------------------------------|----------------------------------|---------|----------------|---------|---------|----------|---------------------|--|-------------------------|-----------------|
|                                     | A1 (4%)                          | D2 (6%) | G1 (13%)       | G2 (7%) | L1 (9%) | L2 (25%) |                     |  |                         |                 |
| <u>BoggsRR x N00-3350 (POP1)</u>    |                                  |         |                |         |         |          | no.                 | %  | g kg <sup>-1</sup>      | wk <sup>Ω</sup> |
| 375-1                               | S <sup>†</sup>                   | S       | 1 <sup>‡</sup> | S       | S       | 1        | 4                   | 26   | 4140<br>(2670-6510)     | 2.4<br>(1-4)    |
| 375-2                               | S                                | 1       | 3 <sup>§</sup> | S       | 1       | 3        | 2                   | 11   | 5760<br>(4630-6420)     | 2.1<br>(1-7)    |
| 480-3                               | S                                | 1       | 3              | S       | 1       | 3        | 2                   | 11   | 5320<br>(4050-6290)     | 3.0<br>(1-6)    |
| <u>H7242RR x N00-3350 (POP2)</u>    |                                  |         |                |         |         |          |                     |  |                         |                 |
| 318-1                               | S                                | S       | S              | S       | S       | S        | 6                   | 64   | 3380<br>(1910-6260)     | 3.1<br>(1-6)    |
| 318-2                               | S                                | S       | S              | S       | S       | S        | 6                   | 64   | 3120<br>(2240-4770)     | 3.2<br>(2-6)    |
| <u>PrichardRR x N00-3350 (POP3)</u> |                                  |         |                |         |         |          |                     |  |                         |                 |
| 380-1                               | 3                                | S       | S              | S       | 1       | S        | 4                   | 51   | 2810<br>(2290-4580)     | 4.8<br>(3-8)    |
| 380-2                               | S                                | 3       | S              | S       | S       | 3        | 4                   | 33   | 3340<br>(2560-4260)     | 5.1<br>(3-8)    |
| 380-3                               | 3                                | S       | 3              | 3       | 1       | S        | 2                   | 51   | 2970<br>(2320-4210)     | 6.2<br>(3-8)    |
| 380-4                               | 3                                | S       | 3              | 1       | 3       | S        | 2                   | 44   | 3120<br>(2570-3730)     | 7.4<br>(7-8)    |

Table 5.4. Mean and range 18:1 concentration and maturity of lines selected by MAS, SPS, and PS. † Means with the same letter are not significantly different based on LSD<sub>(0.05)</sub>. ‡ Mean maturity measured as days after August 31. § Range listed in parentheses

| Cross                        | Mean 18:1 concentration  |                    |                    | Mean days to maturity‡ |         |         |
|------------------------------|--------------------------|--------------------|--------------------|------------------------|---------|---------|
|                              | MAS                      | SPS                | PS                 | MAS                    | SPS     | PS      |
| BoggsRR x N00-3350 (POP1)    | g kg <sup>-1</sup>       | g kg <sup>-1</sup> | g kg <sup>-1</sup> | no.                    | no.     | no.     |
| 375-1                        | 4640a <sup>†</sup>       | 4970b              | 4900b              | 41b <sup>†</sup>       | 40b     | 37a     |
|                              | (4010-5120) <sup>§</sup> | (4150-5460)        | (4340-5120)        | (36-46)                | (29-48) | (29-40) |
| 375-2                        | 5510a                    | 5640a              | 5550a              | 39b                    | 37ab    | 35a     |
|                              | (4850-6160)              | (4950-6430)        | (4950-5800)        | (36-42)                | (28-42) | (32-42) |
| 480-3                        | 5830c                    | 5580b              | 5380a              | 36a                    | 35a     | 34a     |
|                              | (5610-6360)              | (5030-6150)        | (4960-6150)        | (28-40)                | (30-40) | (30-40) |
| Boggs RR                     | 2100                     |                    |                    | 47                     |         |         |
| N00-3350                     | 5650                     |                    |                    | 28                     |         |         |
| H7242RR x N00-3350 (POP2)    |                          |                    |                    |                        |         |         |
| 318-1                        | 4170b                    | 4100b              | 3480a              | 40a                    | 41a     | 41a     |
|                              | (3910-5350)              | (2760-5230)        | (2550-5080)        | (34-50)                | (34-51) | (32-51) |
| 318-2                        | 4520c                    | 4200b              | 3710a              | 41a                    | 41a     | 42a     |
|                              | (3320-5200)              | (2760-5250)        | (2760-5090)        | (25-50)                | (34-51) | (25-51) |
| H7242RR                      | 3620                     |                    |                    | 51                     |         |         |
| N00-3350                     | 5110                     |                    |                    | 29                     |         |         |
| PrichardRR x N00-3350 (POP3) |                          |                    |                    |                        |         |         |
| 380-1                        | 3600b                    | 3480a              | 3420a              | 54a                    | 53a     | 54a     |
|                              | (3110-4310)              | (2900-4600)        | (2900-4600)        | (41-61)                | (43-62) | (43-62) |
| 380-2                        | 4100a                    | 4100a              | 4030a              | 50a                    | 51ab    | 53b     |
|                              | (4190-5110)              | (4600-5960)        | (3530-5280)        | (47-57)                | (43-58) | (43-60) |
| 380-3                        | 4510a                    | 5330b              | 4610a              | 52a                    | 52a     | 51a     |
|                              | (4080-4730)              | (3810-5370)        | (3810-5370)        | (34-57)                | (34-63) | (50-63) |
| 380-4                        | 4270a                    | 4520b              | 4440b              | 50a                    | 53b     | 55c     |
|                              | (3640-4710)              | (3330-4730)        | (3330-4380)        | (39-58)                | (43-58) | (49-58) |
| PrichardRR                   | 2180                     |                    |                    | 58                     |         |         |
| N00-3350                     | 5800                     |                    |                    | 29                     |         |         |
| Grand mean                   | 4570                     | 4660               | 4390               |                        |         |         |

Table 5.5. Mean oleic acid concentration of selected lines separated by SNP genotype at oleic acid QTL L2 and G1.

| Cross                        | L2                 |                    |                    | G1                 |                    |                    |
|------------------------------|--------------------|--------------------|--------------------|--------------------|--------------------|--------------------|
|                              | 1                  | S                  | 3                  | 1                  | S                  | 3                  |
|                              | g kg <sup>-1</sup> | g kg <sup>-1</sup> | g kg <sup>-1</sup> | g kg <sup>-1</sup> | g kg <sup>-1</sup> | g kg <sup>-1</sup> |
| H7242RR x N00-3350 (POP2)    |                    |                    |                    |                    |                    |                    |
| 318-1                        | 3930a <sup>†</sup> | 3900a              | 4370b              | 4270a              | 4080a              | 4150a              |
| 318-2                        | 4310b              | 3900a              | 4800c              | 4290b              | 4140a              | 4610c              |
| PrichardRR x N00-3350 (POP3) |                    |                    |                    |                    |                    |                    |
| 380-1                        | 2940a              | 3480b              | 3780c              | 2910a              | 3710c              | 3450b              |
| 380-2                        | N/A                | N/A                | N/A                | 3610a              | 4340c              | 4020b              |
| 380-3                        | 4030a              | 4280b              | 4720c              | N/A                | N/A                | N/A                |
| 380-4                        | 4000a              | 4480b              | 4420b              | N/A                | N/A                | N/A                |

<sup>†</sup> Means with the same letter are not significantly different based on LSD<sub>(0.05)</sub>

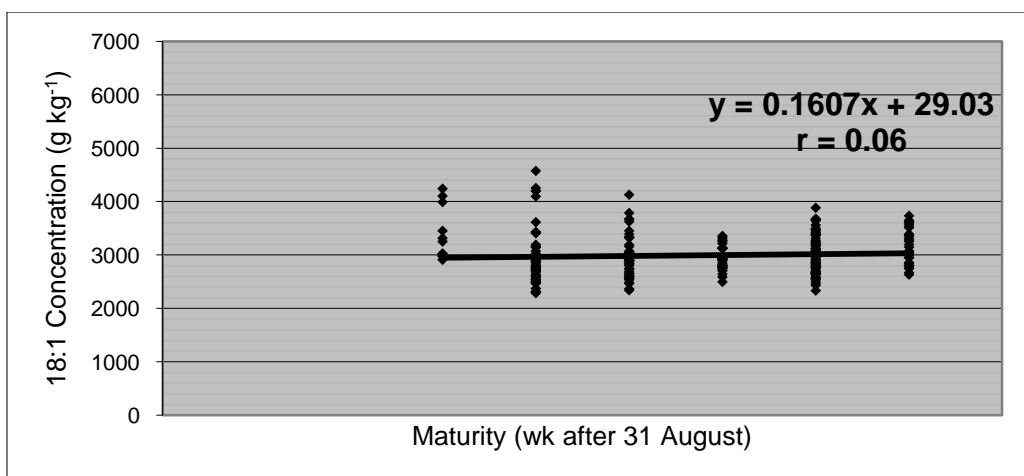
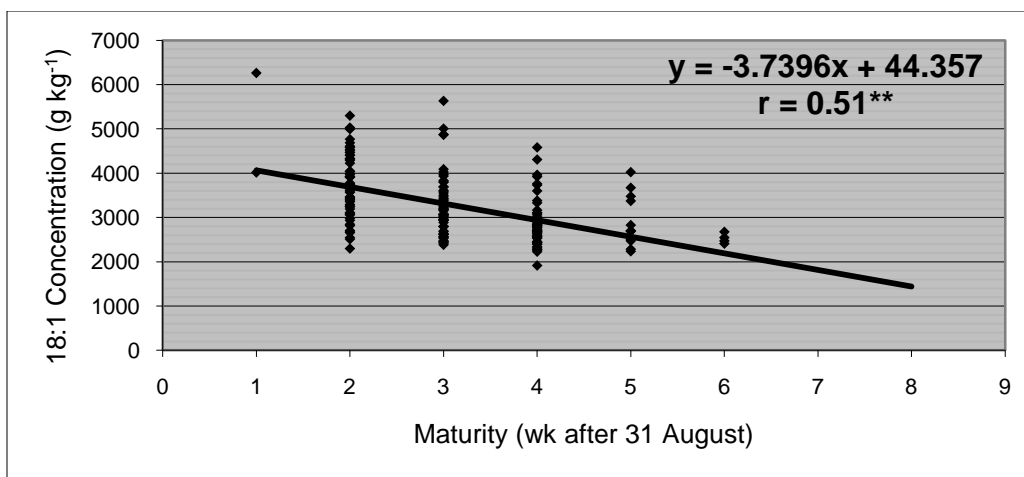
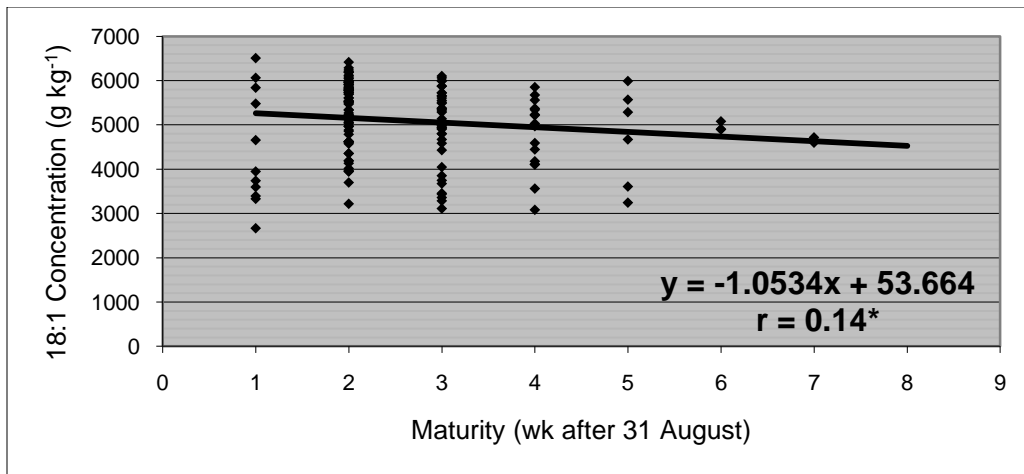


Figure 5.1. Correlations between maturity and oleic acid concentration of single plants grown in 2008 of POP1 (top panel) POP2 (middle panel) and POP3 (bottom panel). Correlations between maturity and oleic acid concentration were significant ( $P < 0.05$ ) for POP1, highly significant ( $P < 0.01$ ) for POP2, and not significant for POP3.

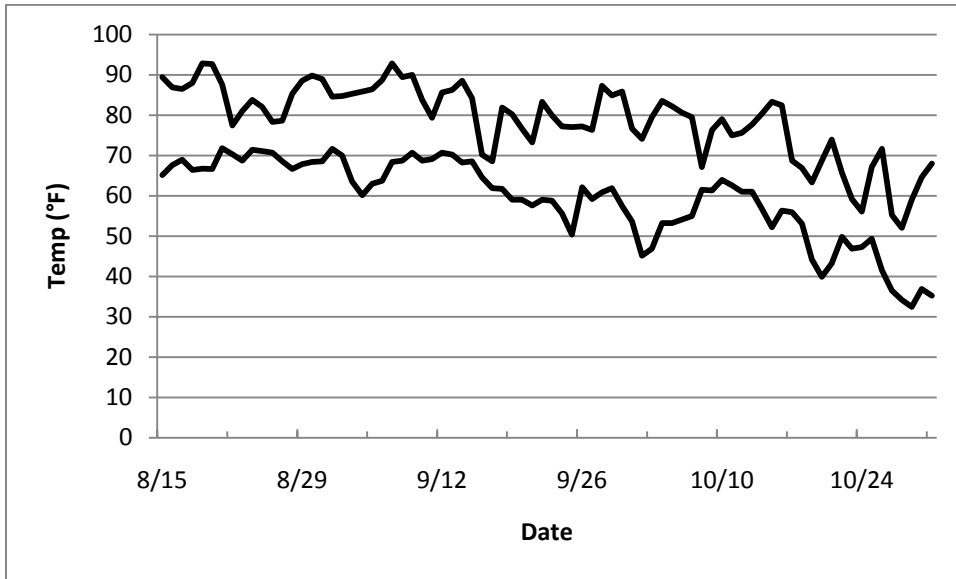


Figure 5.2. Maximum and minimum daily temperature at The University of Georgia Plant Sciences Farm from 15 August, 2008 to 31 October, 2008.

## CHAPTER 6

### CONCLUSIONS

The research presented in the previous chapters has the objectives of enhancing soybean disease resistance and seed fatty acid composition, two economically important aspects of soybean production in the southern USA. In Chapter 4, four southern stem canker resistance genes *Rdc2*, *Rdc3*, *Rdc4*, and *Rdc?*(*PI 398469*) have been mapped to two chromosomes. Two genes, *Rdc2* from D85-10412 and *Rdc4* from Dowling both mapped to Gm02, but are located at different regions of the chromosome. The other two genes, *Rdc3* from Crockett and *Rdc?*(*PI398469*) from PI 398469 mapped to a similar location on Gm14 and appear to be clustered with several genes with known disease resistance motifs. The *Rdc2* gene from D85-10412 mapped to the segment of chromosome Gm02 between Satt141 and 02\_1635. The *Rdc4* gene mapped to the segment of Gm02 between SSR markers BE021153 and Satt157. Both *Rdc3* and *Rdc?*(*PI398469*) mapped to a region on Gm02 between SSR markers Sat\_177 and 14\_0097. Linkage analysis of the SSR and SNP markers used to map *Rdc2*, *Rdc3*, *Rdc4*, and *Rdc?*(*PI398469*) are in reasonable agreement with the USDA consensus soybean genetic linkage map. Mapping stem canker resistance genes allows soybean breeders to more efficiently select for stem canker resistance in their segregating populations and pyramid stem canker resistance genes in improved cultivars. These SNP and SSR markers may ultimately be used for marker assisted selection in breeding programs in lieu of phenotyping for disease resistance using the more traditional greenhouse and field methods which require maintenance and culturing of the



stem canker-causing fungus. Marker assisted selection for these stem canker resistance genes will require confirmation in independent populations to verify the location of each resistance gene. Molecular markers linked to these genes will empower breeders to select homozygous resistant plants and lines in early breeding generations and practice selection for other traits such as seed yield among stem canker resistant lines.

Identifying an effective method to select for elevated seed oleic acid content will allow breeders to more efficiently create soybean lines that carry this trait. Chapter 5 presents strong evidence that marker assisted selection and stratified phenotypic selection can more effectively select lines with elevated oleic acid content than phenotypic selection in populations that are segregating for maturity and oleic acid QTL. In all nine families presented in Chapter 5, average oleic acid concentration among selected lines using either marker assisted selection or stratified phenotypic selection were numerically superior to phenotypic selection. Marker assisted selection and stratified phenotypic selection may avoid the selection bias towards earlier maturity associated with phenotypic selection. This is especially critical in populations segregating for maturity where these earlier maturing lines are exposed to higher temperatures during podfill, an environmental condition that confounds the genetic expression of increased oleic acid content. This is evidenced in Chapter 5 in the BoggsRR x N00-3350 population. This population had the earliest maturing lines, while average maturity of lines selected using phenotypic selection were 2 to 4 d earlier than lines selected using marker assisted selection and 1 to 3 d earlier than lines selected using stratified phenotypic selection.