Investigating the effects of modified oil synthesis genes on carbon and nitrogen partitioning in *Arabidopsis thaliana*

by

Christopher Fitzner

A Thesis Submitted to
Saint Mary's University, Halifax, Nova Scotia
in Partial Fulfillment of the Requirements for
the Degree of Master of Science in Applied Science

September, 2010, Halifax, Nova Scotia

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ABSTRACT

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In recent decades, there has been a dramatic shift in the balance of the natural carbon cycle brought upon by increased anthropogenic greenhouse gas emissions (GHG); most notably, carbon dioxide (CO_2). Biofuels may play a significant role in the preservation of the environment by providing a means of GHG mitigation, as well as a cleaner, renewable energy source.

In this experiment, knock-down mutants of *Arabidopsis thaliana* expressing reduced seed oil content (S-5 ~25.77%; S-6 ~24.89%; AS11~31.23%) were used to determine whether genetically modified oil synthesis genes affected carbon (C) and nitrogen (N) partitioning within the plant. Molecular analyses of bacterial communities in the rhizosphere were also conducted.

Results showed dramatic changes in the progression though specific developmental stages among the S-5 and S-6 genotypes, as well some changes in normal C and N partitioning. It was also observed that the microbial ecology of the surrounding rhizosphere was altered resulting from genetic modification.

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1.0 INTRODUCTION

In recent decades, there has been a dramatic shift in the balance of the natural carbon cycle brought upon by increased anthropogenic greenhouse gas emissions (GHG); most notably, carbon dioxide (CO_2). This has affected our climate and as a result our environment. Reported impacts include the sudden loss of habitats and species (Thomas et al. 2006), accelerated melting of icecaps at the north and south poles (Overpeck et al. 2006), as well as many health and food security issues (McMichael, 2001; Parry et al. 2004). Because of this, it has become increasingly apparent that biofuels may play a significant role in the preservation of the environment by providing a means of GHG mitigation, as well as a cleaner, renewable energy source.

Petroleum dependence can be partially offset by the addition of biofuels derived from several oilseed species such as soybean (*Glycine max*), canola (*Brassica napus*), and sunflower (*Helianthus annuus*) (Yuan et al., 2008). The advantages of utilizing plant based fuels stems from their ability to absorb CO_2 from the atmosphere, and convert it into a source of renewable energy. Since biofuel feedstocks have the ability to capture carbon (fossil fuel) from the atmosphere and act as a carbon sink, it can be possible to have a carbon neutral (carbon released = carbon absorbed) source of energy (Yuan et al., 2008). Some biofuels, such as bio-ethanol, require only minor hardware modifications in existing engines, making it a convenient alternative to fossil fuel.

Since 2002, biodiesel production has increased nearly 15-fold in the U.S., and is currently driving the prices of soy, canola and sunflower oil up to record high prices (Durrett et al. 2008). This has indirectly caused food prices to rise and sparked debate

regarding the displacement of land used for food, in exchange for land used for fuel (Searchinger, et al. 2008). Research on the combustion of fatty acid methyl esters (FAME) biodiesel in northern countries showed many cold-flow problems which caused engine failure and poor performance due to solidification (Dunn et al. 1996). Other issues with current first-generation biofuels include relatively low yield and high production costs that actually result in a net gain of CO_2 into the atmosphere (Mittelbach et al. 1992; Canakci and Van Gerpen, 2001; Kazancev, 2006).

Recent studies (Katavic et al. 1995; Zou et al. 1999; Jako et al. 2001; Cernac & Benning, 2004) have focused on improving biofuel feedstock so that these limitations can be resolved. Much of this work relies on the genetic modification of oil synthesis, plant growth, and development. The majority of this work is conducted on the model species *Arabidopsis thaliana*, which has been highly instrumental in our current understanding of seed oil synthesis and the genetic control of such processes. *Arabidopsis*' role as a model organism in biofuel production is related to its high seed oil content (comparable to other commercial biofuel feedstocks) and the close genetic relationship to *Brassica*, a very important commercial oil crop used in biofuel production (Baud & Lepiniec, 2009).

A significant step in genetic research was accomplished in 2000 by fully mapping the genome of *Arabidopsis* (Arabidopsis Genome Initiative, 2000) which has allowed for the discovery of dozens of gene functions including oil synthesis, disease resistance, and others that code for root growth, chlorophyll production and the development of reproductive organs (Bouche et al. 2001). An important study by Katavic et al. (1995) created a novel *Arabidopsis* mutant (AS11) with an average seed oil content of ~31% -

approximately 75% of average wild type *Arabidopsis* (Katavic et al. 1995). These "knock-down" mutants have been integral in highlighting important genes involved in seed oil synthesis. By identifying and suppressing genes involved in oil production, scientists can first observe the effects of reduced oil synthesis before taking the next step towards over-expressing these genes in an attempt to increase oil production.

The intent of the research presented here is to observe the effects of genetic modification of oil synthesis genes on three mutant genotypes of *Arabidopsis* that have genetically altered oil content (S-5 ~25.77%; S-6 ~24.89%; AS11~31.23%) and compare the results to a wild type (WT~35-37%) control. The objectives are to observe the effects of gene modification on secondary functions such as carbon and nitrogen partitioning; to explore the soil microbiology of the rhizosphere of these plants and determine if it is altered as a result of the genetic modification. Molecular DNA fingerprinting via terminal restriction fragment length polymorphism (T-RFLP) is used to assess whether there are changes in rhizosphere microflora resulting from changes in carbon exudation that may occur as a result of changes in C and N partitioning caused by genetic modification of oil synthesizing genes.

It is hypothesized that modification of genes involved with seed oil synthesis will alter the pattern of C and N partitioning among shoots and roots; and will be most pronounced during the seed filling and maturation stages of development. It is during this time that triacylglycerol (TAG) synthesis uses carbon from various plant tissues for seed filling, therefore, the knock-down mutants may show a significant reduction in carbon transfer to the seeds, in comparison to a wild-type control. It is unknown whether there is

a change in C/N rhizodeposition as a result of gene modification. Since carbon exudation from roots represents a significant factor in microbial proliferation, it is of great importance to understand how genetic manipulation could impact the surrounding microflora through increased or decreased rhizodeposition.

The results obtained from this study can be used to assess the potential impacts of altering these genes on other biofuel feedstocks such as *Brassica*. It is also important to observe whether there are significant changes in carbon and nitrogen exudation as a result of this genetic modification, as it may disrupt the symbiotic relationship with plant growth promoting microorganisms in the surrounding soil. Previous research has shown that increasing C compounds in the rhizosphere may be an undesirable trait as it can enhance competition for nutrients, and likely increase the prevalence of pathogens (Jones et al. 2004). In addition, phenotypic alterations resulting from genetic modification could affect plant growth and development (Boyes et al. 2001). Reduced root system may impede the growth of the plant by restricting the amount of nutrients being taken up through the roots, and inhibit the ability to effectively search out sources of water. There may also be changes in the progression of growth stages, such as delayed maturation, which would be undesirable for biofuel feedstocks.

2.0 LITERATURE REVIEW

The following section highlights the issues currently being faced regarding our rapidly changing environment, depleting fossil fuel reserves, and the use of biofuel as an alternative energy source. All points are considered, including many of the negative problems associated with biofuel use, and ways in which scientists are utilizing genetics engineering as a means of improving their viability.

2.1 Global Warming

According to the Intergovernmental Panel on Climate Change (IPCC, 2007), the global atmospheric concentrations of carbon dioxide, methane, and nitrous oxide are at their highest since 1750, and far exceed the pre-industrial values determined by ice core data from the past several thousand years. These greenhouse gasses absorb outgoing infrared radiation, which results in the raising of Earth's temperature; this is more commonly known as the greenhouse effect. CO_2 remains as the principle anthropogenic gas that is thought to affect the Earth's radiative balance, with the combustion of fossil fuels as a main contributor (IPCC, 2007). Although there is still some debate whether or not human influence can be solely responsible for global warming (Florides & Christodoulides, 2009), the effects of increased CO_2 should not be ignored.

2.1.1 Glacial melting

Recent studies on the Greenland and Antarctic ice shelves has provided some startling results regarding the rate at which they are currently receding (Darnis et al., 2007; IPCC, 2007; Ramanathan & Feng. 2009). According to the IPCC (2009), average arctic temperatures increased at almost twice the global average rate in the past 100 years. As a result, satellite data since 1978 show that annual average arctic sea ice extent has shrunk by 2.7 (2.1 to 3.3) % per decade, with larger decreases in summer of 7.4 (5.0 to 9.8)% per decade. In addition, global average sea level rose at an average rate of 1.8 (1.3 to 2.3) mm per year over 1961 to 2003. The rate was faster over 1993 to 2003: about 3.1 (2.4 to 3.8) mm per year (IPCC, 2007).

Research by Maslanik and colleagues (1996) show a nearly continuous, below normal summer sea ice coverage since 1990, with the decrease accelerating over the period 1987–1994. The extent of the ice pack was reduced by 9% in 1990–1995 compared with 1979–1989. In the Antarctic; of the nine ice shelves examined, the five most northerly shelves have retreated dramatically between 1945 and 1995 (Vaughan & Drake, 1996). Glaciers in the European Alps have lost 30–40% of their surface area and approximately half their volume since the mid-1800s, with an additional loss of 10–20% of their remaining volume since 1980. Since the late1980s, warming of alpine permafrost indicates acceleration by a factor of five to ten. Melting of ground ice also accelerated markedly from 1980–1990 compared with 1970–1980 (Haeberli & Beniston, 1998).

2.1.2 Storm severity

The increase in global temperatures has a wide variety of effects on the weather, and has been attributed to the rise in severe storms such as hurricanes, cyclones, and typhoons. In a study by Levinson (2005), it was shown that record-breaking numbers of hurricanes and cyclones were observed in Florida and Japan recently; almost doubling the current Japanese record of 6 to 10 cyclones in a single season. More frequent and severe storm surges have been linked to increased sea surface temperatures (SST); warmer ocean temperatures essentially fuel the intensity of many ocean borne storms (Arpe & Leroy, 2009). As ocean temperatures increase as predicted, there is increased risk of hurricane frequency and intensity on a global scale.

2.1.3 Ecological Implications

Recent studies on oceanic temperatures have shown that Australians' great coral reefs are already suffering permanent damage due to the increase in temperatures. Mass bleaching and mortality of corals are directly related to sea surface temperatures, with higher SSTs resulting in greater damage (Berkelmans et al, 2004; Goreau et al. 2005). The financial loss that can result from the destruction of the coral reefs is enormous, with an annual income of $30 billion each year from tourism alone. But the real loss would be 25% of the inhabitants of the oceans that spend at least a part of their life cycle in the coral reefs, which range from small dinoflagellates to larger predatory fish (Flannery, 2006; Warner et al. 2006). By removing this integral stage of an organism's life cycle, its natural growth and development is interrupted and may lead to its extinction, or the

extinction of its primary consumer. One such example of a species facing extinction is the *Gibiodon* species C, a small coral fish native to Papua New Guinea. Mass coral bleaching and habitat loss resulted from the 197-98 El Niño, leaving the *Gibiodon* only a small patch of remaining coral to live in (Flannery, 2006; Munday, 2004).

Among the most sensitive to changing temperatures are amphibians, which have already begun to show diminishing numbers as a result of unstable weather conditions leading to longer dry periods and sporadic precipitation (Flannery, 2006; Pounds et al., 2006; Wagner, 1999). The golden toad and the Monteverde harlequin frog are prime examples of species that have been mortally affected by the shifts in precipitation and temperature (Flannery, 2006; Pounds et al., 2006). Extensive observation by scientists (Pounds et al., 2006) in the Monteverde Cloud Forest Preserve in Costa Rica have documented a steep decline in the bird, reptile and amphibians inhabiting the preserve, as well as the complete disappearance of the golden toad and Monteverde harlequin frog. Historical temperature data shows that since the 1970's, average temperatures in the region have raised 0.18°C per decade, which is triple the average rate of warming for the entire twentieth century (Pounds et al. 2006).

2.2 Biofuels and the Carbon Debt

As the price and demand of oil continues to rise, coupled by a limited supply, we are faced with a global energy dilemma. Energy derived from biomass carbon in the form of biofuel has an important role to play in both reducing reliance on fossil fuel consumption, as well as mitigating CO_2 production. The IPCC (IPCC, 2007) maintains

that renewable energy such as biodiesel and bio-ethanol will play an integral role in overcoming the 'carbon debt' that has been incurred in recent decades. The term 'carbon debt' refers to the total amount of carbon (i.e.CO_2) that has been released as a result of human influence, rather than by natural causes. What biofuels aim to do is create a carbon balance, which is calculated as carbon dioxide emitted by biomass production and usage subtracted from the carbon dioxide fixed in the plant material, both above ground and underground (Yuan et al. 2008).

There are currently three classes of biofuel sources that are being pursued; first, second, and third – generation. What we currently recognize as 'biofuel' or plant-based fuel derived from fatty acid methyl esters (FAME) or sugars are first-generation. Other forms of renewable energy that can be derived from food crops, such as waste biomass (lignocellulose) or non-food crops such as switchgrass, are considered second-generation biofuels. Third generation biofuels, which will not be fully covered in this review, include oils derived from microbes and algae.

2.3 First-Generation Biofuels

First generation biofuels, such as bio-ethanol and biodiesel, are made from the extracted oils and sugars of several oilseed crops such as sugar cane, sugar beet, rapeseed, canola and soy. Combustion of first-generation biofuels does release new CO_2 into the atmosphere; however, it is done so at a rate that is comparable to the amount previously absorbed (Peterson & Hustrulid, 1998).

First-generation biofuels, such as biodiesel and bio-ethanol have been around since 1896 when Henry Ford build his first 'quadricycle', and intended it to run on ethanol (Schubert, 2006). The use of fossil fuels, however, began to rise with the automobile and has soared to unprecedented heights. As supply begins to diminish, alternative fuels have become sought after. In 2005, worldwide production of biodiesel had increased by 60%, ethanol by 19% and 'flex-fuel' cars (those capable of running on petroleum / bioethanol blends) became popular again (Schubert, 2006).

The most popular forms of first-generation biofuels are bio-ethanol and biodiesel. Most are derived from food feedstocks (i.e. corn, sugarcane, soy etc.), they offer renewable energy, and lower emissions compared to petroleum. They also have several significant disadvantages that have lead to increased interest in second-generation biofuels, mainly due to the food vs. fuel debate (See section 2.7.2).

2.3.1 Bio-ethanol

Bio-ethanol (ethanol) is produced through the fermentation of sugar derived from various high-sugar or high starch content feedstocks such as corn, sugarcane, sugar beet, and wheat. Production of ethanol is quite simple, requiring few steps to turn sugars and starches into ethanol. In the United States, for example, the main feedstock is corn. Grains are processed with the assistance of specific enzymes that help convert the starch into sugar, and then yeast ferments this sugar into ethanol (Schubert, 2007). Currently, 90% of the ethanol used for transportation in the U.S. is obtained from American grown corn (Van Gerpen, 2005). In 2003, the United States produced 3 billion gallons of

ethanol, which approximately made up 11% of the total US corn production (Van Gerpen, 2005).

2.3.2 Biodiesel

Biodiesel is a product of vegetable oil or animal fat that is chemically reacted with an alcohol and strong base such as sodium or potassium hydroxide, forming new compound called fatty acid methyl esters (FAME) (See figure 1). Current research has shown that waste oil from restaurants can even serve as a feedstock for biodiesel (Canakci, 2006). Canakci reports that waste cooking oils are an inexpensive alternative to conventional biodiesel feedstock (i.e. canola, soy, rapeseed, sunflower, etc.). Evidence from this study (Canacki, 2006) supports the notion that most diesel engines can run using waste oils, potentially offering a cheap, efficient alternative to feedstocks in direct competition with human consumption.

2.4 Second – Generation Biofuels

Second-generation biofuels differ from their first-generation counterparts in that they are derived from lignocellulosic biomass, a non-edible feedstock for humans, made by complicated physical and enzymatic processes. Switchgrass, for example, is a lignocellulosic feedstock that is of particular interest to scientists. Recent studies have shown that switchgrass can produce 540% more renewable than nonrenewable energy consumed, and estimates the GHG emissions from cellulosic ethanol produced by

switchgrass to be 94% lower than gasoline (Schmer et al. 2007). The technology to break down the lignocellulose effectively and efficiently is still being developed.

2.5 Third-generation Biofuels

The most recent sources of biological renewable energy are known as third-generation biofuels. These include algae and cyanobacteria that are genetically engineered to produce large quantities of oils. These feedstocks are considered to be far superior to first and second- generation biofuels because of their high yield and low land use; approximately 0.4% of the earth's arable land devoted to algae / cyanobacteria production sites could meet the current world fuel demand (Gressel, 2008). This technology is, however, still at the research stage, and far from becoming a viable option for fuel production.

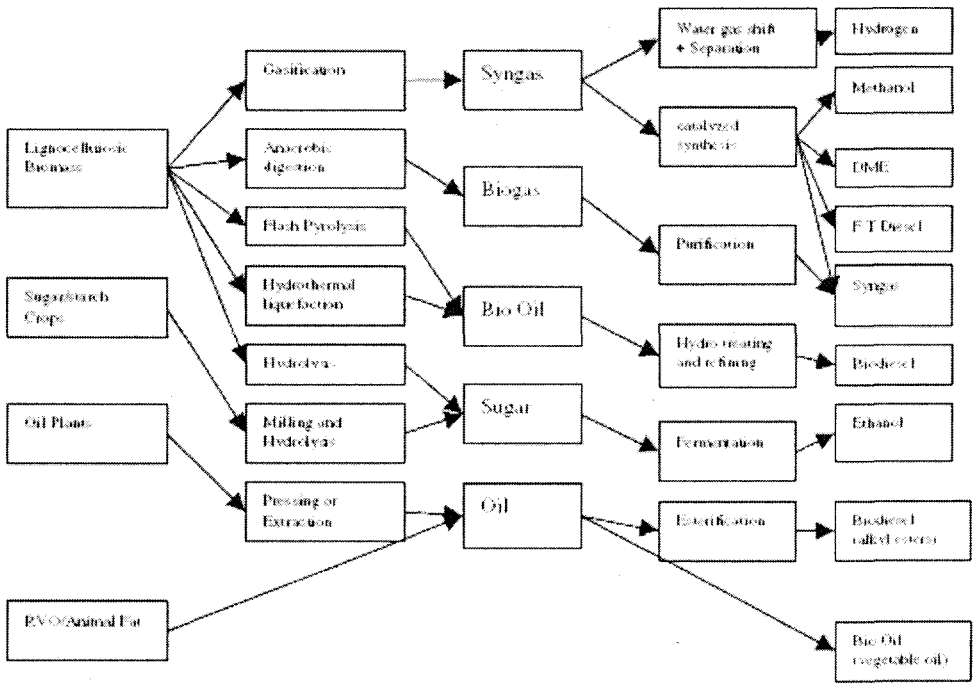

Figure 1: Conversion of source materials into biodiesel or bio-ethanol. (Agarwal, 2007)

2.6 Advantages of Biofuels

The advantages of utilizing biofuels for fuel and energy are numerous. Most importantly, biofuel is a renewable energy source with the ability to reduce current atmospheric CO_2 levels and offset some of the reliance on fossil fuels as a universal source of energy. Table 1 summarizes some of the most recent discoveries regarding biofuel type (platform) and potential ecological benefits, net energy balance, CO_2 balance and the energy required to harvest biofuel (agricultural practice) (Yuan et al. 2008). The study by Yuan et al. (2008) illustrates the importance of biofuels, particularly second-generation platforms such as lignocellulosic ethanol derived from *miscanthus* and switchgrass.

2.6.1 Reduced CO_2

The advantages of increasing production of biodiesel in the United States and Canada include a 78% reduction in CO_2 emissions when compared to petroleum-based diesel fuel (Coronado et al. 2009; Sheehan et al. 1998). Biofuels also have the ability to capture carbon (CO_2) from the atmosphere, and act as a carbon sink. A good example of this capacity to act as a carbon sink was demonstrated using a *Miscanthus* x *giganteus* genotype to fix CO_2. It was estimated that 5.2 – 7.2 t C/ha/yr was fixed, which results in a negative carbon balance in which more carbon was fixed than emitted (Clifton-Brown et al., 2008).

Table 1:Comparison of different platforms and bioenergy crops (Yuan et al. 2008)

Platforms[a]	Feedstock[a]	NEB[b] GJ/ha/yr	NER[b]	CO_2 balance	Annual feedstock	Establishment	Germplasm	Agricul. practice[d]	Ecological benefits	Refs
Ethanol from starch or sucrose	Maize	10–80	1.5–3.0	Positive	Yes	+++[c]	+++	+++	+	[4,28,30,31]
	Sugarcane	55–80	3.0–4.0	Positive	No	+++	+++	+++	+	[4,28,30,31]
	Sugar beet	40–100	2.5–3.5	Positive	Yes	+++	++	+++	+	[28]
	Sweet sorghum	85–300	5–10	Positive	Yes	+++	++	++	++	[28,92]
Ethanol from lignocellulosic feedstocks	Miscanthus	250–550	15–70	Possibly negative	Yes/No	+	+	+	+++	[28]
	Switchgrass	150–500	10–50	Possibly negative	No	+	+	+	+++	[4,28,30,31,93]
	Poplar	150–250	10–20	Possibly negative	No	+	++	++	+++	[24,30]
Biodiesel	Soybean	−20–10	0.2–0.6	Positive	Yes	++	+++	+++	+	[28]
	Canola	−5–2	0.7–1.0	Positive	Yes	+++	+++	+++	+	[28]
	Sunflower	−10–0	0.3–0.9	Positive	Yes	+++	++	+++	+	[28]

[a]Multiple platforms and crops are compared in a synthesis integrating information from multiple studies.

[b]Abbreviations: NEB, energy balance; NER, net energy ratio, which is the ratio of output to input energy needed to produce a fuel from a feedstock.

[c]Favorable features are indicated by + symbols, with +++ being the most favorable.

[d]Agricul. Practice, agricultural practice: how advanced is the current states of farming, harvesting, and processing.

2.6.2 Increasing automotive efficiency

In a recent study (Agarwal, 2007) the use of ethanol in conventional compression ignition (CI) diesel engines was assessed for its performance, long-term wear on the engine, overall emissions, and economic feasibility. Minor hardware modifications in existing engines would allow for the combustion of ethanol, which has a higher octane level than gasoline, delivering more power efficiently and economically (Agarwal, 2007). Ethanol burns cleaner, produces less carbon monoxide / dioxide, and oxides of nitrogen (Agarwal, 2007), however, there is a greater prevalence of aldehyde emissions when burning ethanol which contributes to the formation of phytochemical smoke (Agarwal, 2007).

Ethanol also resulted in an improvement in thermal efficiency and fuel consumption and a 20% ethanol-diesel blend was shown to be fully functional in a stock engine, and demonstrated a significant reduction in CO_2 and NOx emissions as well as an increase in fuel efficiency, with some minor problems resulting from viscosity (Harwood, 1984; Ma, 1999). Long-term endurance tests showed positive results on engine performance, condition, and emissions. Significant reductions in harmful green house gasses with no decrease in engine power or endurance were observed for both bio-ethanol / diesel and FAME biodiesel / diesel mixtures, and would greatly improve the current environmental conditions (global warming, acid rain, smog, etc.) that result from fossil fuel powered transportation (Harwood, 1984; Ma, 1999).

2.7 Disadvantages of Current Biofuels

Although the prospect of switching over to ethanol or animal / plant derived FAME biodiesel seems ideal, there are some significant drawbacks as well. High production costs for relatively low yield, offsetting arable land used for fuel instead of food (Mittelbach et al., 1992; Canakci and Van Gerpen, 2001; Kazancev, 2006; Searchinger et al., 2008), and the tendency for biodiesel to be problematic when left in temperatures colder than -10 °c (Chandler et al. 1992., Dunn et al., 1996., Lewtas et al. 1991; Kazancev, 2006) have all affected their viability as a replacement for petroleum. The study by Yuan et al. (2008) highlights other drawbacks of current first-generation biofuels, especially biofuels derived from canola and soy. In their study, some current biofuels show a positive CO_2 balance, meaning they contribute to atmospheric CO_2 rather than capture it. This is mainly due to the carbon cost of growing, harvesting, and transportation of biofuels (Fargione et al., 2008).

2.7.1 Cold flow properties

Factors relating to the poor results found in sub-zero temperatures stem from the physical nature of the vegetable derived fatty acid methyl esters (FAME), primarily its high viscosity, low cloud point (temperature at which waxy solids first appear during the cooling of diesel fuel), and mostly the formation of wax-like crystals in cold temperatures. Although not a serious problem to warmer countries, cold-flow inefficiencies of FAME biodiesel can be problematic for many northern regions of Europe, and North America. Engine failure, poor performance, and the addition of

chemical additives to allow for winter driving are some drawbacks to 100% biodiesel fuel in winter climates (Dunn et al., 1996).

2.7.2 Food vs. Fuel

The food vs. fuel debate has greatly impeded political and public support for biofuels, and has undermined their importance as a viable alternative energy source (Fargione et al., 2008; Koh & Ghazoul, 2008; Searchinger et al., 2008; Srinivasan, 2009). The "carbon debt" of land conversion for biofuel crops is the amount of CO_2 released during the first 50 years of development – it is during this time that biofuels "repay" their debt through atmospheric CO_2 sequestration and production of oils used as biofuel (Fargione et al., 2008). In their study, Fargione (2008) attribute a larger carbon debt to biofuels vs. fossil fuels because of factors such as land conversion and crop displacement (moving pre-existing crops in order to grow biofuel crops such as corn or sugarcane). Since there is only a limited area of arable land, biofuel producers have used a small proportion of food grain as biofuel feedstock which has lead to an increase in grain prices in recent years (Gressel, 2008).

The food vs. fuel issue has been the catalyst for much of the research conducted on first-generation biofuels, as improving biofuel feedstock efficiency and yield could minimize the problems associated with land allocation and food prices (Yuan et al. 2008). This issue has also been the driving force behind second-generation biofuel research, by offering bioenergy derived from non-food feedstocks (i.e. *Miscanthus*) thus eliminating the food factor all together.

2.8 Improving Biofuels

In order to effectively utilize biofuels and other plant-based renewable energy, significant changes must be made to improve feedstock yield and problems associated with the physical properties of fatty acids in sub-zero temperatures. Cold-flow solutions have been made in recent decades, and ongoing genetic research is bringing biofuels closer to becoming a viable option for renewable energy.

2.8.1 Cold-flow Solutions: Winterization

Two approaches for dealing with cold-flow inefficiencies were aimed at increasing the cold point, cold filter plugging point (CFPP), viscosity, and low temperature flow (LTF) (Dunn et al., 1996). The first of these approaches conducted by Dunn et al. (1996) was filtering off the solids in a large cylinder during an initial cold treatment (winterization), thereby refining the FAME before being used as in the engine. Results of this preliminary examination showed that winterizing FAME derived biodiesel can greatly improve the cold point, CFPP, viscosity and LFT problems allowing for use in colder temperatures and greater efficiency (Dunn et al., 1996). The only setback to this pretreatment process is the high reduction in yield, with up to 75% less with complete removal of the saturated methyl esters. Therefore a more feasible alternative would be a semi-filtering routine which would allow for a higher yield and greater ignition quality (Dunn et al., 1996).

2.8.2 Cold-flow solutions: Additives

The second alternative would be altering the physical chemistry of the FAME biodiesel with the application of additives. The role of additives in biodiesel has been worked on extensively, with several specific 'combos' demonstrating promising results (Dunn et al., 1996; Kazancev, 2006; Agarwal 2007). The function of these additives changes the properties of the thick portions of the fuel, co-binding to the sticky paraffin molecules that clog filters and plugs in engines at low temperatures (Dunn et al., 1996; Kazancev, 2006). The result is a crystallized, increasingly soluble paraffin molecule that effectively reduces the effects of low temperature coagulation (Dunn et al., 1996; Kazancev, 2006). On a comparative scale, results of winterization verses the application of additives shows that additives did not significantly reduce the cloud point or the viscosity of the FAME, while filtering greatly altered both (Dunn et al., 1996; Kazancev, 2006). Additives did, however, significantly reduce the LTF, but not in conditions below -5° C. It was concluded by Dunn et al. (1996) that winterization was most effective, but the great reduction in yield would be a major obstacle to overcome if applied on a mass scale. Also, additives show a great reduction in low temperature flow with an increase from 5°C to -5°C, which may be beneficial to areas that rarely dip below that temperature (Dunn et al., 1996)

2.9 Genetic Modification

There have been significant advances in our understanding of the factors related to seed oil synthesis, and ways at improving the viability of biofuels through genetic

manipulation. Most genetic research is conducted on *Arabidopsis thaliana*, a good model species for biofuel feedstock plants such as *Brassica*. The focus of this research is mainly altering plant growth and development, with an emphasis on increasing seed oil yield and land use efficiency.

2.9.1 The Role of Arabidopsis

Arabidopsis thaliana has been significant in the pursuit for key genes involved in fatty acid synthesis and storage, and has been instrumental in the development of several important species (Wallis and Browse, 2002). *Arabidopsis*'s role as a model organism in biofuel production is related to its high oil producing seeds (comparable to other commercial plants) and close genetic relationship to *Brassica*, a very important commercial oil crop used in biofuel production (Baud & Lepiniec, 2009). Great advances in understanding the location and functionality of *Arabidopsis* genes have been made, which is setting the stage for molecular manipulation of a variety of plants (Murphy, 1996; White et al. 2001). It has several important factors that make it an ideal model organism; these include its ease to grow and manipulate in a laboratory setting, a small genome amenable to detailed analysis, high mutation rate, versatility (research on Arabidopsis spans over several fields including physiology, biochemistry and developmental biology), funding (one of the most funded plants to research in the world), and a strong community of researchers with a commitment towards free exchange of data whenever it becomes available (Meinke, et al. 1998; Meyerowitz, 2001; Leonelli and Sabina, 2007).

2.9.2 Seed Oil Synthesis

Triacylglycerol (TAG) is the major lipid reserve in plants and animals. Nearly all the commercially important fats and oils of animal and plant origin consist almost exclusively of this simple lipid class, with a composition of roughly 95% TAG (Buchanan et al. 2000). This includes all the vegetable oils, such as those from corn (maize), olive, palm, and sunflower, and animal fats, such as tallow, lard and butter. The assembly of TAG occurs in the endoplasmic reticulum (ER), and is also known as the Kennedy pathway (Ramli, et al. 2002). Four consecutive reactions are catalyzed by ER membrane bound enzymes. The two intermediates, phosphatidate and 1, 2-diacylglycerol, are also substrates for the synthesis of membrane lipids glycosylglycerides and phosphoglycerides. The third acyltransferase, diacylglycerol acyltransferase (DGAT) which esterifies a fatty acid at the *sn*-3 position, is unique to TAG biosynthesis. Therefore, the last step in the pathway is the only dedicated step in triacylglycerol synthesis (Buchanan et al. 2000).

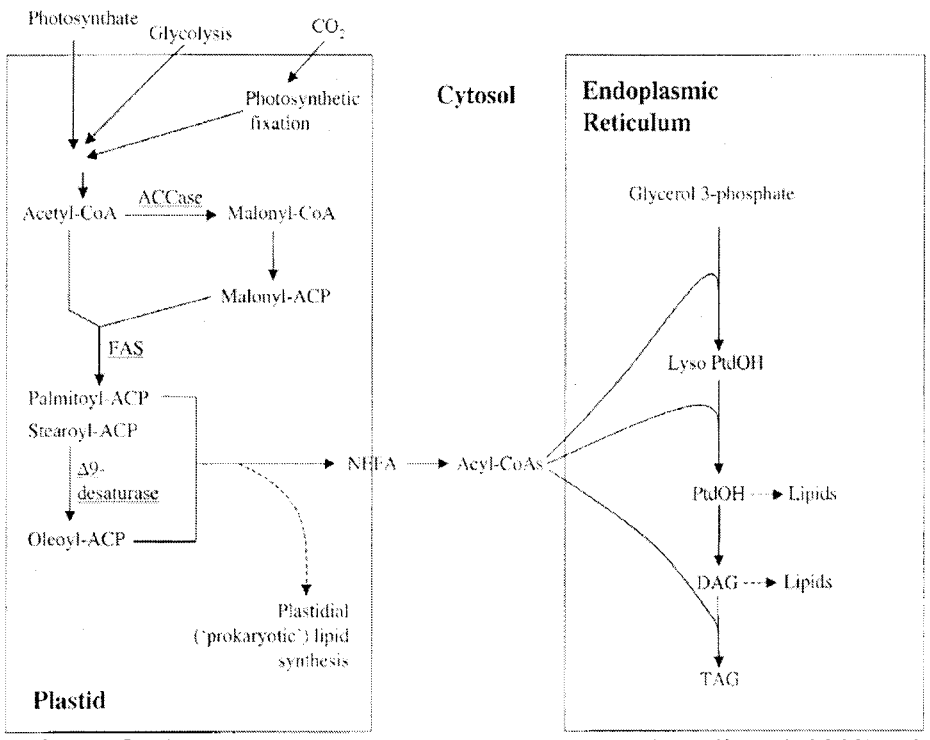

Figure 2: TAG biosyntheis via the Kennedy pathway (Ramli et al. 2002)**.** Most lipids are produced by the Kennedy pathway, however, only the final step is unique to TAG biosynthesis.

2.9.3 FAD genes

Specific locations such as the fatty acid elongation gene (FAD 2 and FAD 3) loci were shown to play a significant role in the production of fatty acids. It was observed that genetic modification of specific genes (FAD 1, 2) in *Arabidopsis* resulted in significantly altered seed oil composition, and several mutants containing a range of seed oil have been produced (Arondel et al. 1992; Okuley et al. 1994; James et al.1995).

2.9.4 Seed Oil modification

Katavic et al. (1995) demonstrate the importance of diacylglycerol acyltransferase (DGAT) in seed development and the pattern of fatty acid biosynthesis of *Arabidopsis*. A novel Arabidopsis mutant (AS11) was created via EMS mutation at a locus on chromosome II designated as *Tag1* which altered DGAT activity and caused delayed seed development, reduced triacylglycerol (TAG) content, as well as a repressed very long chain fatty acid (VLCFA) biosynthesis resulting in an average seed oil content of ~31% - approximately 75% of average wild type *Arabidopsis* (Katavic et al. 1995). The results therefore indicate that an overexpression of DGAT activity could significantly increase seed oil content by increasing VLCFAs and TAGs and may also have important implications for biotechnology through the use of DGAT manipulation in oilseed crops such as *Brassica* (Katavic et al. 1995).

2.9.5 *wrinkled*1 Genotype

To further explore oil deposition in developing seeds of Arabidopsis, Focks (Focks & Benning, 1998), engineered a novel Arabidopsis mutant with 80% reduction in seed oil content named *wrinkled*1. This mutant was successful at identifying a genetic locus (*wri*1) that is either responsible for encoding a regulatory protein which governs carbohydrate metabolism during seed development, or controls activity / expression of other glycolytic enzymes by a novel hexokinase acting as a sugar sensor (Focks & Benning, 1998). These gene knockout mutants have been integral in understanding TAG biosynthesis and the regulatory genes responsible for oil concentration and content (Bouche and Bouchez, 2001).

2.9.6 Identification of the QTL

Work done by Hobbs et al. (2004), located multiple quantitative trait loci (QTL) which are stretches of DNA that are closely linked to genes responsible for the inheritance of phenotypic characteristics, that are thought to control both seed oil and fatty acid composition, and that accounts for 43% of the variation in oil content in the population. It was shown that several QTL, two major and two minor, each individually control the production of linoleic and linolenic acids, oleic acid, stearic and palmitic acids. The most significant QTL was identified at the bottom of chromosome 2 accounting for 17% of the genetic variation, as well as two important QTL located on the upper and lower arms of chromosome 1 accounting for an additional 19% of the variation. This work has highlighted the most significant regions of the genome

responsible for fatty acid synthesis, and allowed scientists to mark these particular genes enabling geneticists to breed new crops that have enhanced traits (i.e. increased seed oil content, drought resistance).

2.10 Carbon and Nitrogen Partitioning

The effect of genetic modification of oil synthesis genes on C and N partitioning has not been fully explored. Throughout normal plant development, C and N are transported throughout the plant based on many factors (i.e. plant age, growth stage, nutrient abundance / deficiency), but the effects of genetic modification on this process are not fully understood. Nitrogen is mostly transported to areas that are undergoing growth and development, and may also act as a signaling molecule throughout the plant (Ford, 2002). Carbon is also utilized throughout the plant, and is the building block for many carbohydrates, lipids and carboxylic acids. For example, the developing embryos of *Arabidopsis* accumulate lipids in the form of triacylglycerols as the major carbon and energy reserves, which are then used for germination and growth of the young seedling. The triacylglycerols are stored in oil bodies that occupy close to 60% of the cell volume of the cotyledons in mature embryos (Focks and Benning, 1998).

2.10.1 The Rhizosphere and Carbon Efflux

The rhizosphere is nutrient rich region surrounding the roots of a plant. This area contains a high density of microbial biomass that feeds on various root exudates, comprised mainly of carbohydrates, carboxylic acids and amino acids (Baudoin et al.,

2005). Plants normally release root exudates during growth and development, and have been shown to play a significant role in many plant – plant, plant – microbe interactions (Bais et al., 2006). Organic carbon is considered as the limiting factor for microbial density and activity (Bowen and Rovira, 1999; Lugtenberg and Dekkers, 1999), and factors such as plant genotype, age and nutrition level can all affect carbon rich root exudation, thereby altering microbial populations (Marschner and Timonen, 2005).

Genetic manipulation of oil synthesizing genes may play a role in altering the soil carbon levels and thereby affecting neighboring microbial populations. Because root exudation is a determining factor for microbial density, it is of great interest to investigate how altered carbon and nitrogen partitioning may affect this balance.

2.11 The use of T-RFLP Analysis

Recent advances in the methods used to identify microbial communities, such as Polymerase Chain Reaction (PCR), Amplified Ribosomal DNA Restriction Analysis (ARDRA), Thermal / Denaturing Gradient Gel Electrophoresis (TGGE / DGGE) and Terminal Restriction Fragment Length Polymorphism (T-RFLP), have been showing great promise in the detection of novel soil microorganisms. The most recent of these methods, T-RFLP, has been particularly useful in identifying microbial communities in the soil, and has been utilized in several laboratories (Chin et al., 1999; Fey et al., 2000; Lueders et al., 2000) since its discovery a decade ago. T-RFLP differs from other methods of soil microbial analysis by avoiding some of their inherent limitations (i.e. limited resolution, detection of only dominant bacteria species, etc). It utilizes the same

principles as RFLP (Restriction Fragment Length Polymorphism), by identifying changes in the 16s rRNA gene fragment common to all bacteria; however, it simplifies complex community analysis through the addition of a fluorescently labeled PCR primer. Because of the complexity of RFLP profiling of diverse communities, the development of terminally labeled PCR products enables for a robust, yet simplified method for investigating changes in the quality and quantity of microbial populations (Liu et al., 1997). The greatest advantage of utilizing T-RFLP for community profiling of bacteria stems from its ability to be fully automated, and could lead to significant advances in our understanding of soil microbes (Liu et al., 1997).

2.11.1 Minimizing Bias and Other TRFL-P Hazards

Although the method provides a reliable means of assessing bacterial presence in soil samples, several important disadvantages can often skew results. PCR bias may occur, providing inaccurate estimates of organism abundance due to differences in gene copy number (Kitts, 2001). It is important to pool samples and maintain 10-100ng of template DNA at 30 PCR cycles to minimize PCR bias and obtain the most accurate depiction of microbial populations in each sample (Kitts, 2001). Another potential hazard while using the T-RFLP method arises with restriction enzyme use. It is maintained in the literature that 4-6 restriction enzymes are sufficient to obtain good resolution (detection of separate bacterial populations in a sample) (Kitts, 2000). While it is possible to use a greater number of enzymes, therefore increasing resolution, it is not advisable to use any less than four. Finally, analyses of T-RFLP peaks are subjective. There are many pitfalls

that must be taken into account during data analysis, as several interpretations of a single peak may be made. The contribution of a single peak may be from several bacterial species, or a single dominant species (Kitts, 2000). Therefore, it is essential to remove excess 'noise' from peak charts, so that analysis errors can be minimized. The most frequently used method is by establishing a variable threshold, which standardizes the results and eliminates peaks that fall below a particular percent area of the chart.

3.0 MATERIALS AND METHODS

3.1 Experimental Design

Four genotypes of ecotype Columbia *Arabidopsis thaliana* were used to observe the effects of genetic modification of oil synthesizing genes on C and N partitioning at specific growth stages. ^{13}C and ^{15}N isotope labeling was used to trace the flow of C and N through various regions of the plant, and rhizosphere. Molecular analysis of the rhizosphere via T-RFLP was conducted at final harvest to explore whether any changes in C and N partitioning altered the bacterial microflora resulting from increased or decreased root exudation.

A total of 160 pots (experimental units) consisting of 7 plants per pot were used for this experiment. The experimental group consisted of 100 total pots (n= 100), that was then divided into 5 sampling periods (n=20 per sampling period), and 4 genotypes (see section 3.2) (n=5 per genotype). The experimental group was given both $^{13}CO_2$ and ^{15}N isotope labeling, and sampled at specific growth stages (see section 3.5.1 – 3.6). The control group of 60 pots (n=60) served as an environmental control (no isotope labeling) and was also subdivided by 5 sampling periods (n=12 per sampling period) and 4 genotypes (n=3 per genotype). Additionally, two spare pots of each genotype were grown (unlabeled) to be used for T-RFLP analysis of the rhizosphere soil.

3.2 *Arabidopsis* Genotypes

Three knock-down *Arabidopsis thaliana* (ecotype Columbia) mutants expressing reduced seed oil content were used in comparison to a wild-type control. S-5 and S-6 are T-DNA insertion mutants expressing 25.77% (SE +/- 0.84) and 24.89% (SE +/-0.84) seed oil, respectively. Insertions were made by the Salk institute in the LACS4 gene, thereby disrupting the LACS4 enzyme involved in activation of fatty acids to coenzyme-A (CoA) during lipid metabolism (Chong et al. 2008).The mutants also possess a speckled seed coat phenotype, that accompanies the reduction in seed oil. Katavic suggests that the decrease in seed oil content and speckled seed coat may be caused by an unknown gene(s), designated as "Gene-X", because during reciprocal crosses with the mutants and wild-type, the co-segregation of the two phenotypes yielded progeny that still contained the T-DNA insertion yet did not display the phenotypic characteristics of reduced oil content and a specked coat (Katavic, personal communication). The loci of the gene(s) are currently being pursued by Dr. Katavic at UBC.

The AS11 genotype is an EMS (ethyl methanesulfonate) induced mutant containing 31.23% (SE +/- 1.48) seed oil. The use of EMS is regarded as a 'shotgun' approach to molecular research, as it produces random mutations in genetic material, often leading to a variety of genotypes (Mayer et al. 1991). In this case, the AS11 genotype has been characterized as having TAG and VLCFA deficiencies resulting from EMS mutations on a region of chromosome II designated as *Tag1* caused by disruptions in DGAT activity (Katavic et al. 1995). These were then compared to a wild-type control

(ecotype Columbia), which normally contains 35-37% seed oil content. All Genotypes were provided by Dr. Kunst at the University of British Columbia.

3.3 Soil

Soil used for this experiment was obtained in the summer of 2007 and 2008 from a private farm in Wolfville, Nova Scotia, Canada. The soil was manually excavated and transported to the greenhouse at Saint Mary's University where it was spread out thinly on a tarp and dried naturally in the sun for several days. The dried soil was then sieved to 2mm and mixed (50/50) with coarse sand for improved drainage. An analysis of the soil constituents including nutrients and physical / aggregate properties was conducted by Bodycote Testing Group ® (see appendix 7.1)

3.4 Growth Conditions

All genotypes of *Arabidopsis* were grown in the Saint Mary's University (SMU) greenhouse for the entirety of the experiment, only to be removed momentarily for ^{13}C labeling. One liter pots were filled with 1570g's soil, watered and *Arabidopsis* seeds were then added to the surface of the soil to germinate. Shortly after germination, pots were thinned to contain only seven plants that were evenly spaced (2-3 inches apart). In early stages of development, plants were given approximately 100 ml of 50% Hoagland's solution when needed (2-3 times per week). At 50% dilution, the Hoagland's solution contains sufficient nutrient content to maintain healthy growth and development, without the risk of toxicity sometimes occurring without dilution (Leggett, 1971). At later stages

of development plants were supplied with 100 ml Hoagland's solution every day. Photoperiod of supplemental lighting was set to16 hours on and 8 hours off at an intensity of 200-340 µmol/m^{-2}/s^{-1}. Once a week, pots were randomly rearranged to ensure even light distribution.

3.5 Isotope Labeling

3.5.1 $^{13}CO_2$ Labeling

Pulse-chase $^{13}CO_2$ labeling was conducted at principal growth stage 5.1 (day 27) (See Figure 4). This stage is characterized by completion of rosette growth, and the concurrent development of a bud at the apical meristem. Once the plants were situated within the labeling chamber, the internal CO_2 was measured by a gas analyzer and allowed to lower to the predetermined compensation point. The ambient CO_2 was ~485 ppm. The CO_2 compensation point was identified in an earlier experiment to be155 ppm. Once the chamber reached this 155 ppm, 1 M $Na_2{}^{13}CO_3$ was applied into 200 ml of 3 M H_2SO_4 solution in the chamber by a peristaltic pump to bring the CO_2 concentration back up to ~500 ppm. After the CO_2 in the chamber declined to the compensation point, additional $Na_2{}^{13}CO_3$ was added. This continued until 100 ml of ^{13}C was absorbed.

3.5.2 $^{13}CO_2$ labeling chamber

The labeling of plants with $^{13}CO_2$ took place in a specially constructed labeling chamber (see Figure 3). The light source was supplied by two 1000 W high pressure sodium (HPS) lights situated above the chamber. This provided a light intensity of 350-

370 umol/$m^{-2}s^{-1}$. The temperature within the labeling chamber was controlled by a cooling system and was maintained at 30-32° C. The relative humidity within the chamber was approximately 85%. See Figure 3 for detailed schematics of labeling chamber.

3.5.3 $^{15}NO_3$ labeling

$^{15}NO_3$ labeling was conducted in the SMU greenhouse one week prior to the $^{13}CO_2$ labeling. Thirteen liters of 15% $^{15}NO_3$ labeling solution was created by adding 11.05 g of KNO_3 and 1.95 g $K^{15}NO_3$ to 13 L of water. Each plant was given 100 ml of the solution in the morning and was not watered again until the following day. Watering resumed the next day, but only 50 ml of Hoagland's solution was used to ensure watering would not wash out the labeling solution. Careful attention was made to ensure no water flowed out the bottom of the pots.

3.6 Sampling for C/N partitioning

Sampling was conducted at five times, which represented distinct growth stages reported by Boyes (Boyes et al. 2001). Plants were sampled at day 27 (stage 5.1), day 36 (stage 6.0), day 43 (stage 6.5), day 50 (stage 6.9) and day 65 (stage 9.7) (See Figure 4). These sampling times were selected to encompass the late vegetative – fully mature stages of development of wild-type *Arabidopsis* plants, and therefore serves as a template to compare affects of genetic modification on phenotype growth and development. Plants were harvested at each sampling time and prepared for analysis. On day 27, the plants

were harvested immediately after $^{13}CO_2$ labeling. All dried, crushed and weighted samples were sent to the Stable Isotope Facility at the University of Saskatoon (Saskatchewan, Canada) for analysis.

3.6.1 Shoots

The entire shoot was cut from plant, dried in an oven at 80°C for 3 days, then ground into a fine powder using a mortar and pedestal. A conventional coffee grinder (Lancaster® Coffee Grinder) was used to grind shoots of plants during sampling periods 3-5 to compensate for the larger, tougher stems at these growth stages. A subsample of 50 mg was then sent for analysis.

3.6.2 Roots

The pots containing the roots and soil were immersed in 2 L of water, gently isolating the root system. The roots were then rinsed twice more in 0.5 L of clean water. The cleaned roots were dried in the oven at 80°C for 3 days, and then ground into a fine powder using a mortar and pedestal. The dried matter was weighed and a 50 mg subsample was sent for analysis.

3.6.3 Soil

After teasing away the roots, the soil (1570g's) and 3 L of water remaining, were put into 8 L carboys, shaken for ~10 minutes then filtered with Whatman® #1 filter paper. A volume of 100 ml of the filtrate was collected and dried in the oven at 80°C. The

dried matter containing exuded water-soluble and microbial C and N was washed out with 5 ml distilled water and centrifuged at 1,000 rpm for 5 minutes. To prepare for analysis, 125 µl of the supernatant was pipetted into small tin cups and placed into the oven at 50°C until evaporated. This continued until 1.4 ml of supernatant was evaporated, leaving residual C and N in the cups. The tin cups were then folded closed, and put into an Elisa plate and sent for analysis.

3.6.4 Seeds

The seeds were carefully collected by placing the cut shoots into a paper bag and shaking them loose from the siliques. They were then put through a mesh screen and collected in a container. At this point they were weighed and stored. For analysis of C and N content, the seeds were placed in a chilled mortar containing a small amount of liquid nitrogen. Once completely frozen, they were crushed into a fine powder using a pedestal and a 2 mg subsample was sent for analysis.

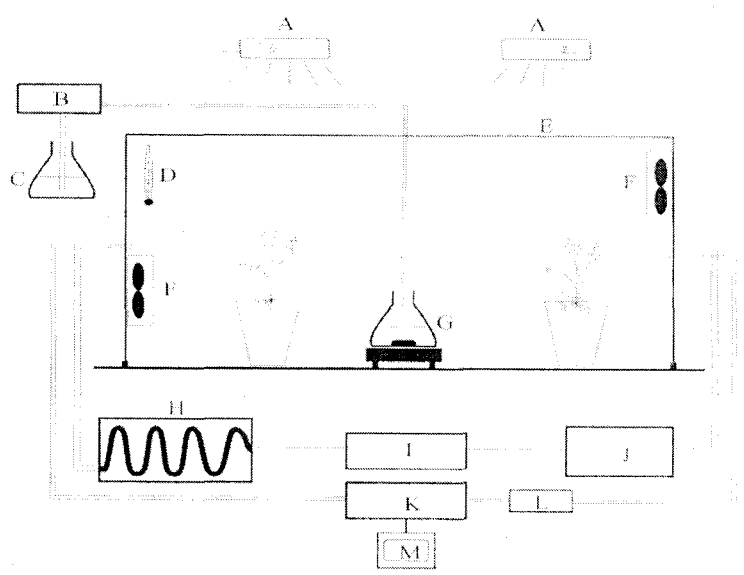

Figure 3: Diagram of ^{13}C labeling system. A: HPS lights (1000 W each), B: peristaltic pump, C: $Na_2{}^{13}CO_3$ solution, D: temperature and relative humidity indicator, E: acrylic labeling chamber, F: circulation fans, G: H_2SO_4 solution, H: cooling system, I: condenser, J: air pump, K: CO_2 monitor, L: air pump, M: computer.

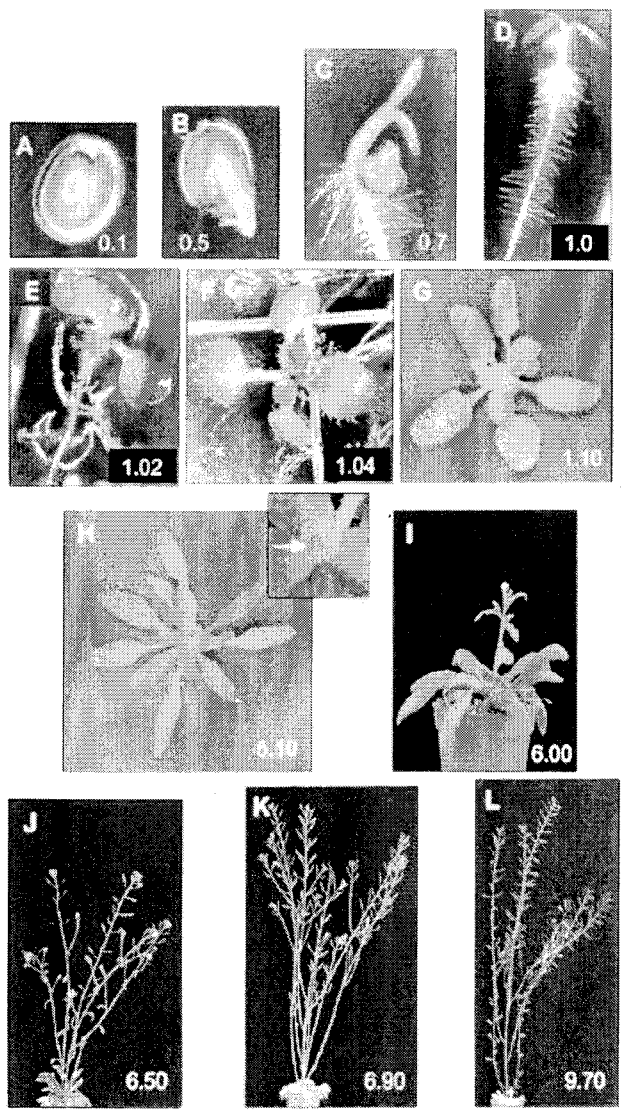

Figure 2. Arabidopsis Growth Stages

Figure 4: Growth stages of Arabidopsis thaliana (Boyes et al. 2001).
Sampling periods were selected at growth stages 5.10, 6.00, 6.50, 6.9 and 9.7

3.7 Molecular Methods for T-RFLP Analysis

3.7.1 DNA extraction

Five 1 g rhizosphere soil samples (randomly selected from pots of each genotype, and unpotted bulk soil) were carefully collected by exposing the roots and gently teasing away the thin layer of soil adhering to the roots. This soil was then treated with the UltraClean® Soil DNA Isolation Kit (Mo Bio Laboratories, Inc., Solana Beach, CA) to isolate the bacterial DNA from the soil samples. The Alternative Protocol was followed in accordance to Mo Bio Laboratories, Inc. for maximum yields. To ensure DNA purity and record nucleic acid concentration, 1.0 µl of the extract was analyzed by a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific Inc). Purity can be affected by RNA contamination and residual salts / solutes from the DNA extraction process. A 260/280 reading of 1.8 +/- 0.1 indicates a relatively pure sample. See table 3 of results of NanoDrop.

3.7.2 Amplification of 16S rRNA genes

A segment of the 16S rRNA gene with an approximate length of 527 base pairs (bp) was amplified using a pair of universal bacteria primers; a fluorescently labeled forward primer, BSF 8/20 (6-FAM-5' – AGAGTTTGATCCTGGCTCAG – 3') and unlabelled reverse primer, BSR 534/18 (5' – ATTACCGCGGCTGCTGGC -3'). Each 25µl reaction mixture contained 17.1 ml of ultra purified water treated with 0.1% DEPC (Diethylpyrocarbonate), 2.5 µl 2 mM dNTP (Qiagen, Mississauga, Ont., CA), 2.0 µl Magnesium, 2.5 µl buffer, 0.2 µl Taq DNA polymerase (Fermentas Life Sciences,

Burlington, Ont., CA), and 0.5 µl of each aforementioned primers (New England Biolabs Ltd., Pickering Ont., CA).

Amplified reactions were carried out in a Bio-Rad My-Cycler thermal cycler (Bio-Rad Laboratories, Inc., Hercules CA) using the following protocol: three minutes of initial denaturation at 95°C, 40 cycles of 30 seconds at 95°C for denaturation, 30 seconds at 56.8°C for annealing, and 45 seconds at 72°C for extension, followed by a final 10 minute primer extension at 72°C. Seven PCR reactions from each sample were pooled together to minimize PCR-induced random biases and then purified using the QIAquick purification kit (Qiagen, Mississauga, Ont., CA).

3.7.3 Restriction endonucleases and TRF peaks

Four restriction endonucleases (REs), *BstUI, HaeIII, HinfI, and MspI* (New England Biolabs Ltd., Pickering, Ont., CA), were selected due to their success in previous experiments used isolating bacterial populations in soil samples (Zhang et al, 2009). To obtain four different terminal restriction fragment (TRF) profiles for each sample, 10 µl of purified PCR product from each sample was treated with 1 µl of each RE, 34ml of dH_20, and 5 µl of #2 buffer (provided with REs). Each 50µl reaction mixture was then incubated overnight; *BstUI* at 60˚C and the rest at 37˚C. For each reaction, three replicates were made and pooled together to minimize artificial bias. To stop the enzyme digestion, all samples were then put through the QIAquick Nucleotide Removal Kit (QIAGEN Inc., Mississauga, Ont., CA). Finally, 6-FAM-TRFs (6-FAM labeled terminal restriction fragments) in digested amplicons were separated and recorded by a model

ABI3730 DNA sequencer (Applied Biosystems, Foster City, CA) at the University of Calgary Core DNA Services lab (Calgary, AB, Canada).

3.7.4 TRF Peak Generation

Gene Marker software was used to generate the TRF profiles, which showed both the fragment length in base pairs, and the peak height or intensity (see section 7.2 in appendix). Gene Marker also provided a partially completed allele report, a binary grid of 1s, 0s, and question marks indicative of the presence or absence of peaks at specific base pairs (bps). Several steps are required to complete the allele report; normalization of TRF profiles helps eliminate false positives, and a variable percentage threshold is used to complete the identity of the question marks.

3.7.5 Standardization of TRF Profiles

After TRF peak generation, the data was standardize using a percentage threshold limit in order to eliminate some of the background 'noise'; insignificant peaks that do not contribute significantly to the overall TRF profile. As reported by Osborne et al. (2006), a variable percentage threshold effectively sets a limit unique to each TRF profile, thereby minimizing error caused by noise. Establishing a threshold was done by using a divisor to divide the total area of each profile. The divisor was calculated using a custom Matlab program called TRFLPdemo, written by F. Lou (M.Sc. Computer Science student, SMU) and Zhang, Y.(M.Sc. Applied Science, student SMU). Total size and area of each TRF profile was loaded into the program, and created a curve with the R square (R^2) value. In

order to obtain the best divisor containing the most random distribution of all points illustrated by a straight line across the grid, divisor (set at 100x the mean total area of all profiles) and interval (set at $1.00*10^6$) values were adjusted. The best threshold (see table 2) was then generated using this method, and used as the divisor for normalizing each TRF profile.

3.7.6 Establishing VPT for TRF peaks

Results of the normalization of TRF peak data allowed for the generation of a variable percentage threshold (VPT), which is used to minimize the prevalence of false peaks and reduce background noise. The VPT was then calculated using the following formula:

VPT = Total Area / Optimal Divisor *100

This calculation provided unique cut-off points for each TRF profile, and allowed for completion of the binary grid (allele report). First, the total area of each TRF profile was calculated, and then the percent area of each peak was calculated. By using the VPT calculation, a threshold % was established and any peak area % under this threshold % was considered insignificant and removed.

3.7.7 Generation of Dendrograms

The following commands were used in Matlab to create the dendrograms:

```
S={'AS11', 'S5', 'S6', 'Wild', 'Bulk soil'}
        data1=data';
Dist= pdist(data1,'jaccard');
link = linkage(Dist,'average');
c = cophenet(link,Dist);
[H,T] = Dendrograms(link,'colorthreshold','default','orientation','left', 'labels', S)
```

3.8 Statistical Analysis

Statistical analysis was conducted using GraphPad PRISM® software (version 5.0). A two-way repeated measures ANOVA was used to test for differences in carbon and nitrogen partitioning between genotype, as well as the sampling period. When significant differences were found, a Bonferroni post-test test was used to determine the location of the differences. Results were considered significant at a P value <0.05.

Table 2: Optimal divisors generated by Matlab used to normalize data

Data Set	*BstUI*	*HaeIII*	*HinfI*	*MspI*
Optimal Divisor	$5.08*10^{7}$	$2.18*10^{7}$	$3.36*10^{7}$	$6.28*10^{7}$
R^2	$6.6*10^{-3}$	$2.1*10^{-3}$	$7.2*10^{-6}$	$2.9*10^{-6}$

Table 3: NanoDrop Results. Samples used for T-RFLP appear in bold. A 260/280 at 1.8 +/- 0.1 is considered pure and was therefore used for subsequent PCR reactions

Sample ID	Nucleic Acid Concentration	Unit	A260	A280	260/ 280	260/ 230
a1	66.6	ng/µl	1.332	0.736	1.81	0.65
a1	**54.0**	**ng/µl**	**1.080**	**0.578**	**1.87**	**0.56**
a2	48.4	ng/µl	0.968	0.591	1.64	1.00
a2	46.6	ng/µl	0.933	0.543	1.72	0.97
a3	39.8	ng/µl	0.797	0.452	1.76	1.13
a3	40.1	ng/µl	0.803	0.459	1.75	0.89
blank1	0.6	ng/µl	0.011	0.007	1.48	1.77
b2	**57.8**	**ng/µl**	**1.156**	**0.638**	**1.81**	**0.93**
b2	57.2	ng/µl	1.143	0.665	1.72	0.90
b3	25.0	ng/µl	0.500	0.292	1.71	0.63
b3	32.8	ng/µl	0.656	0.383	1.71	0.75
blank2	0.3	ng/µl	0.007	0.014	0.47	0.52
c2	40.3	ng/µl	0.805	0.424	1.90	0.49
c2	**52.0**	**ng/µl**	**1.039**	**0.600**	**1.81**	**0.78**
c3	78.3	ng/µl	1.566	0.859	1.82	0.96
c3	78.3	ng/µl	1.565	0.869	1.80	0.97
c4	23.4	ng/µl	0.468	0.277	1.69	0.63
c4	23.2	ng/µl	0.465	0.280	1.66	0.63
Bulk 1	**26.4**	**ng/ul**	**0.528**	**0.284**	**1.86**	**0.53**
Bulk 2	26.3	ng/µl	0.509	0.298	1.76	0.51
Bulk 3	30	ng/µl	0.6	0.315	1.9	0.47
d1	48.7	ng/µl	0.975	0.549	1.78	0.89
d1	39.4	ng/µl	0.789	0.446	1.77	0.83
d3	**58.9**	**ng/µl**	**1.178**	**0.655**	**1.80**	**0.62**
d3	53.8	ng/µl	1.075	0.598	1.80	0.59

4.0 RESULTS

4.1 Growth and Development

Although no quantitative data was recorded, it was observed that the development of *Arabidopsis* genotypes were significantly different. Upon germination, the *Arabidopsis* mutants AS11, S-5, and S-6 appeared to display higher seedling mortality rates, in comparison to the wild type control. This was accompanied by an apparent decreased resistance to environmental stressors (drought, heat), which was shown by an increased prevalence of purpling plants amongst the S-5 and S-6 genotypes.

Progression through specific life stages were also significantly different based on genotype. The S-5 and S-6 genotypes exhibited delayed vegetative growth prior to the first sampling period (late vegetative / early reproductive), and were shown to reach the budding / bolting stage before rosette growth was complete. This was further verified upon analysis of shoot dry weight (see section 4.1.1). These plants, however, appeared to catch up to the wild type / AS11 genotypes and progress to the fourth growth stage (late reproductive) at a similar rate. Upon entering final harvest (fully mature), it was clear that the S-5 and S-6 genotypes had not yet fully matured, and still contained many developing siliques. Meanwhile, the AS11 and wild type genotypes had completely matured and all siliques were brown / rupturing. However, it was also observed that the AS11 had reached full maturity approximately 1 week after the wild type.

4.1.1 Shoots

The total dry weight (Figure 5) of *Arabidopsis* shoots was not significantly different based on genotype ($F_{(3, 64)} = 0.40$, P=0.7574); however, AS11 and S-5 were found to have significantly different final harvest (Growth stage 5) mass ($p<0.05$). Overall, genotype did not significantly affect shoot mass ($F_{(3, 64)} = 0.86$, P=0.4807); however there were significant differences in final harvest (Growth stage 5) dry weight between AS11 and wild type ($p<0.05$); S-5 and wild type ($p<0.001$); S-6 and wild type ($p<0.01$) (Figure 6).

4.1.2 Roots

Root mass was shown to be significantly different based on growth stage ($F_{(4, 64)} = 0.86$, $P<0.0001$), and genotype was found to be an insignificant factor (p=0.0620). Bonferroni post-tests showed significantly different root mass ($p<0.05$) in sample period 3 between AS11 and S-5; AS11 and S-6 ($p<0.01$); in growth stage 2 and 5 between AS11 and wild type ($p<0.05$; $p<0.01$); and finally at growth stage 5 between S-6 and wild type ($p<0.05$) (Figure 7).

4.1.3 Seed Yield

There were no significant differences (P=0.3916) in seed yield based on genotype (Figure 8).

4.2 ^{13}C excess

^{13}C excess (mg) was used to identify the flow of ^{13}C isotopes throughout the plant at any given sampling period. This was calculated by first subtracting the ^{13}C percentage found in the labeled samples by the unlabeled control samples (i.e. natural abundance of ^{13}C in the sample). This gave the percentage of ^{13}C in excess of that found in unlabeled samples at each growth stage (shown as At excess %). To trace the content rather than the concentration, the following calculation was used:

^{13}C excess (mg) = (dry weight * elemental C %) / At excess % *10

To further explain this formula, the dry weight of each sample is multiplied by its measured carbon content and then divided by the percentage of ^{13}C (At excess %) and multiplied by 10, to give it a measurement in milligrams. The resulting number is the amount of 13C labeling (mg) that is present, above the natural abundance (control).

4.2.1 Shoots

There were significant differences in the mean ^{13}C content of the shoots of *Arabidopsis* based on genotype and growth stage. Growth stage accounted for 77.32% of the total variance ($F_{(4, 64)} = 83.34$, $P<0.0001$). Genotype was found to account for 2.10% of the total variance, and significantly affected carbon levels ($F_{(3, 16)} = 4.65$, $P<0.0160$). Bonferroni post-tests showed significantly different carbon content ($p<0.05$) in growth stage 1 between AS11 and wild type; S-5 and wild type ($p<0.01$); as well as S-6 and wild type ($p<0.001$) (see Figure 9).

4.2.2 Roots

There were significant differences in the mean ^{13}C content of the roots of *Arabidopsis* based on genotype and growth stage. Growth stage accounted for 26.05% of the total variance ($F_{(4, 64)} = 9.97$, $P<0.0001$). Genotype was found to account for 11.43% of the total variance, and significantly affected ^{13}C levels ($F_{(3, 64)} = 6.36$, $P<0.0048$). Bonferroni post-tests showed significantly less labeled carbon ($p<0.05$) in S-5 and S-6 genotypes during growth stage 1, and significantly less ^{13}C in growth stage 5 for the S-6 genotype (see Figure 10).

4.2.3 Seeds

There were no significant differences ($P=0.3916$) in ^{13}C content based on genotype (Figure 11).

4.2.4 Soil

Significant differences in the mean ^{13}C content of the soil were observed based on growth stage, and genotype. Growth stage accounted for 7.54% of the total variance ($F_{(4, 64)} = 2.51$ $P=0.05$). Genotype was found to account for 5.31% of the total variance, and did not significantly affect soil ^{13}C levels ($F_{(3, 64)} = 2.46$, $P=0.1003$). A Bonferroni post-test revealed significant differences in ^{13}C content ($p<0.05$) in growth stage 1 between S-6 and the wild-type control; as well as in growth stage 5 ($p<0.01$) between S-6 and the wild-type, and AS11 and the wild-type (see Figure 12).

4.3 ^{15}N excess

^{15}N excess was determined by using the same formula as ^{13}C (see section 4.2)

4.3.1 Shoots

There were significant differences in the mean ^{15}N content of the shoots of *Arabidopsis* based on growth stage, but not genotype (see Figure 13). Sample period accounted for 50.97% of the total variance ($F_{(4, 64)} = 22.45$, $P<0.0001$). Genotype was found to account for 1.01% of the total variance, and did not significantly affect ^{15}N levels ($F_{(3, 64)} = .88$, $P=0.4729$)

4.3.2 Roots

There were no significant differences in the mean ^{15}N content of the roots of *Arabidopsis* based on growth stage or genotype (see Figure 14). Sample period accounted for 7.16% of the total variance ($F_{(4, 64)} = 2.25$, $P=0.731$) and the effect is considered not quite significant. Genotype was found to account for 5.74% of the total variance, and did not significantly affect ^{15}N levels ($F_{(3, 64)} = 1.42$, $P=0.2740$).

4.3.3 Seeds

There were no significant differences ($P=0.3916$) in ^{15}N content based on genotype (Figure 15).

4.3.4 Soil

There were significant differences in the mean ^{15}N content of the soil based on growth stage and genotype. Growth stage accounted for 90.86% of the total variance ($F_{(4,64)} = 256.66$, $P<0.0001$). Genotype was found to account for 0.44% of the total variance, and did not significantly affect ^{15}N levels ($F_{(3,64)} = 1.42$, $P=0.2229$). A Bonferroni post-test revealed a significant difference in ^{15}N ($p<0.01$) in growth stage 1 between AS11 and S-5; and between S-5 and S-6 (see Figure 16 - 18).

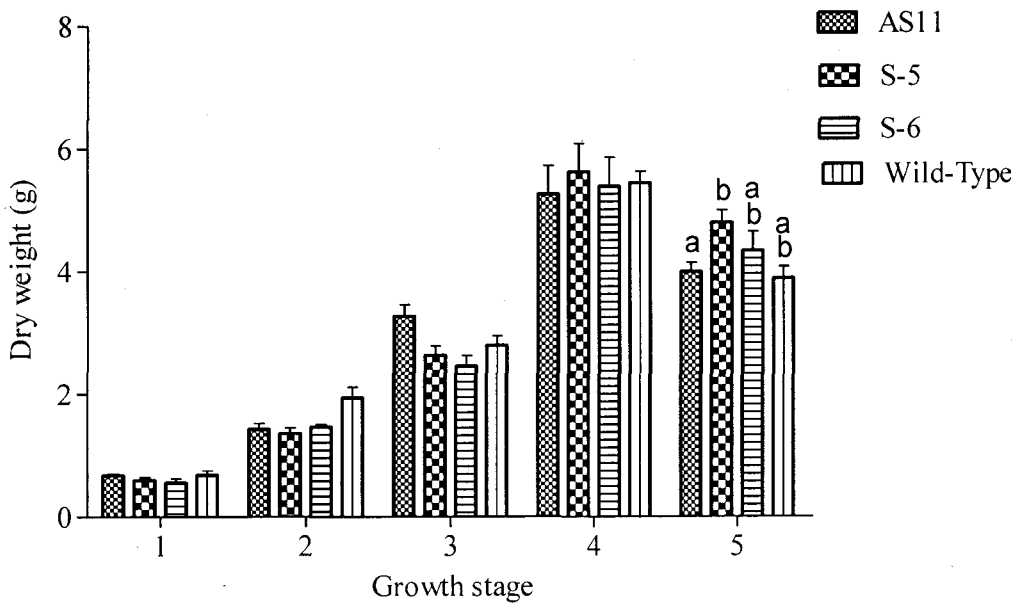

Figure 5: Total plant growth (root and shoot combined) of *Arabidopsis* genotypes at each growth stage. Significant differences ($p<0.05$) in total dry weight were observed in growth stage 5, between the AS11 and S-5 genotypes. Bars indicate standard error of the mean (SEM).

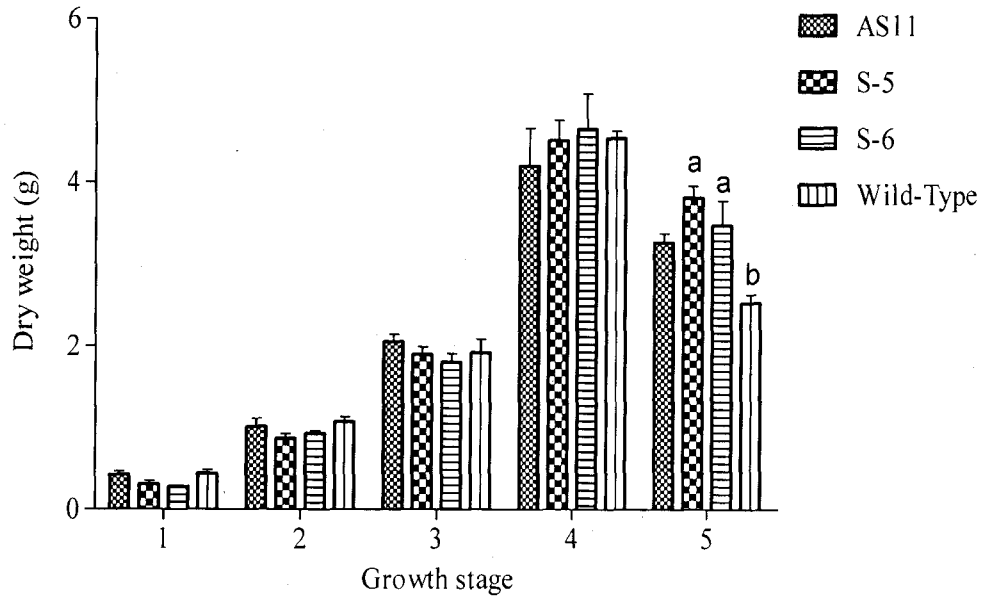

Figure 6: Shoot dry weight (g) of *Arabidopsis* genotypes at each growth stage. Shoot dry weight was found to be significantly different between the wild-type genotype and S-5 & S-6 genotypes at growth stage 5. Bars indicate standard error of the mean (SEM).

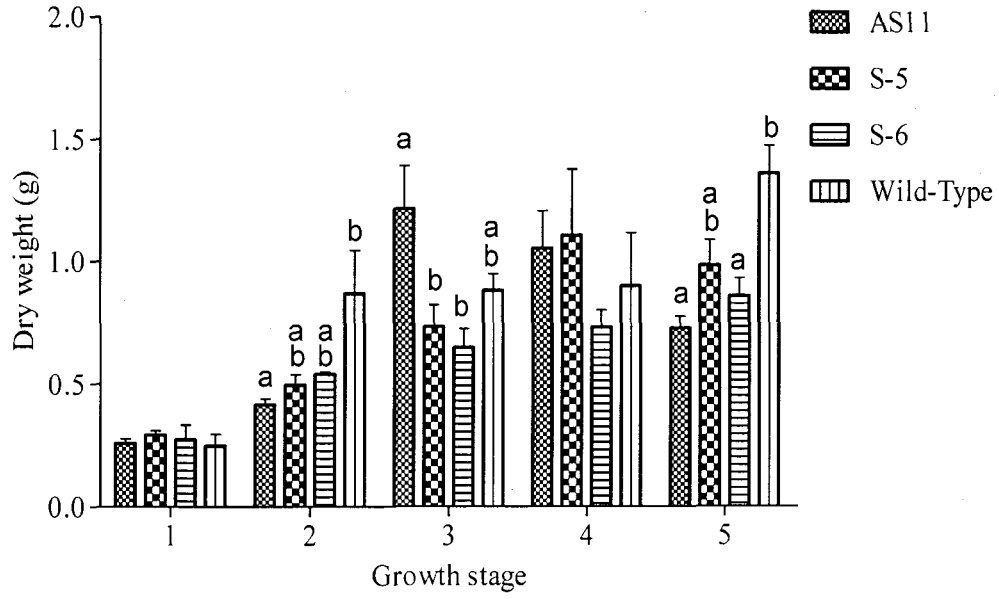

Figure 7: Root dry weight (g) of *Arabidopsis* genotypes at each growth stage. There were significant differences in root mass at growth stage 2 between AS11 and wild-type genotypes; growth stage 3 between AS11 and S-5 & S-6 genotypes; and growth stage 5 between AS11 and wild-type, and S-6 and wild-type. Bars indicate standard error of the mean (SEM).

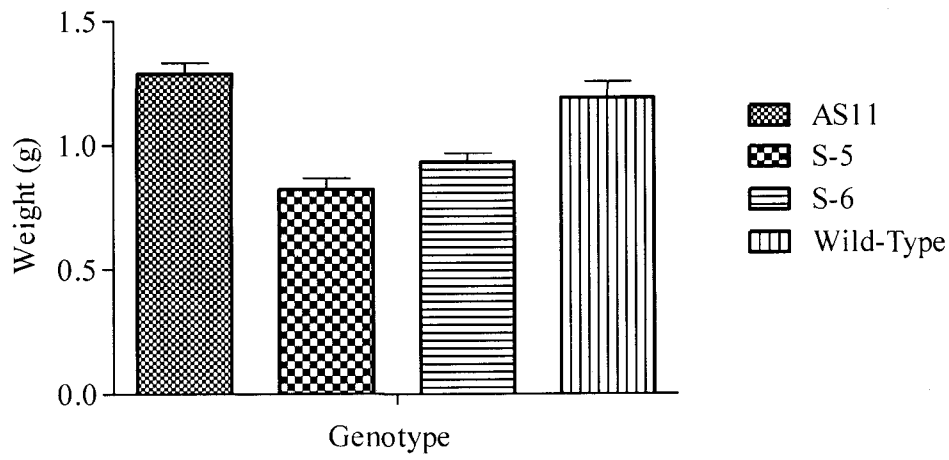

Figure 8: Total seed yield of *Arabidopsis* genotypes. There were no significant differences in yield based on genotype. Bars indicate standard error of the mean (SEM)

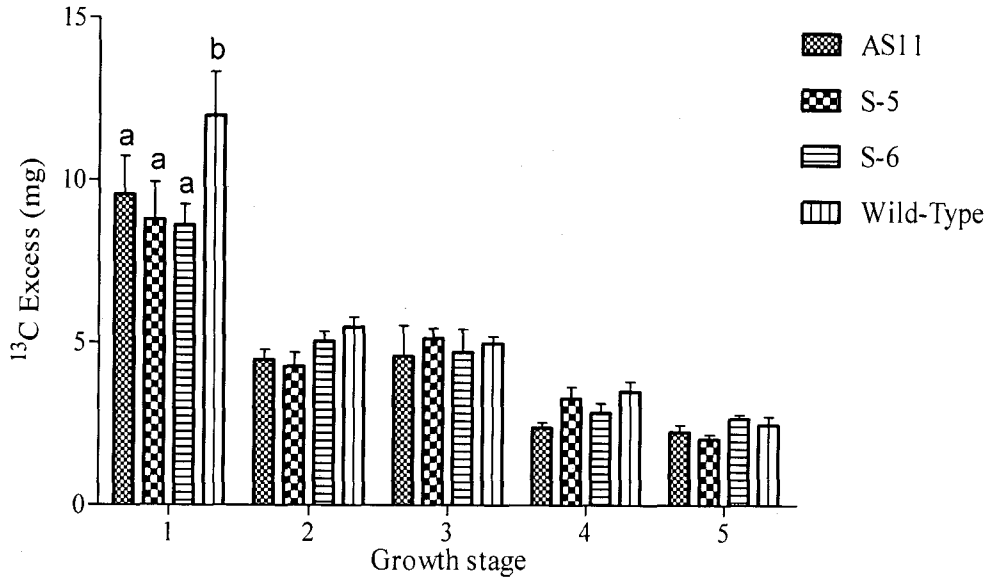

Figure 9: ^{13}C excess in shoots of *Arabidopsis* genotypes at each growth stage. In growth stage 1, significantly less ^{13}C was observed in AS11, S-5, S-6 genotypes, in comparison to the wild-type control. Bars indicate standard error of the mean (SEM)

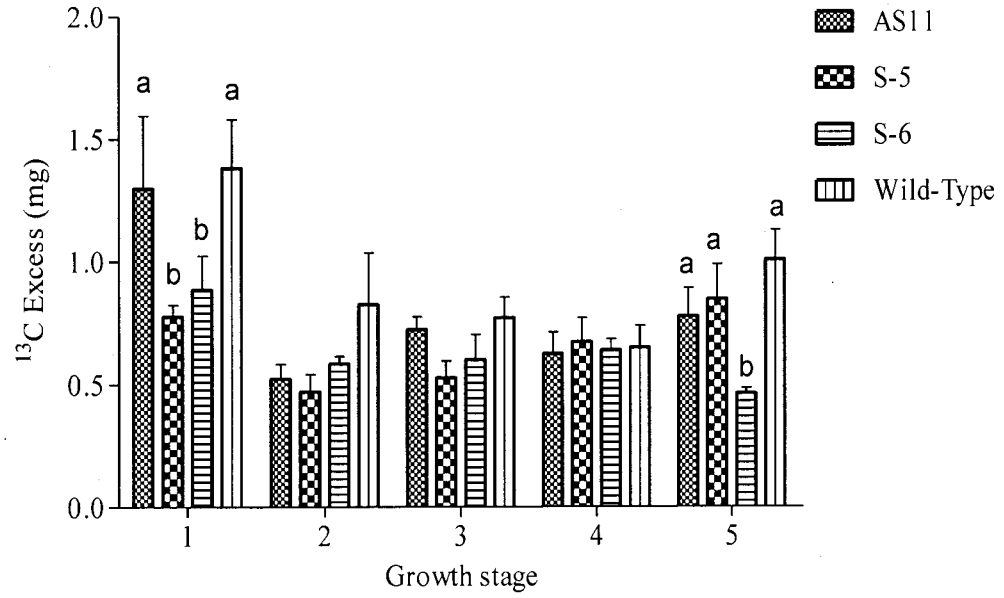

Figure 10: ^{13}C excess in roots of *Arabidopsis* genotypes at each growth stage. There were significant differences in ^{13}C content during growth stages 1 and 5. Bars indicate standard error of the mean (SEM)

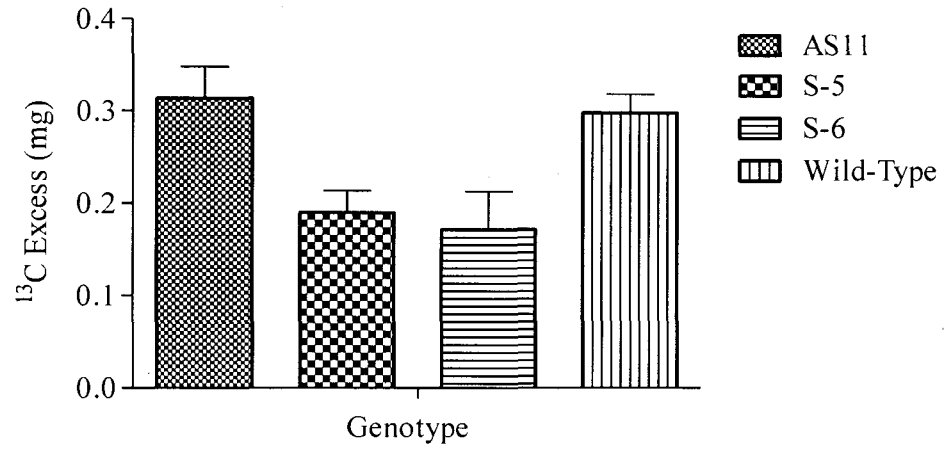

Figure 11: ^{13}C excess in seeds of *Arabidopsis* genotypes at final harvest. Genotype did not significantly affect ^{13}C content of seeds. Bars indicate standard error of the mean (SEM)

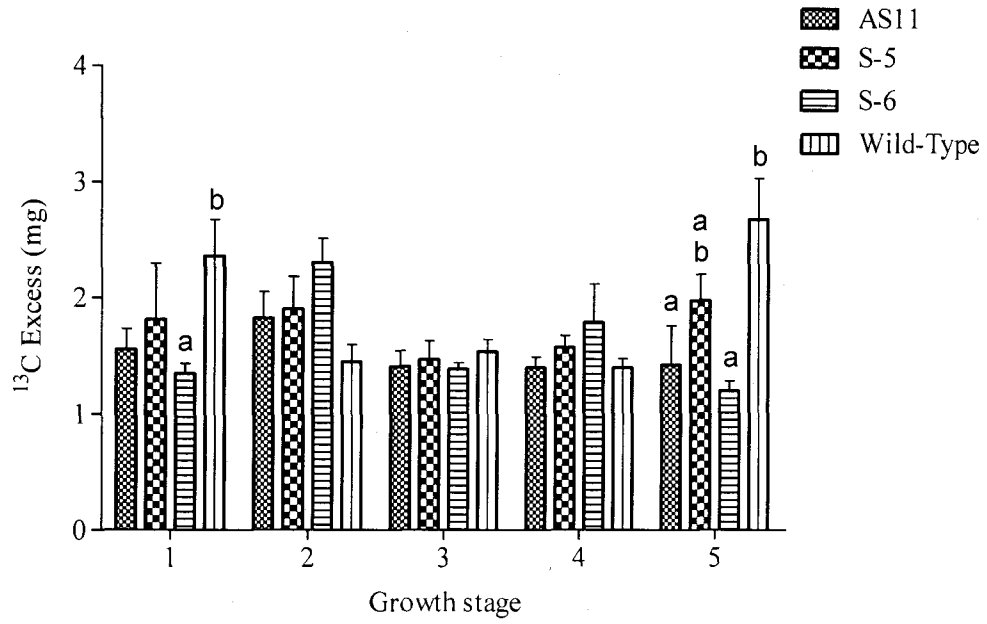

Figure 12: ^{13}C excess in soil of *Arabidopsis* genotypes at each growth stage. Significant ^{13}C differences were observed in growth stages 1 and 5. Wild type *Arabidopsis* displayed significantly higher ^{13}C than S-6 in growth stage 1, as well as AS11, and S-6 in growth stage 5. Bars indicate standard error of the mean (SEM).

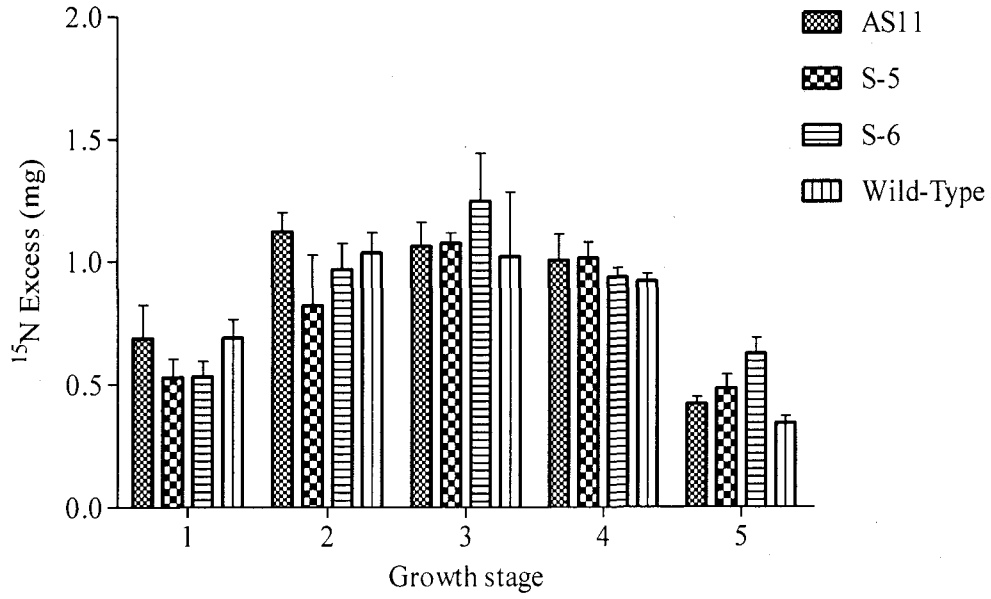

Figure 13: ^{15}N excess in shoots of *Arabidopsis* genotypes at each growth stage. ^{15}N content of the shoots was significantly different ($p<0.001$) based on growth stage, but was not significantly different based on genotype. Bars indicate standard error of the mean (SEM)

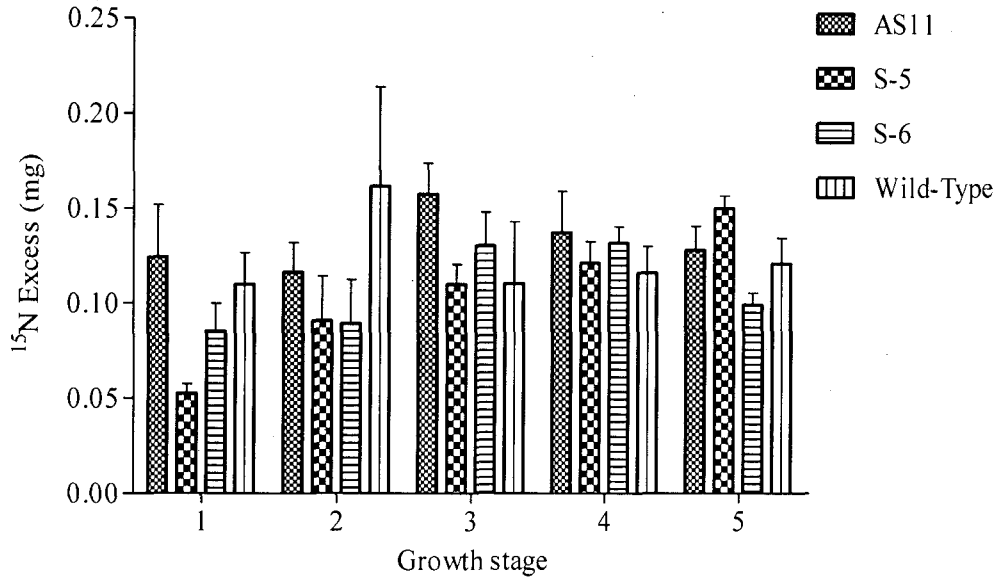

Figure 14: ^{15}N excess in roots of *Arabidopsis* genotypes at each growth stage. No significant differences in ^{15}N content in roots were observed. Bars indicate standard error of the mean (SEM)

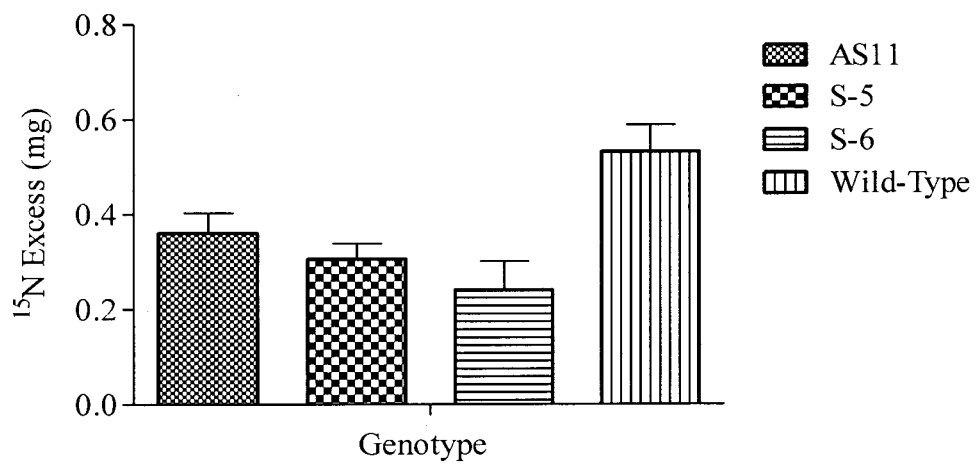

Figure 15: ^{15}N excess in seeds of *Arabidopsis* genotypes at final harvest. Genotype did not significantly affect ^{15}N content of seeds. Bars indicate standard error of the mean (SEM)

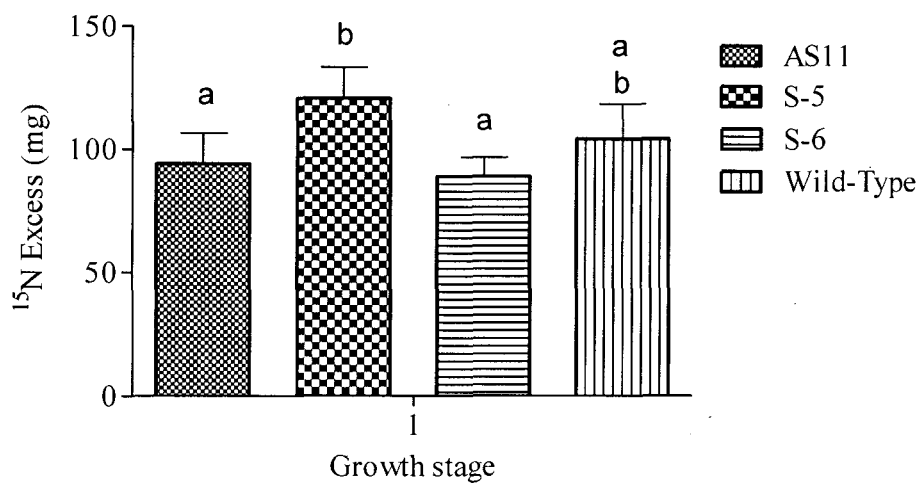

Figure 16: ^{15}N excess in soil of *Arabidopsis* genotypes at growth stage 1. N content was significantly different ($p<0.05$) based on genotype, with S-5 displaying the highest amount of ^{15}N, followed by wild-type, AS11 and S-6. Bars indicate standard error of the mean (SEM).

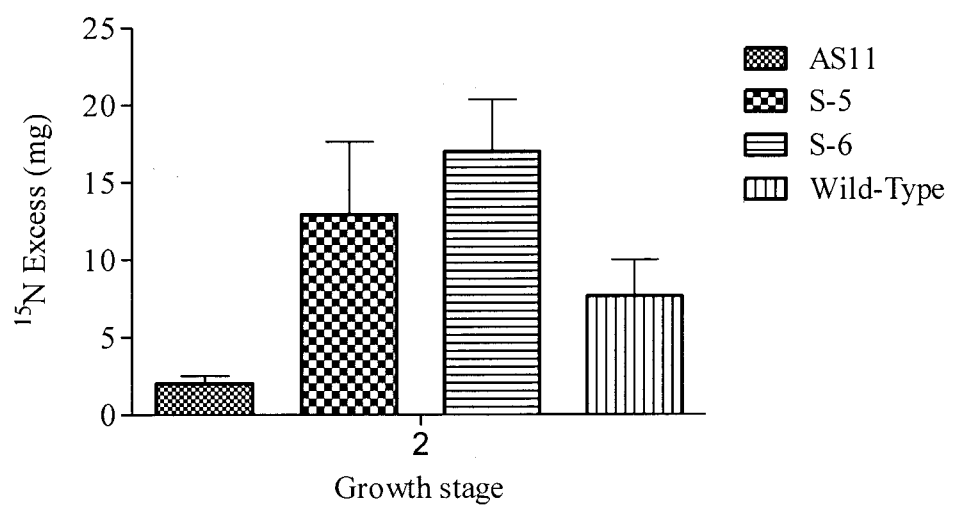

Figure 17: ^{15}N excess in soil of *Arabidopsis* at growth stage 2. There were no significant differences in ^{15}N content among the genotypes during growth stage 2. Bars indicate standard error of the mean (SEM)

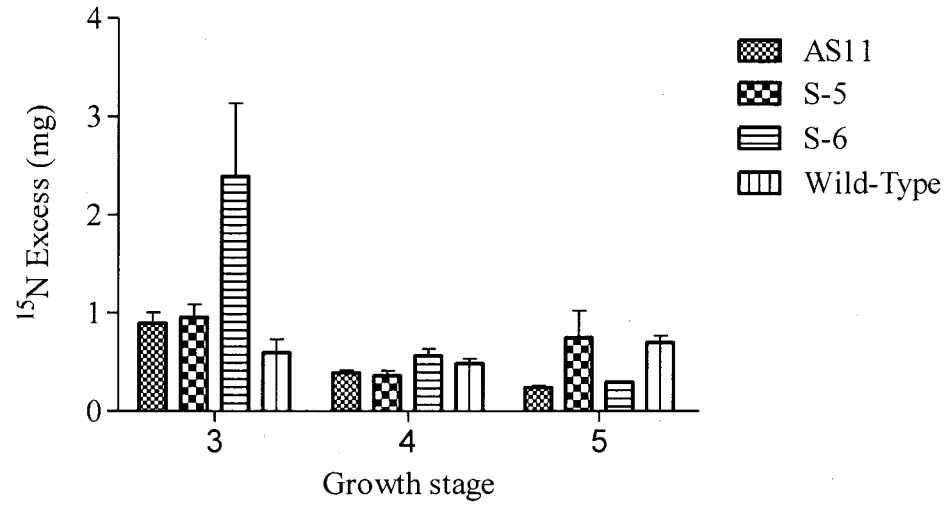

Figure 18: ^{15}N excess in soil of *Arabidopsis* genotypes at growth stages 3, 4 and 5. There were no significant differences in ^{15}N content among the genotypes during growth stages 3 – 5. Bars indicate standard error of the mean (SEM)

4.4 Results of T-RFLP Analyses

4.4.1 Normalization and Generation of VPT

Optimal divisors were obtained (see table 2) using the size and area data provided by the peak chart made by Gene Marker. The curves of the size and area data were optimized in Matlab to become horizontal lines, with R^2 linear power curves as close to zero as possible. Each restriction endonuclease was calculated separately, and generated unique percentage thresholds and graphs (see figures 19 - 22).

4.4.2 TRF Peak analysis

After normalization of peak charts based on the establishment of the variable percentage threshold, allele reports were corrected to contain the appropriate binary scores for each peak. Question marks were either replaced by a positive peak (1) or a negative peak (0), and some false positives were removed according to the threshold. A full report on the peaks generated by Gene marker software can be reviewed in section 7.3 of the appendix.

4.4.3 Genetic Similarity

Dendrograms were created using Matlab software illustrating the genetic relationships of each TRF profile (see figures 23 – 27).

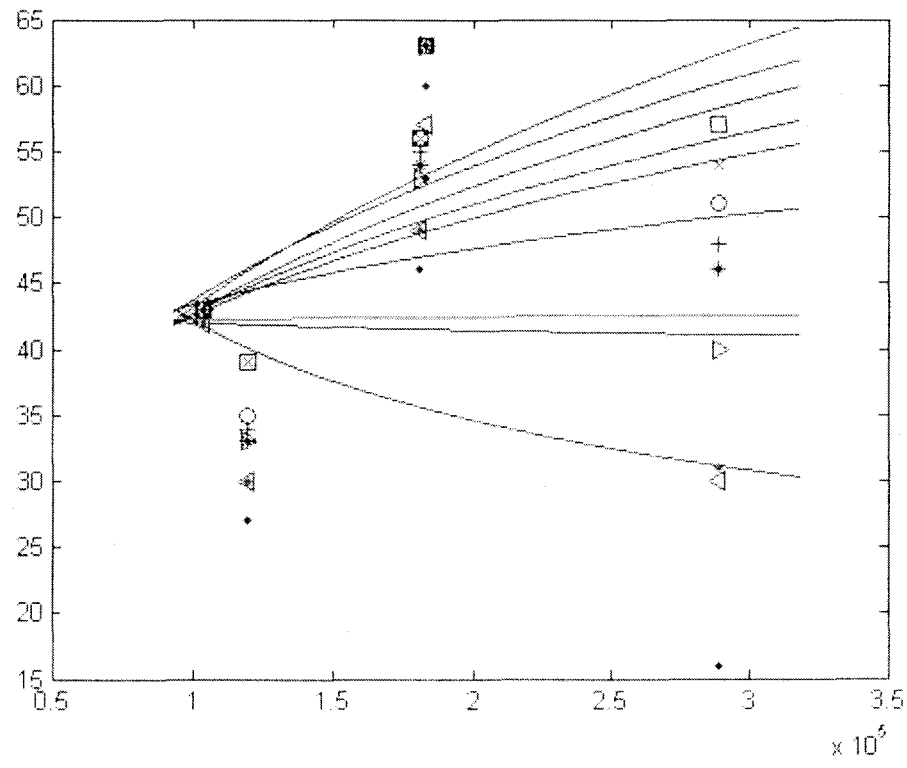

Figure 19: *BstuI* optimal divisor estimation. The curve of the R square is closest to zero when shown as a straight line. The X axis represents the total area of each *BstuI* T-RFLP profile, while the Y axis illustrates the number of peak remaining.

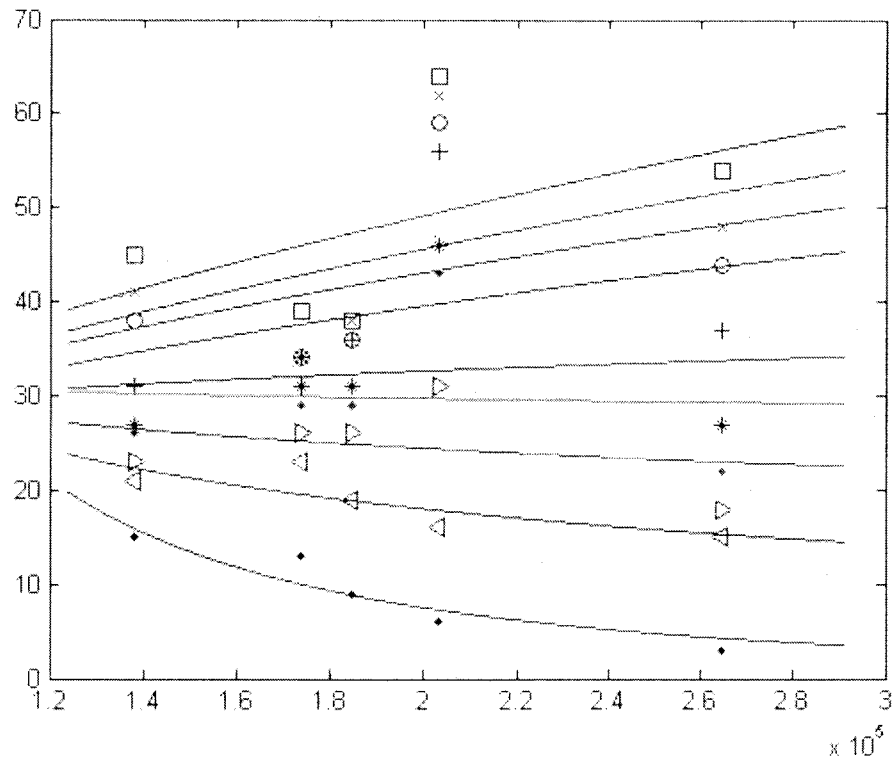

Figure 20: *HinfI* optimal divisor estimation. The curve of the R square is closest to zero when shown as a straight line. The X axis represents the total area of each *HinfI* T-RFLP profile, while the Y axis illustrates the number of peak remaining.

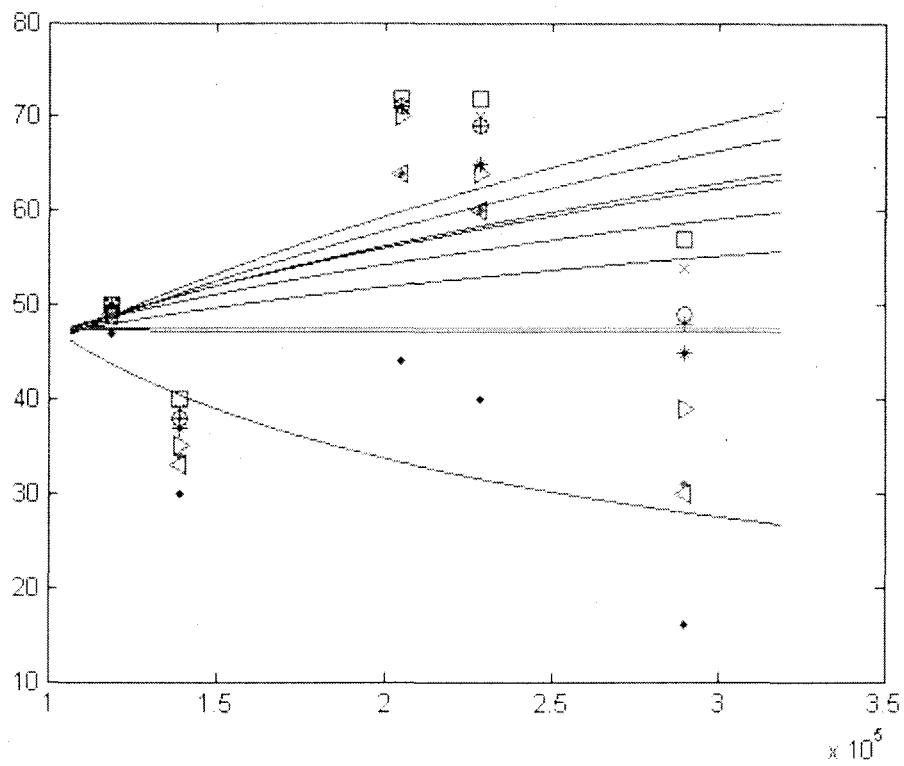

Figure 21: *HaeIII* optimal divisor estimation. The curve of the R square is closest to zero when shown as a straight line. The X axis represents the total area of each *HaeIII* T-RFLP profile, while the Y axis illustrates the number of peak remaining.

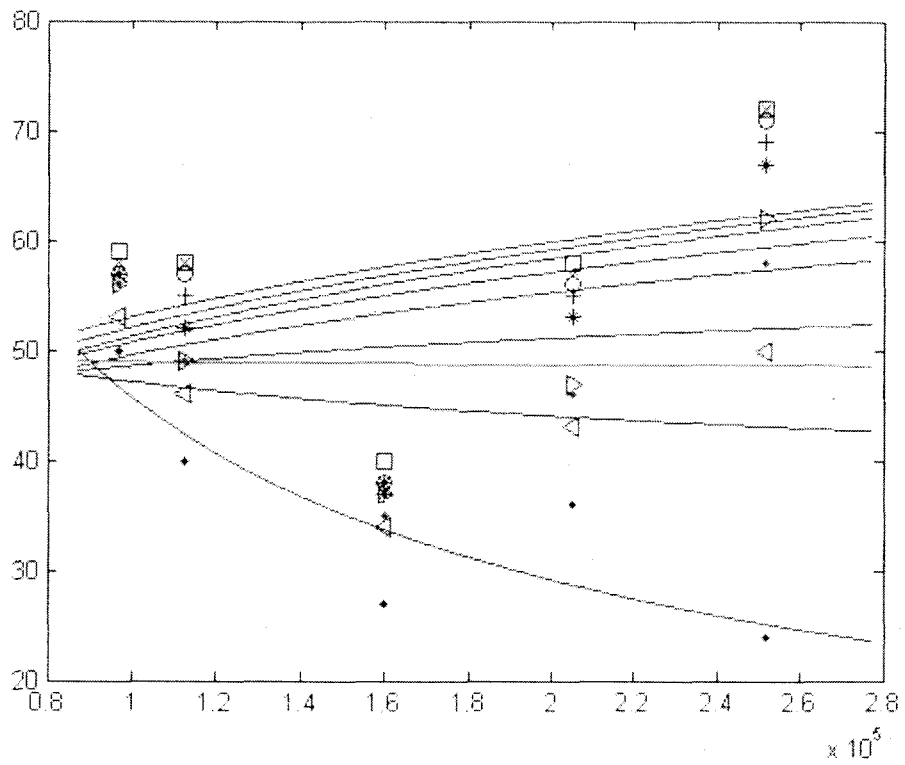

Figure 22: *MspI* optimal divisor estimation. The curve of the R square is closest to zero when shown as a straight line. The X axis represents the total area of each *MspI* T-RFLP profile, while the Y axis illustrates the number of peak remaining.

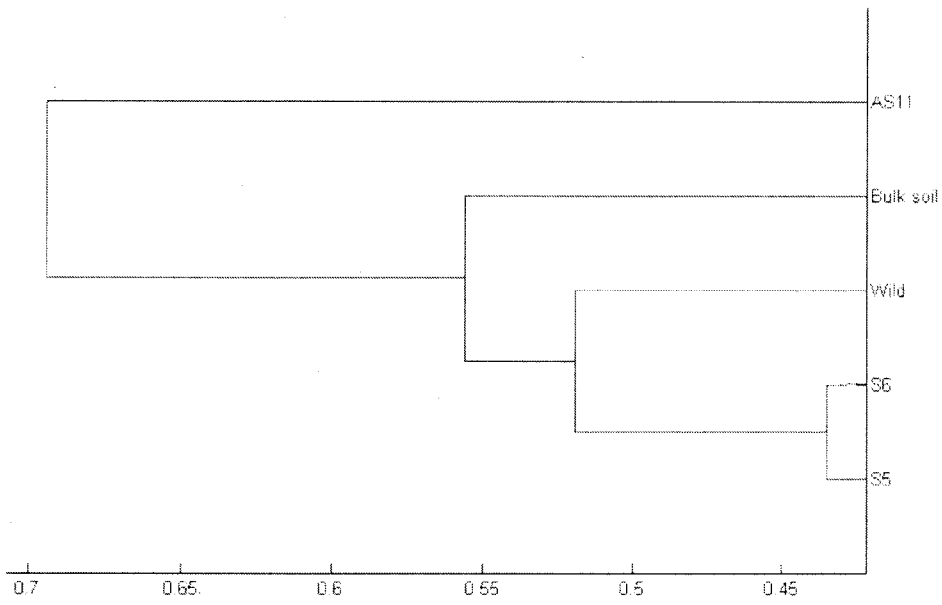

Figure 23: Dendrogram of *BstuI* data set. Copehentic correlation coefficient = 0.86.

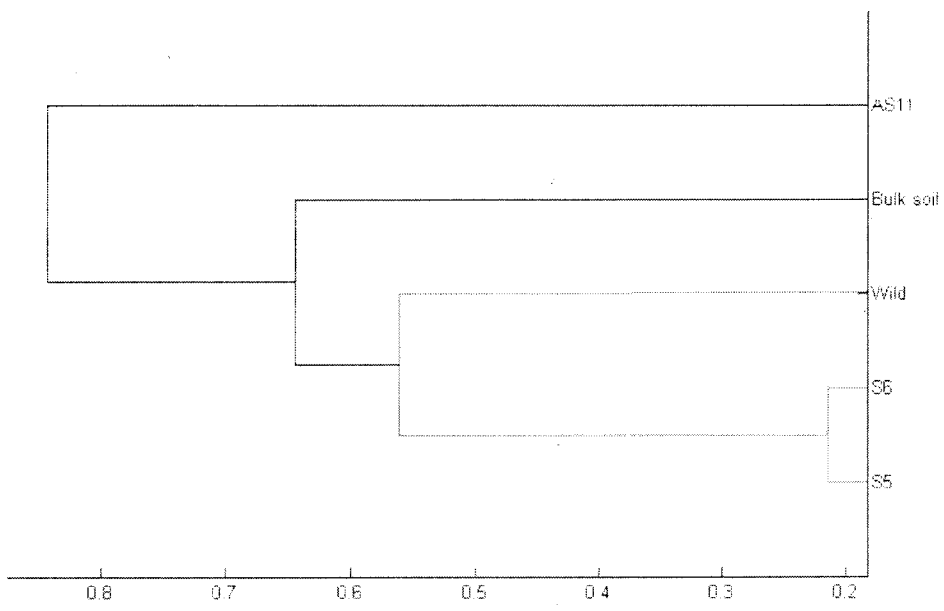

Figure 24: Dendrogram of *HinfI* data set. Copehentic correlation coefficient = 0.85.

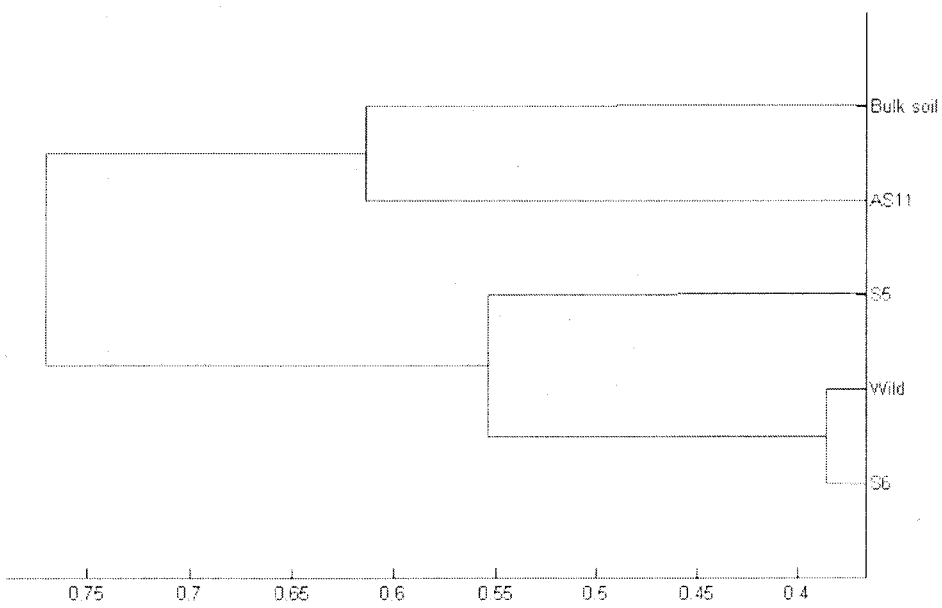

Figure 25: Dendrogram of *HaeIII* data set. Copehentic correlation coefficient = 0.86.

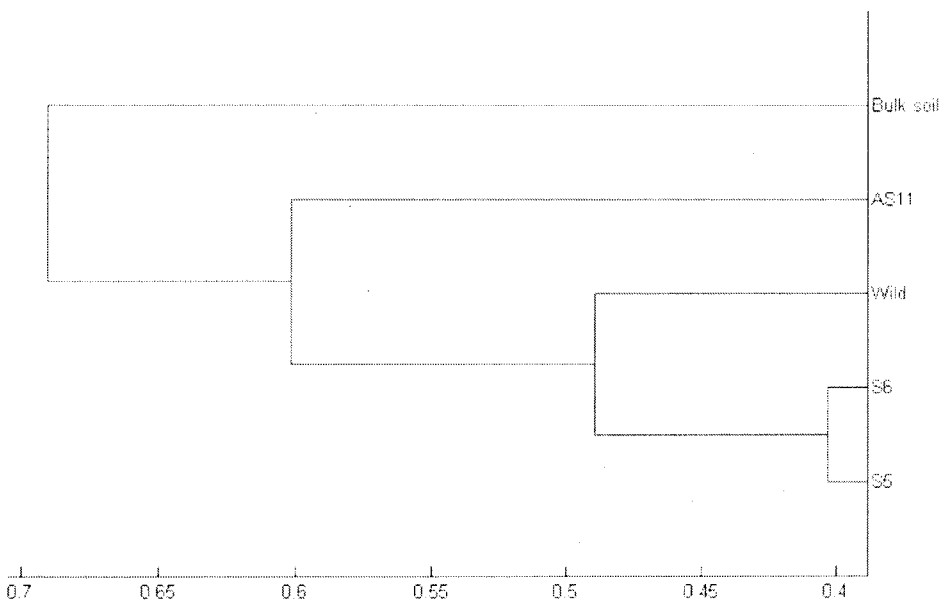

Figure 26: Dendrogram of *MspI* data set. Copehentic correlation coefficient = 0.85.

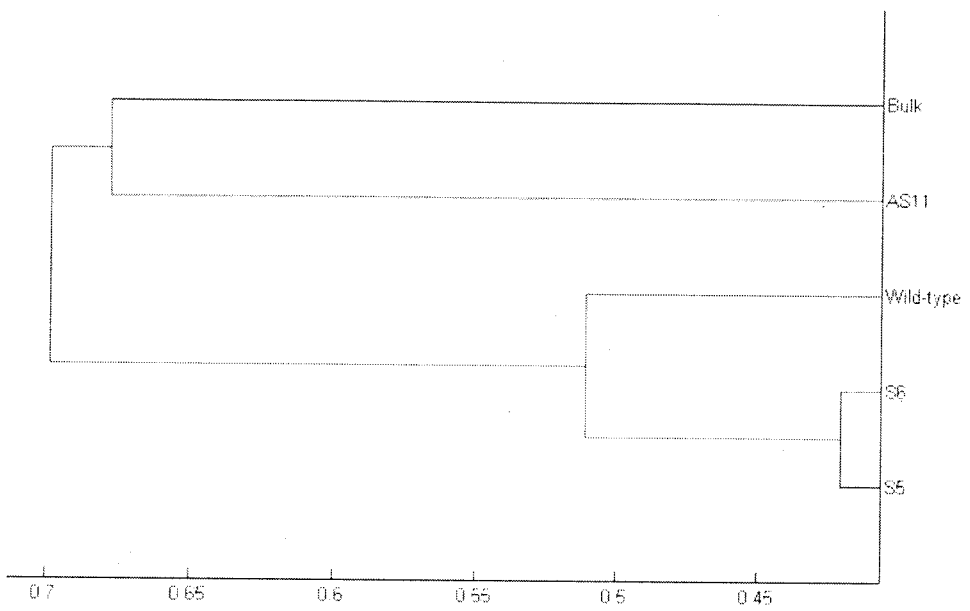

Figure 27: Dendrogram of combined RE data set. Copehentic correlation coefficient = 0.82.

5.0 DISCUSSION

5.1 Growth and development

The results show that genetic manipulation of "Gene-X" (S-5 / S-6) and *Tag1* genes (AS11) had a significant growth effect resulting in a delayed onset of senescence, which explains the differences in shoot mass at growth stage 5 (Figure 6, and section 4.1). The lack of silique shattering and delayed seed maturation at day 65 of genotype S-5 and S-6 was in sharp contrast to the wild type control, which at the same time, had fully matured. Altered developmental timing was observed among all experimental genotypes (AS11, S-5, and S-6) compared to the wild-type control during the first and final sampling periods, however, AS11 appeared to catch up quicker to the wild-type and seed maturation was only delayed by about a week; S-5 and S-6 genotypes did not reach full maturation by day 65. Similar findings were reported by Boyes et al. (2001), in which an *Arabidopsis* mutant designated as *fae1-1*, a seed-specific LCFA deficient genotype, showed delayed rosette leaf production and prolonged flowering period.

The relationship between genetic modification of oil synthesis genes and plant development is still not fully understood, however several papers (Lock et al. 2009; Lu and Hills, 2002; Routaboul et al. 1999; Katavic et al. 1995) report developmental abnormalities and sensitivities in *Arabidopsis* mutants with modified oil content. It was observed that manipulating DGAT activity causes delayed seedling growth and disrupted development in *Arabidopsis* mutant AS11 (Katavic et al. 1995), and our results confirm these observations. Other research (Lock et al. 2009) revealed developmental abnormalities in DGAT deficient *Brassica*. The plants produced fewer siliques, some of

which failed to fully develop or had phenotypic alterations such as thicker, hollow siliques and orange secretions on the stem during flowering (Lock et al. 2009). These results highlight a fundamental misunderstanding of the complexity of genes involved in oil synthesis, as evidence begins to mount regarding the role of DGAT and other enzymes in normal plant development. It is important to note that these effects are not present in all plants, the aforementioned morphological changes were not observed in tobacco lines with suppressed DGAT activity (Zhang et al. 2005). Lock et al. (2009) suggests that such reports might indicate that some plants may have a different preference for the last step in TAG biosynthesis, and that the effects of DGAT1 enzyme in TAG biosynthesis could be masked by the expression of different genes with overlapping functions.

In regards to total seed yield reported in this study, we did not find any significant differences in overall yield at the time of harvest. There were, however, notably lower values for seed yield among the S-5 and S-6 genotypes. This can be explained by the rate at which these genotypes produced seed, and matured, in comparison to the AS11 and wild type genotypes. It was observed that at growth stage 4 (late reproductive), the AS11 and wild type plants had already begun silique shattering, a process that occurs once the seeds within the siliques have fully matured. It was estimated that approximately 10% – 20% of the AS11 and wild type plants had begun this process, yet the S-5 and S-6 genotypes were still green and undeveloped. By final harvest (growth stage 5), the AS11 and wild type genotypes were fully mature and many of the siliques were rupturing or had already ruptured. At the same time, the S-5 and S-6 lines maintained many

undeveloped siliques; and did not appear to be at the same developmental stage as the AS11 and wild type genotypes.

5.2 Carbon partitioning

Carbon is a significant component in the production of triacylglycerol (TAG) in seed oil. This experiment was designed to observe how limiting seed oil synthesis could disrupt the natural flow of carbon throughout the plant, at many stages of growth and development. We had selected a broad range of developmental stages to encompass the majority of Arabidopsis' life cycle, with a focus on the reproductive and seed maturation stages of development. It was at these stages that we suspect large fluctuations in carbon partitioning would occur, especially during seed filling. Since seed filling requires a large quantity of fixed carbon from the plant, the genotypes used in this study that have been shown to be oil deficient (through various genetic modification) should exhibit decreased carbon partitioning – especially to the seeds.

^{13}C content was shown to be significantly different at specific growth stages, particularly growth stages 1 and 5 (late vegetative and fully mature). There are several factors which may be responsible for these differences in ^{13}C content amongst each *Arabidopsis* genotype. Throughout the experiment, several observations were made regarding the developmental timing of each genotype. Although no measurements were taken, it was apparent that a significant delay in rosette growth was occurring amongst the S-5 and S-6 genotypes. Much like the oil deficient *fae1-1* mutant reported by Boyes (Boyes et al. 2001), S-5 and S-6 genotypes reached stage 5.1 (budding) before

completing stage 1.10 (rosette growth). The resulting lack of vegetative growth and shoot biomass could explain the significant differences in ^{13}C content during growth stage 1. The larger vegetative biomass of the wild type genotype (see Figure 6), would likely have an increased capacity to absorb $^{13}CO_2$ from the labeling chamber, and possibly absorbed more ^{13}C, as was demonstrated by Butler et al. (Butler et al. 2004) during a ^{13}C pulse-chase labeling experiment on recently fixed photosynthate in ryegrass.

During growth stages 2-4, there were no significant differences in ^{13}C content of the roots, shoots or rhizosphere. This could be explained by a few different factors. Because it is during these stages that extensive growth and development of reproductive organs is taking place (Boyes et al. 2001), a dilution effect of ^{13}C labeling could be responsible. Over time, the initial dose of ^{13}C labeling is likely to be replaced by unfixed carbon fixed during respiration, and it is expected that a percentage may be lost during respiration as well (Butler et al. 2004). There may be significant partitioning of recently fixed unlabeled CO_2 that was not measured, possibly confounding the true flow of carbon to the shoots during these developmental stages. This was not, however, observed in the roots or soil samples, as they maintained a relatively stable level of ^{13}C throughout the experiment. It is during the first and final sampling periods that significant ^{13}C mobilization was observed; with the S-6 and wild genotypes showing increased disparity (Figures 10 and 12). Although each genotype demonstrated initial peaks and subsequent stabilization over time (caused by the initial pulse of ^{13}C labeling), the S-6 genotype was quite variable in its allocation of ^{13}C to the roots and soil, ending up with a significant drop in ^{13}C at final harvest (Figures 10 and 12). It is unclear why this was not also

observed in the sibling line, S-5, however it may simply be due to increased plant or microbial respiration of $^{13}CO_2$ causing a dilution effect over time.

^{13}C flow to the seeds was expected to be significantly different depending on *Arabidopsis* genotype. It was hypothesized that the experimental genotypes (AS11, S-5 and S-6) selected for oil deficiency would contain significantly less ^{13}C than the wild type control. This, however, was not shown to be true. S-5 and S-6 genotypes did have less ^{13}C than the wild type, but AS11 had the same amount of ^{13}C as the wild type (Figure 11). It is likely that at this stage of development (day 60), much of the ^{13}C labeling had either been partitioned to the soil via rhizodeposition, or more likely, respired. Therefore, there wasn't sufficient ^{13}C labeling remaining in the plant to accurately represent the total flow of carbon to the seeds. The raw data (not presented in this thesis) also shows significant differences in elemental C (unlabeled carbon) between the wild type and knock-down mutants. For example, there is nearly a 10% reduction in total elemental C present in the seeds of the S-5 genotype vs. the wild type; with numbers ranging from 64% (wild type) to 55% (S-5 genotypes). These numbers suggest that there are in fact significant oil deficiencies in the experimental genotypes, however, the lack of remaining ^{13}C labeling by the end of the experiment do not accurately demonstrate this.

5.3 Nitrogen Partitioning

Nitrogen partitioning remained relatively unchanged as a result of genetic modification of oil synthesis genes. There were no significant changes in ^{15}N content of the roots and shoots based on genotype. Growth stage showed mobility of ^{15}N to the

shoots during flower production (growth stages 2 – 4) which represented itself as a typical bell curve (see Figure 13), meanwhile the ^{15}N content of the roots maintained a near constant level (see Figure 14). There were some significant differences in ^{15}N among genotypes at growth stage 1, with S-5 having significantly higher ^{15}N content than AS11 and S-6 (Figure 16). This can be explained by looking at the ^{15}N content in the shoots and roots of each genotype during this stage. It appears that ^{15}N was actively partitioned to the roots (Figure 14) and shoots (Figure 13) by the AS11 and wild type genotypes, causing a significant reduction in soil ^{15}N. It was also observed that each genotype had slightly different partitioning patterns, illustrated by peaks and dips of ^{15}N content at various developmental stages.

Because nitrogen is not a major component of seed oil, we had been correct in assuming that there would be no significant change in N partitioning resulting from the genetic manipulation of genes involved in this process. It was interesting to observe some changes in N partitioning between genotypes at specific growth stages, illustrating subtle changes in growth patterns resulting from gene modification; however, it did not appear that N partitioning was significantly affected, overall.

5.4 Effects on soil microflora

The rhizosphere is a dynamic environment surrounding the roots of plants, where biologically and chemically diverse interactions take place between the plant roots, and soil biota (Hartmann et al. 2009). It is known that organic carbon is considered the limiting factor for microbial density and activity (Bowen and Rovira, 1999; Lugtenberg

and Dekkers, 1999), therefore, plant genotypes which mobilize a greater proportion of their organic C to the roots and surrounding rhizosphere may trigger the proliferation of microbial communities in response to the increased carbon influx.

The amount of bacterial DNA isolated from the 1g rhizosphere samples ranged from 52 – 58.9 ng/µl (see table 3). As expected, the bulk soil did not contain as much bacterial DNA as the rhizosphere samples, and contained approximately 26 ng / µl. After the application of several restriction endonucleases (RE) (*HinfI*, *HaeIII*, *BstuI*, *MspI*), an interesting correlation was observed. The genetic composition of bacterial DNA of the rhizosphere was most similar between S-5 and S-6 genotypes, in all RE profiles except *HaeIII*. Because these two genotypes are more closely related than the other genotypes tested, it was surprising to find that the bacterial communities were also so closely related, with distinct peaks at around 500bp not found in other TRF profiles. The wild type genotype was shown to share some genetic similarities to the S-6 line when cut with *HaeIII*, however, overall it appears to be distinctly separated from the S-5 / S-6 lines as well as the AS11 genotype. The AS11 genotype had little relationship to any other genotype, and was found to either branch off independently from the group (see *Hinfi* and *BstuI* data sets, figures 23 / 24), or share a distant connection to bulk soil (figure 27).

Based on Nanodrop results (see Table 3), the total extracted bacterial DNA was not significantly different based on genotype. Bulk soil showed the least amount of extracted DNA, however, this was expected as bacteria abundance is significantly higher within the rhizosphere (Bais et al. 2006; Jones et al. 2004). This could explain why bulk soil was the least related among the 5 soil samples tested. Based upon this ^{13}C data, the

wild type genotype had shown significantly higher ^{13}C excess in the soil at various stages of development (see Figure 12), in comparison to AS11 and S-6. Dendrograms also showed the wild type as being distinctly different from the other groups, perhaps as a result of increased C content found in the soil. Since the molecular analysis of rhizosphere soil was conducted at the final sampling period (day 65), and carbon / nitrogen content in the soil is in a constant state of flux, the results of this experiment only show the similarities at the time of harvest. It could be that as plants age, and conditions change, so to do the bacterial communities that colonize the rhizosphere.

By the second - third growth stage, we began noticing some stress related purpling of the leaves, and discovered these plants were infected with insects believed to be thrips (*Thysanoptera thripidae*). This was most apparent in the AS11 and wild-type genotypes; however, S-5 and S-6 genotypes were also affected. The infestation did not progress beyond these stages, due to the lack of a food source caused by the senescing leaves. It is known that plants can release root exudates that induce a defense response in neighboring plants during an herbivore attack (Bais et al., 2006), therefore it is conceivable that during this time an influx of these plant-plant signaling molecules were present in the soil. Because plant roots initiate cross talk with soil microbes by producing signals that are recognized by the microbes, which in turn produce signals that initiate colonization (Bais et al., 2006); distress signals sent by plants may also affect colonized bacteria. It is unclear how soil microbes react to plant-plant signals, as more research in this area is needed.

It is well known that bacteria and plants can form both positive relationships, in the case of plant growth promotion rhizobacteria (PGPR); and negative relationships, observed during continuous attacks from pathogenic microbial infection. Plants, such as *Arabidopsis*, rice, corn, soybean, and the model legume *Medicago truncatula*, have been studied extensively for their antimicrobial defense mechanisms which include antimicrobial indole, terpenoid, benzoxazinone, and flavonoid/isoflavonoid natural products (Bais et al. 2006). Because the soil used for this experiment was taken from a farm and was not sterilized, it is possible that pathogenic bacteria were present in the soil causing the release of antimicrobial root exudates in the case of an infection. The effects of genetic modification of oil synthesizing genes on the production of these secondary root exudates has not been studied, and the role of these genes on normal plant development is still not fully understood. It has been suggested that DGAT, an enzyme once believed to be involved solely on TAG synthesis, actually plays a significant role in plant development, and excessive accumulation of DAG and/or its precursors in developing seeds deficient in DGAT activity could lead to disturbances in signaling pathways (Lock et al., 2009). The resulting differences in microbial populations may be contributed to the impaired ability to produce antimicrobials, which could be an unknown byproduct of genetic manipulation of "Gene-X".

Another factor that may explain the differences in bacterial communities is plant age. According to Baudoin et al (2003), research conducted on maize showed bacterial community structure differed quantitatively (densities) and qualitatively (metabolic potentialities, genetic structure), with plant developmental stage. This research suggested

that exudate diversity and availability changes in response to plant age (Baudoin et al. 2003). Also in this study, variations in C/N ratios did not affect bacterial proliferation, and increased C content influenced bacterial densities and genetic structure significantly (Baudoin et al. 2003). Considering the evidence provided above, it is likely that plant age may have played a significant role in altering the genetic profile of the rhizosphere. It is unknown whether modification of the "Gene-X" has secondary effects on root exudates, however, it was obvious that normal growth rates were delayed resulting in a prolonged flowering period / incomplete seed maturation at the time of harvest. Since the soil collected for T-RFLP analysis was taken at the end of the experiment, only bacteria that had either established a competitive advantage throughout the experiment, or those of which were present at full maturity vs. early – mid seed maturation stages, were observed.

5.5 Limitations and Directions for Future Studies

The goals of these experiments were to monitor changes in C and N partitioning resulting from genetic modification of oil synthesis genes resulting in reduced TAG content, and to characterize microbial populations of the rhizosphere. The scope of this microbial characterization is quite broad, and is limited to the genetic similarity / dissimilarity of each sample. This, although an important first step in observing changes in microbial populations due to manipulated oil synthesis genes, paints an incomplete picture. It is of great interest to continue onto the next steps and sequence the peaks unique and conserved to the S-5 and S-6 genotypes, to better understand what types of

bacteria are taking advantage of the apparently altered growth conditions offered by these mutants. An analysis of the rhizosphere soil could also yield results pertaining to why specific bacteria were found in some soil samples, but not others. It is also of interest to observe changes in bacterial community structures at each growth stage, rather than at the end of the experiment. Research by Baudoin et al. (2003) show plant age significantly affects bacterial density and genetic diversity due to fluctuations in root exudation. By sampling at each growth stage, we might observe significantly different bacterial communities depending on the rate of exudation at that specific growth stage.

The S-5 and S-6 mutants used in this study have genetic manipulation in unknown regions of the genome. At the start of this experiment, it was believed that the LACS4 T-DNA insertions were causing the speckled coat and reduced seed oil phenotypes. However, recent evidence suggests that an unknown gene(s), "Gene-X", may be responsible (Katavic, personal communication). Because of the mysterious nature of this discovery, it is difficult to pin-point the exact mechanisms behind the developmental abnormalities and altered rhizosphere ecology resulting in significantly different bacterial populations. It is a logical assumption that the gene(s) affected are important in seed oil synthesis, but beyond that there is only speculation. Continued research on the exact location of these genes is imperative.

Because this experiment used knock-down mutants, results only indicate changes resulting from decreased TAG synthesis. It would be ideal to test for C and N partitioning changes resulting from over-expressing these genes in *Arabidopsis* mutants as well. Considering the effects on growth and development observed in S-5 / S-6, it would be

interesting to observe whether such changes are present after over-expressing this gene. Similarly, it is unknown how other plant species may react to genetic manipulation of "Gene-X", therefore continued research using other species such as Brassica would be an important step in identifying the actions of this gene on other species.

There were also some difficulties in sampling the extensive root systems of *Arabidopsis*, especially in a soil medium where they must be gently teased away from soil particles they are clinging to. It was quite difficult to isolate the entire root system from the soil; therefore a small amount of variability resulting from sampling error could not be avoided. Although the majority of the tap root system was successfully collected, a small percentage of the fibrous roots may have broken up in the process, unable to be retrieved. We had conducted several experiments prior to this one, testing different sand / soil compositions in order to minimize root loss through sampling, and it was found that the 50% sand 50% soil mix provided the best growth, and root retrieval in comparison to using a potting soil mix (unable to retrieve roots, although excellent growth), or pure sand medium (poor growth, excellent root retrieval).

Finally, because this experiment could not be duplicated due to time constraints, our study is limited to one growth season. A second and perhaps third trial would establish more robust and compelling evidence regarding the C/N partitioning, developmental abnormalities, and bacterial communities affected by the modification of oil synthesis genes.

6.0 CONCLUSION

Biofuels may play a significant role in the conversion from fossil to renewable energy, as well as help mitigate CO_2 emissions; however continued research on improving biofuel feedstocks is imperative. Our current research was aimed at exploring the unknown physiological effects of genetic modification of oil synthesis genes on carbon and nitrogen partitioning, as well as the interactions between these genotypes and the surrounding bacterial microflora. The implications of this study offer novel insights of the effects of gene modification on the whole plant, and highlight some important side effects.

The results of the carbon / nitrogen partitioning experiment indicate that genetic modification of these specific oil synthesis genes resulted in reduced TAG content and significant changes in developmental timing, but did not significantly affect the normal flow of carbon or nitrogen. Significant delays in seedling growth and seed maturation were observed in S-5 and S-6 genotypes, and AS11 showed slight delays (~1 week) in seed maturation as well.

In regards to the T-RFLP analysis, there are several things to consider when interpreting TRF peaks. It has been suggested that that a single gram of soil may contain over 4000 bacterial species (Kirk *et al.*, 2004) and most of these have not yet been identified (Singh *et al.*, 2006). In order to help illustrate the diversity of bacteria in the soil, 4 RE's were used to help eliminate the possibility of several species comprising the same length in profiles of each soil sample, a problem commonly observed when using fewer than 4 RE's (Kitts et al., 2003). Although this method helps in distinguishing

between groups of bacteria, it is still likely that more than one group of bacteria comprise a single peak (Kitts et al., 2003). However, it is impossible to know what groups of bacteria are present without further studies such as sequencing the 16s rRNA.

There is an interesting correlation between genotype and bacterial composition in the rhizosphere. It was shown that up to 80% of the microbial DNA found in the S-5 rhizosphere was also found in the S-6 rhizosphere (See Figure 24). AS11 on the other hand, merely shared 20-30% of the DNA found in the S-5 / S-6 rhizosphere, and the wild type shared ~50%. It is unclear what the exact reasons for this relationship are. It has been shown that carbon availability in the rhizosphere is a limiting factor for bacterial colonization and proliferation (Bais et al. 2006; Jones et al. 2004; Baudoin et al. 2003), however, ^{13}C content in the soil and roots were significantly different between S-5 and S-6, suggesting other factors were involved.

Seed oil synthesis is a complex and relatively misunderstood phenomenon. Once believed to be a linear process, we are only recently discovering its truly dynamic nature involving a variety of genes with multiple functionalities. To our knowledge, this is the first study to explore the physiological effects of "Gene-X" modification. Because of the significant reduction in seed oil content (~10%), we speculate that the gene(s) involved are highly important in seed oil synthesis, and also play a role in normal plant development. It could be a possible candidate gene for increasing oil yield via over-expression; however, the prolonged seed maturation observed in the knocked-down mutants would not be a desirable trait for commercial oilseed crops.

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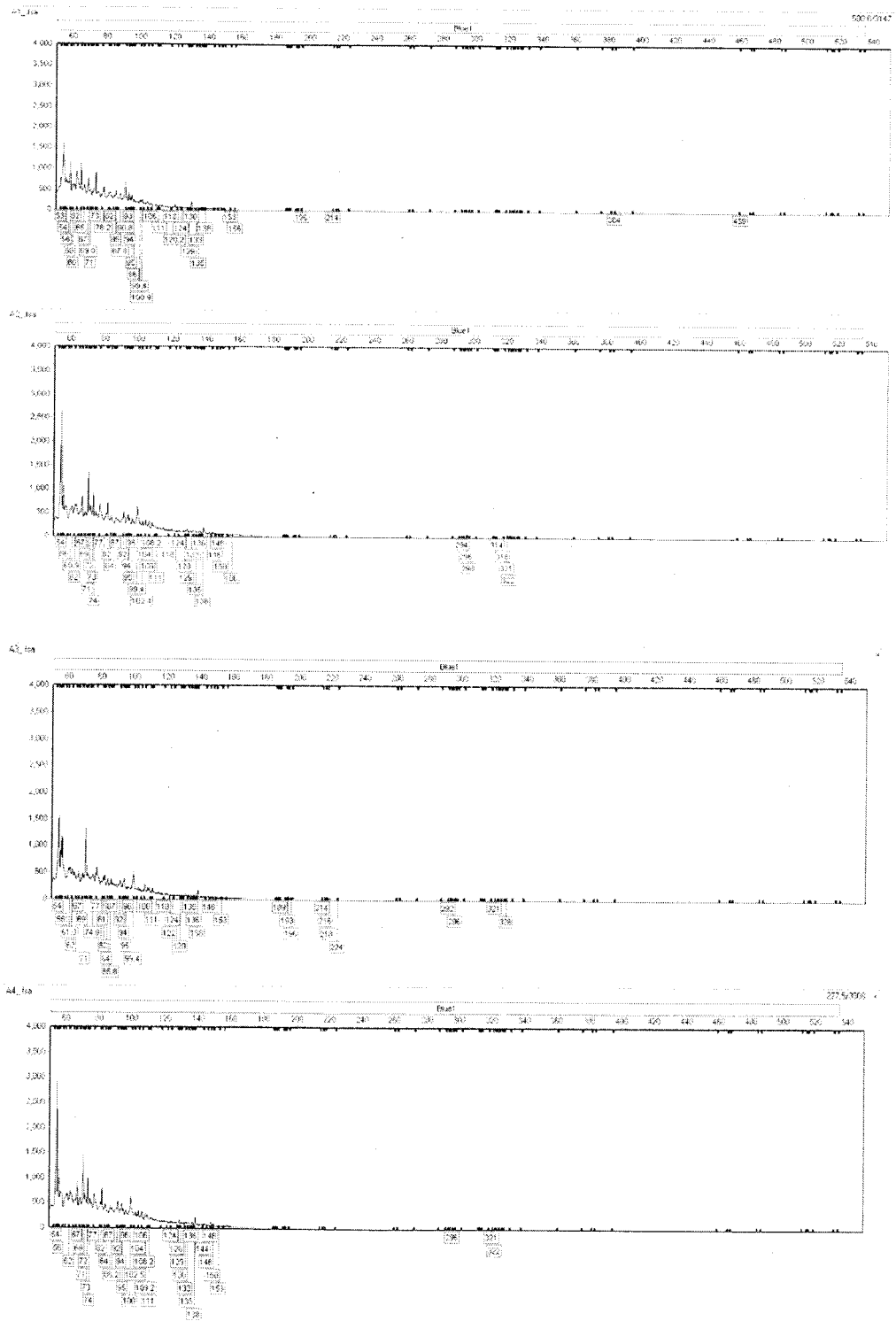
7.0 APPENDIX

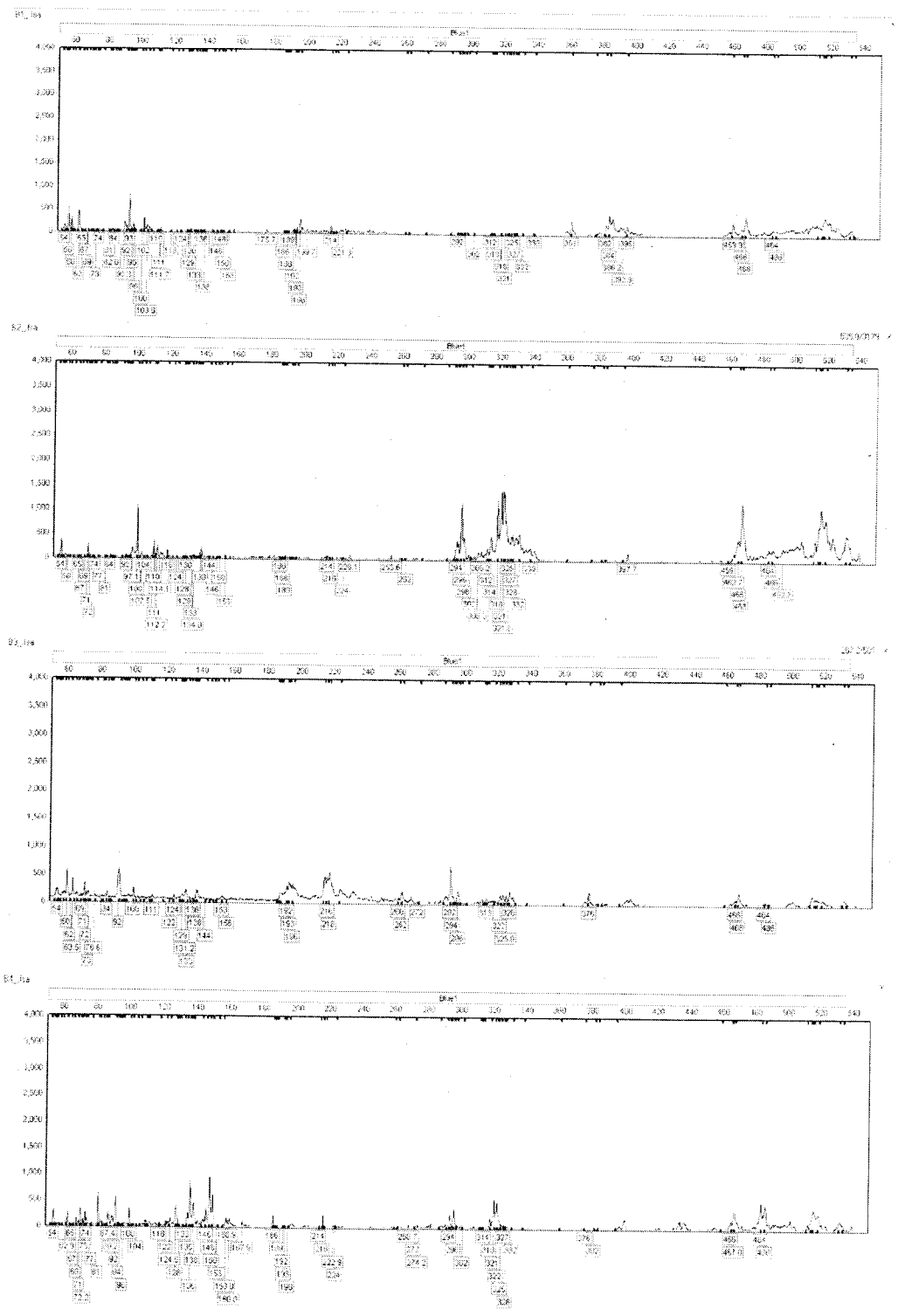
7.1: Analytical report of soil used for experiment

Analyte		Units	Results
Organic Matter		% weight	2.4
Available Nutrients			
Nitrate – N	Farmsoil	ppm	14
Phosphorus	Farmsoil	ppm	>60
Potassium	Farmsoil	ppm	106
Sulfate – S	Farmsoil	ppm	3
Copper	FS micro-nutrients	ppm	24.7
Iron	FS micro-nutrients	ppm	55.4
Manganese	FS micro-nutrients	ppm	5.86
Zinc	FS micro-nutrients	ppm	5.50
Base Saturation	FS Base-Saturation	%	91.2
Calcium	FS Base-Saturation	%	66.0
Magnesium	FS Base-Saturation	%	21.4
Sodium	FS Base-Saturation	%	0.8
Calcium	FS macro-nutrients	ppm	1200
Magnesium	FS macro-nutrients	ppm	236
Sodium	FS macro-nutrients	ppm	16
Boron	FS macro-nutrients	ppm	0.4
Physical and Aggregate Properties			
Silt	Soil Texture	%	23.4
Clay	Soil Texture	%	8.6
Sand	Soil Texture	%	68.0
Texture			Sandy Loam
Soil Acidity			
pH	1:2 Soil:Water	pH	6.9

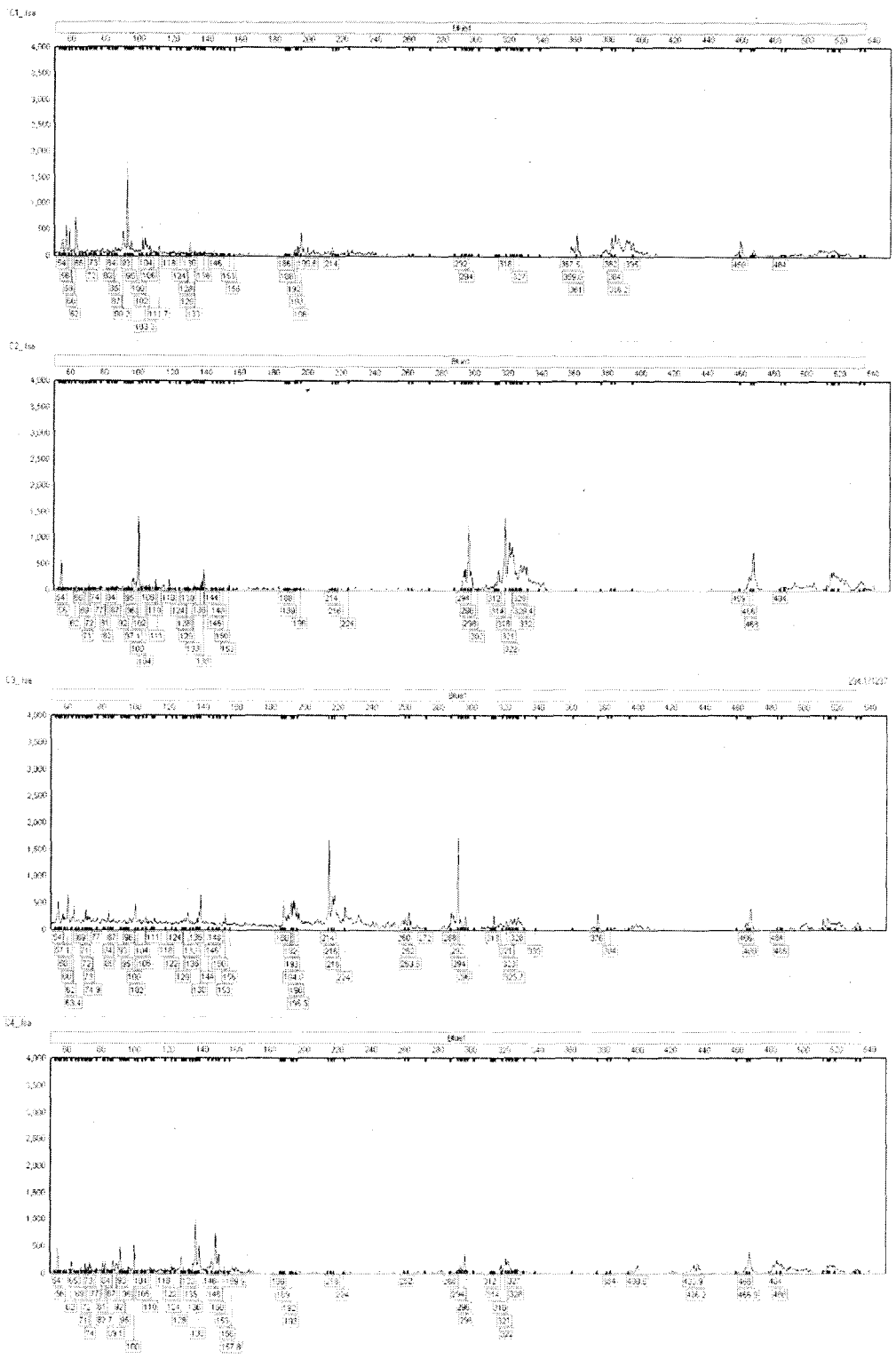
7.2.1 Electropherograms of RE derived TRF profiles from AS11 soil sample. A1 digested by *BstUI*, A2 digested by *HinfI*, A3 digested by *HaeIII*, and A4 digested by *MspI*.



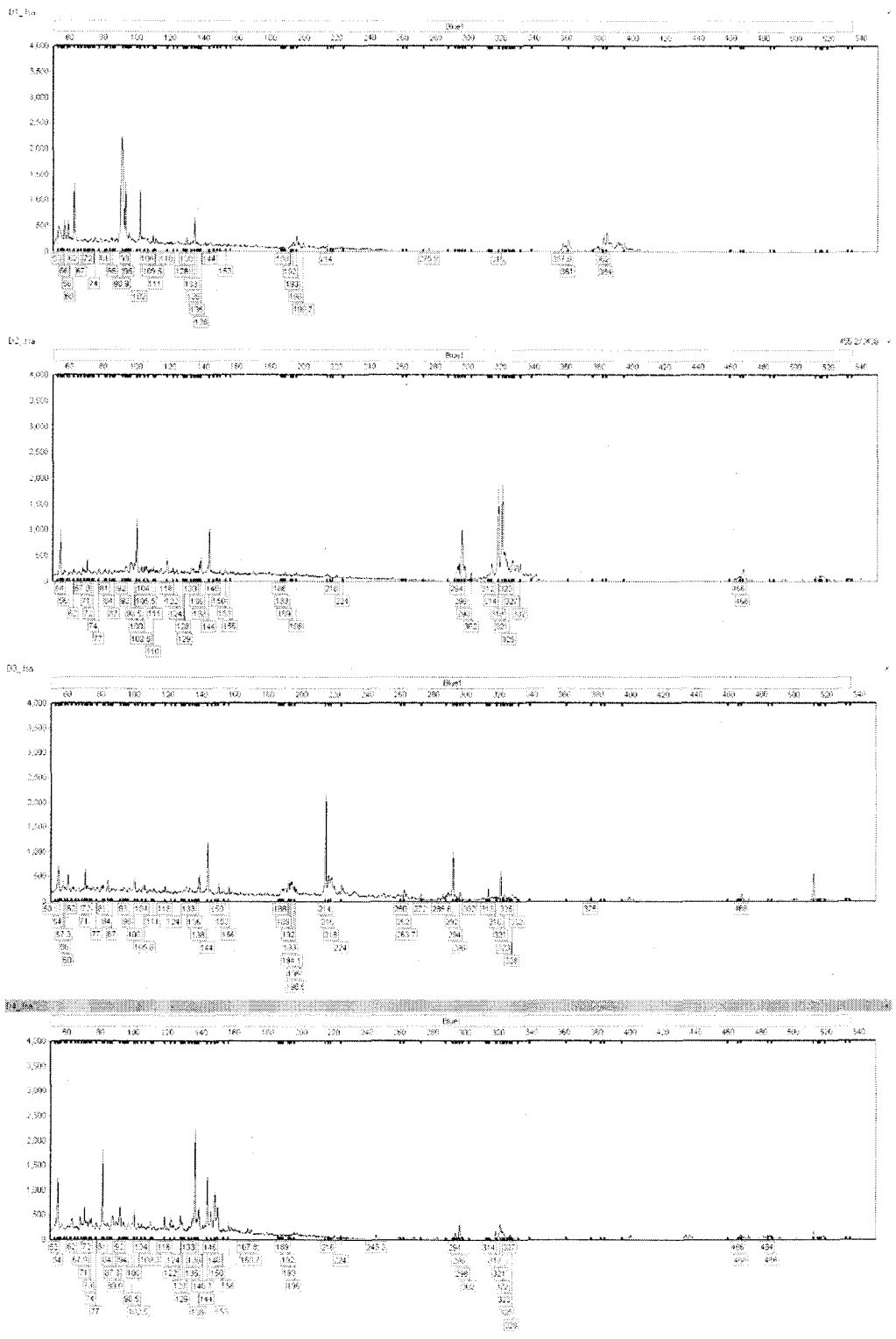
7.2.2 Electropherograms of RE derived TRF profiles from S-5 soil sample. B1 digested by *BstUI*, B2 digested by *HinfI*, B3 digested by *HaeIII*, and B4 digested by *MspI*. Size (bp) and area of the peaks shown by the X and Y axis.



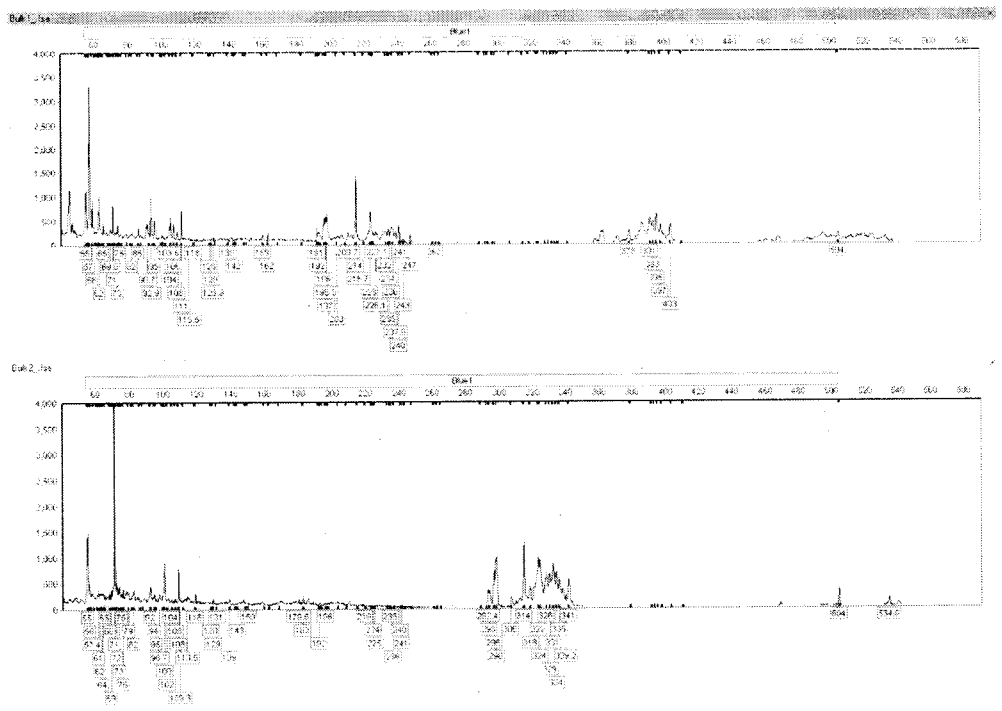
7.2.3 Electropherograms of RE derived TRF profiles from S-6 soil sample. C1 digested by *BstUI*, C2 digested by *HinfI*, C3 digested by *HaeIII*, and C4 digested by *MspI*. Size (bp) and area of the peaks shown by the X and Y axis

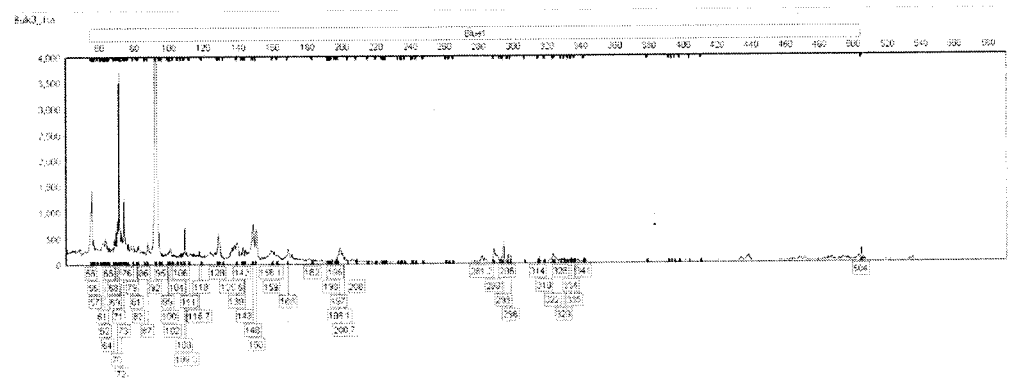


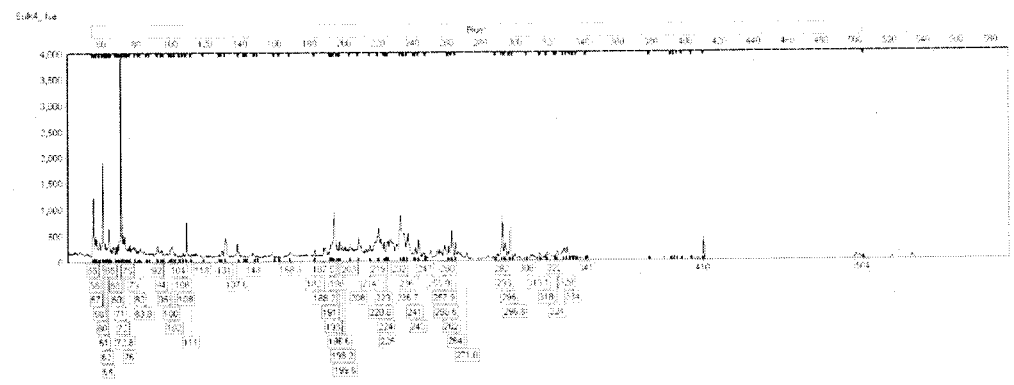
7.2.4 Electropherograms of RE derived TRF profiles from Wild-type soil sample. D1 digested by *BstUI*, D2 digested by *HinfI*, D3 digested by *HaeIII*, and D4 digested by *MspI.* Size (bp) and area of the peaks shown by the X and Y axis



7.2.5 Electropherograms of RE derived TRF profiles from bulk soil sample. Bulk1 digested by *BstUI*, Bulk2 digested by *HinfI*, Bulk3 digested by *HaeIII*, and Bulk4 digested by *MspI*. Size (bp) and area of the peaks shown by the X and Y axis







7.3 Original data of RE derived TRF profiles.

***BstUI* digested TRF profiles. AS11 genotype.**

Size (bp)	Area	Size (bp)	Area
52.8	4983	100.9	2786
54.3	12875	106	1703
55.6	4956	108.2	1057
56.3	5882	109.2	1776
58.2	6948	111	1051
59.9	7217	118.2	792
62	7466	120.2	862
64.7	7275	123.9	477
66.7	10090	128.8	431
69	5073	129.9	1196
70.2	4424	133.2	349
73.3	5881	134.5	522
75.2	7536	138.4	479
78.2	4756	153.1	187
81.9	3153	156.2	201
85.3	5000	195.5	522
87.8	3325	197	302
90.8	4472	214.3	133
93	3055	224.8	78
93.9	1823	384.2	259
94.9	3149	392.9	169
96.3	2540	403.5	102
99.4	1898	459.5	138

BstUI digested TRF profiles. S-5 genotype.

Size	Area	Size	Area
54.2	1736	187.5	109
56.5	3145	189	136
58.5	1830	191.2	396
62.6	3673	192	1035
64.6	137	193.5	818
66.5	257	195.6	3145
68.3	202	196.9	1370
73.1	125	199.7	563
74.6	324	202.9	615
75.9	219	208	370
81.1	155	214.4	1331
82.6	434	221.3	581
83.7	422	223.1	1340
85.9	346	224.8	678
87.6	178	239.9	715
90.3	1808	241.1	177
91.6	776	242.7	197
93	4970	247	535
95.1	1394	291.9	47
96.3	630	293.9	26
100.3	124	301.9	31
101.8	1656	312.1	120
103.6	1012	318.4	369
108.4	175	320.4	50
109.6	141	325.3	70
111	380	331.6	151
111.7	487	334	35
118.2	84	361.1	3015
124.1	179	382.2	3083
128.7	174	384.2	4167
129.8	418	386.2	5121
132.7	382	391.3	2941
136.4	290	392.9	1807
138.2	505	394.9	2630
145.4	119	403.5	863
147.7	120	459.9	3970
149.7	234	465.4	1679
153	190	467.5	5575
175.7	385	484	2075
186	75	486.5	2220

***BstUI* digested TRF profiles. S-6 genotype.**

Size (bp)	Area	Size (bp)	Area
54.4	3117	168.2	117
56.5	3661	186.1	114
58.5	2832	187.4	118
59.9	1070	191.1	448
62.3	5276	192	1329
64.7	524	193.7	1336
72.1	799	195.6	3764
73.3	543	197.1	1492
75.1	1929	199.6	945
81.8	2425	202.9	780
84	948	208.1	207
85.6	1330	214.3	1350
87.5	1379	219.8	654
90.2	3569	223.2	1342
92.9	9738	225	431
95.1	2173	239.9	702
99.5	1197	241	87
101.7	1994	247	508
103.3	2504	292	87
104.3	2189	293.1	166
106.1	1876	294.8	179
108.3	882	318.4	281
111.7	2053	357.5	1517
118	654	361.1	3545
123.9	620	381.9	3109
127.8	449	384.1	3940
128.7	447	386.2	3737
129.9	1539	390.9	4908
131.1	397	392.7	3652
132.6	1331	394.7	3227
138.4	723	403.4	1202
145.6	339	410.2	123
153.1	364	459.2	4106
156.1	193	483.9	641
162.2	155		

***BstUI* digested TRF profile. Wild genotype.**

Size (bp)	Area	Size (bp)	Area
53	9324	138.6	1476
56.5	3995	143.9	2286
58.6	4039	153.2	918
59.9	2780	161.8	1186
62.4	8614	182.5	733
67	4011	187.5	809
71.4	2058	192.2	1831
74.7	2281	193.7	1462
81.2	3888	195.7	3027
85.6	2155	197.1	2226
87.6	2526	199.7	1536
90.9	35634	203	1243
93	8143	214.3	1150
95.1	3145	223.1	1157
101.7	6241	240.1	749
106.3	2540	242.8	284
108.4	2172	275.9	359
109.5	2027	318.5	234
111.2	2806	357.6	1544
118.1	3454	361.1	2728
127.5	2486	382.2	2911
129.9	2351	384.3	4034
133.2	1698	390.9	3121
134.5	3751	393	1746
135.9	2013		

BstUI digested TRF profiles. Bulk soil.

Size (bp)	Area	Size (bp)	Area
54.5	10081	186	924
56.5	17669	188.9	833
58.3	5713	191.2	2023
62.2	6300	192.1	4030
64.7	3045	195.6	6589
66.5	3819	196.3	3601
69	4097	197.1	6562
70.4	4820	203.2	2445
73.3	2657	209.7	2283
74.7	4224	214.4	8331
81.8	2528	215.7	1298
85.7	3427	222.9	7194
90.7	6089	226.1	2017
92.9	5527	227.1	1888
95.1	4378	231.9	3866
103.6	2551	233.6	2427
104.3	3341	234.5	1578
106.2	3670	235.6	6115
108.4	2262	237.9	2887
111	4021	240.1	3623
115.5	1145	241.4	513
118.2	961	242.6	1218
123.8	1073	247.1	1800
127.4	1177	261.7	549
128.6	1174	378.3	3838
129.9	1154	381.8	1719
134.1	1256	391	9217
137.7	1080	392.7	4548
145.9	1192	394.6	6615
153.1	839	397.1	4862
159.1	1524	403.1	5246
162.2	1542	467.6	3140
168	1568	486	3025

HinfI digested TRF profiles. AS11 genotype.

Size (bp)	Area	Size (bp)	Area
54.3	17200	111	1576
55.6	6654	118.2	2638
56.8	10588	124.1	1828
60.9	14261	127.7	1215
62.5	12822	128.8	1347
66.7	5389	130.8	1605
68.5	5891	132.7	1463
70.5	8626	134.2	1028
71.7	7163	136.4	1234
73.2	6182	138.5	1294
74.4	5811	145.8	695
77.3	6607	147.7	689
81.9	4961	149.5	527
83.9	4246	155.9	427
91.6	4465	162.2	259
93.9	4043	293.5	217
94.9	3378	294.3	212
96.5	3592	296.3	721
99.4	5950	297.4	599
102.4	2472	314.4	272
104.3	2783	318.1	314
106.2	2898	320.6	704
108.2	2412	322.1	674
109	3796		

HinfI digested TRF profiles. S-5 genotype.

Size (bp)	Area	Size (bp)	Area
54.5	2429	197.5	359
56.9	836	214.7	900
58.2	223	216.6	181
63.3	211	224.5	218
64.8	106	228.1	702
66.6	138	232.5	65
68.6	251	253.6	518
69.3	170	260.8	144
70.5	1545	262.5	84
71.5	392	264.4	66
74.7	314	293.5	2752
77.3	432	294.4	2624
81.1	174	296.3	9468
83.8	576	297.8	4522
87.6	157	301.9	558
97.1	1960	306.3	1678
100	6231	308.2	1367
102.5	696	312.2	1942
104.3	342	314.3	5115
109.3	708	318.3	9021
110.1	1913	320.6	11244
111.1	689	321.8	20825
112.2	1551	325	4246
114.1	1265	326.7	4507
118.2	1153	327.6	4140
123.8	377	329.5	8239
127.8	304	331.6	6300
129	469	333.4	3368
129.8	204	335.7	4178
133	516	338.5	2319
134	597	341.2	3246
138.4	1659	397.7	1189
143.7	246	403	60
145.9	211	459.4	246
150	226	462.7	1425
153.1	476	465.6	9466
182.2	652	467.5	15614
186.4	136	484	2407
187.6	254	486.5	4223
189	574	492.7	3386
190.9	301		

HinfI digested TRF profiles. S-6 genotype.

Size (bp)	Area	Size (bp)	Area
54.4	3398	146	294
55.7	100	147.7	240
56.9	637	150	324
62.5	419	153.1	856
63.3	445	167.6	249
64.8	127	187.6	153
68.6	388	189.2	406
69.6	126	191.4	323
70.6	742	195.6	80
71.8	528	208.1	80
74.5	348	214.4	597
77.3	880	216.7	88
80.9	342	224.5	289
81.9	260	293.6	4940
84	582	294.5	2868
86.8	576	296.3	9634
91.5	476	297.8	3439
95.1	326	301.8	399
96.3	1286	306.4	1054
97.1	1822	312.2	2147
100	8305	314.3	4054
101.5	610	318.2	9938
104.4	606	320.7	12779
106.1	698	322.4	6849
108.3	377	327.6	4108
110	1054	329.4	8425
111.2	273	331.5	5276
118.1	1441	333.5	1886
123.9	465	335.4	3413
127.6	300	340.9	2377
128.9	454	390.7	131
129.8	246	409.9	196
131	246	459.1	136
132.8	737	465.3	5247
136.4	634	467.4	10855
138.5	2561	484	772
143.7	211		

HinfI digested TRF profiles. Wild genotype.

Size	Area	Size	Area
54.4	6830	153.1	2060
156.8	2194	156	1632
62.4	2446	182.1	1597
67.8	1911	186.3	963
70.5	2812	187.5	1219
71.6	1641	189	1726
74.3	4292	190.8	1137
77.2	3623	195.6	1633
81	1955	197.5	1683
83.9	2409	217.7	780
91.5	1368	224.2	884
93.4	4095	232.1	645
97	7509	236.3	1119
98.5	2787	293.5	2589
99.9	7516	294.4	2424
102.5	2767	296.2	9012
104.4	2378	297.8	2878
105.5	2318	301.9	1141
108.2	1979	306.3	1406
110	1918	312.3	2271
111.1	1735	314.3	3838
118.2	3357	318.3	12875
121.5	1789	320.9	14437
123.9	1922	323.5	4846
127.7	1703	325	2301
128.9	1380	326.9	7437
130.8	1939	329.8	5399
132.8	2846	331.6	3689
136.4	1755	333.6	1406
138.4	3705	335.1	2938
143.4	6363	341.2	2354
145.9	2367	465.3	1837
150	1953	467.5	3505

HinfI digested TRF profiles. Bulk soil.

Size (bp)	Area	Size (bp)	Area
54.5	12162	136.3	1518
55.6	3199	138.6	2436
57.4	2753	143.3	1413
60.9	2544	149.6	1502
63.6	2598	152.9	1227
64.7	2108	156.1	1022
66.7	5078	179.5	1263
67.6	2107	182.1	1703
68.6	3083	186.2	1115
70.5	21318	187.2	1009
71.7	3586	189.3	901
73.2	3578	191.9	1034
74.6	2538	195.7	2542
75.6	3162	217.7	768
77.7	6024	223.9	812
78.8	3583	225	519
81.9	3198	236.5	435
84	2485	239.7	515
91.6	3876	240.7	616
93.9	2356	292.4	2456
95.1	1494	293.4	2433
96.7	3139	296.2	7589
100	6484	297.6	14563
101.8	2860	306.2	2147
104.2	1574	314.2	10423
106.1	1778	317.8	5025
108.2	4111	322.4	9385
109.3	1536	323.5	9732
113.5	2103	327.5	7010
118	2109	329.4	7598
121.4	1517	331.4	12516
124.2	1869	333.5	7438
127.7	1363	335.3	6028
128.8	1878	339.2	2167
131.1	1331	341	7694
134.1	1451		

HaeIII digested TRF profiles. AS11 genotype.

Size (bp)	Area	Size (bp)	Area
54.1	14120	109.1	1740
55.5	6892	111.1	1526
56.2	7499	118.2	1551
61	14530	122	1437
62.4	4672	123.7	993
63.6	5505	128.5	762
66.3	4416	134.2	569
67.6	2853	136.4	658
68.6	5047	138.5	1110
70.5	8837	146.1	615
74.9	5658	153.1	363
77.3	4209	189	195
81.2	3621	193.4	376
82	3529	195.6	194
83.8	3606	197.3	125
85.8	2943	214.5	189
86.8	2534	216.6	381
91.4	3295	217.7	252
93.8	5587	219.3	162
95.2	1885	222.5	83
96.4	4636	224.1	175
99.4	4826	291.7	579
106.3	1860	296.2	211
108.3	2087	320.8	125

***HaeIII* digested TRF profiles. S-5 genotype.**

Size (bp)	Area	Size (bp)	Area
54.3	4299	192.1	3586
60	3661	193.4	3691
62.3	1026	195.5	3142
63.5	3020	216.6	4886
68.6	979	218.2	6566
70.6	2100	232.4	6902
71.8	1429	240.7	1418
73	1805	260.2	1138
75.1	1377	262.2	2389
76.6	1263	264.3	753
84	1808	272.1	315
91.1	9617	291.7	6827
97	2165	293.3	1558
99.9	2024	296.4	3132
111.2	938	306.4	455
121.8	1154	313.6	1301
124.1	1145	322	1854
129	2214	325.9	1943
131.2	3138	327.9	2508
132.8	1182	329.7	1839
136.2	948	376.2	2563
137.7	1724	465.5	1730
143.5	756	467.5	3202
153.2	2850	483.9	578
156.1	676	486.5	996

***HaeIII* digested profile. S-6 genotype.**

Size (bp)	Area	Size (bp)	Area
54.3	4747	182.5	1491
57.1	2331	187.7	3762
58	1723	192.1	5948
59.9	4786	193.3	4023
62.4	1999	194	5550
63.4	3061	195.5	2838
68.7	1936	196.5	3248
70.5	2383	207.6	2490
71.7	1878	214.4	11101
72.9	2389	216.5	7675
74.9	1536	217.8	8923
77.3	1580	224.1	4261
84	2854	232	3698
85.9	1479	240.8	1782
92	2166	242.7	1486
93.2	2666	260.2	1982
95.1	1538	262.1	3286
96.4	4155	263.5	1202
99.9	4359	272.4	125
101.6	1784	287.7	2351
104.5	1357	291.7	11384
106.2	2000	293.3	2167
108.3	1738	296.3	2175
109.2	1364	306	490
111.1	1643	313.3	2075
118.3	1526	320.9	1508
121.9	952	323.3	2929
123.9	1701	325.7	1845
128.9	1723	327.9	2530
131	4457	329.6	1901
132.9	1939	334	167
134.2	1514	375.9	2668
136.5	1553	378.5	124
138.5	4589	383.9	79
143.3	1238	392.5	119
145.9	1023	410.1	346
147.7	1036	465.4	2405
150.1	1179	467.6	5344
153.1	1992	486.2	302
156.2	996		

HaeIII digested TRF profiles. Wild genotype.

Size (bp)	Area	Size (bp)	Area
50.1	1743	193.3	2769
54.4	6469	194.1	3004
57.3	4576	195.6	2413
58.3	3232	196.6	1827
60	4198	207.5	2890
62.5	2349	214.5	13659
63.5	2610	216.4	6809
69.4	5686	218.2	6661
70.5	4479	219.7	4811
71.6	3403	222.9	2491
74.9	3105	224.2	2857
77.4	5891	225.1	2604
81.1	3116	232.1	4787
83.9	4152	240.9	1808
85.8	1882	242.7	1620
86.8	3337	260.5	1307
93.4	3125	262.3	2284
96.3	5570	263.7	1040
99.9	4930	272.4	965
104.5	1972	285.6	925
105.6	2033	291.7	6907
108.4	2252	293.3	1465
109.3	2129	296.2	2006
111.2	2222	302	574
118.2	3000	306.1	562
123.8	1969	313.4	1949
131.1	4438	318.1	973
132.8	3366	320.9	4180
136	3713	323.3	1989
138.3	4519	325	657
143.5	7576	327.8	1775
150	2719	329.5	1434
153	2195	331.3	456
156.1	2210	341.4	186
168.2	1476	376	909
187.7	3764	410.2	237
189.1	2112	467.6	1869
192.1	4909		

HaeIII digested TRF profiles. Bulk soil.

Size (bp)	Area	Size (bp)	Area
54.6	17165	134.7	2006
55.6	3602	135.6	2882
56.7	7018	138.2	6977
62.6	5198	141.9	3685
63.7	2599	143.3	2697
64.7	2357	147.9	10286
66.3	3346	149.6	8151
67.5	3316	153	854
68.5	4523	158.1	2031
69.6	4895	168	2530
70.5	19485	182.2	773
71.7	4772	187.4	884
73.3	7397	189	368
75.7	3165	193	841
77.2	4942	195.6	1078
78.7	3232	197.4	1964
81	2927	198.1	2940
82	3027	200.7	1123
83.9	2476	207.7	886
87.2	4692	281.2	1642
91.7	79306	288.1	2494
95.1	1918	293.6	3434
96.7	1549	296.3	1155
99.9	3512	297.8	1236
101.6	1659	314.2	805
104.2	1347	322.4	2270
106.3	1601	327.5	994
108.3	3764	329.1	940
109.3	1816	333.5	917
111.2	2259	335.4	1157
116.7	2070	340.9	1358
118.1	1138	467.1	532
127.7	5345	483.5	524

MspI digested TRF profiles. AS11 genotype.

Size (bp)	Area	Size (bp)	Area
54.4	23363	106.3	2574
55.6	7345	108.2	2687
62.4	13652	109.2	2156
66.8	5814	111.1	1677
68.6	6009	124	1423
70.6	9365	127.9	1240
71.7	7734	128.9	1172
73.3	6207	130.1	714
74.5	6282	131	683
77.4	5500	132.9	1197
82	5907	134.2	1219
84.1	4719	136.4	1100
86.8	4300	138.5	1540
88.2	2749	143.8	870
91.6	5047	145.8	485
93.9	4240	148	1213
94.9	4012	149.7	743
96.4	4656	153.1	692
99.5	4204	296.3	310
102.5	2633	320.5	228
104.3	2620	322.2	303

***MspI* digested TRF profiles. S-5 genotype.**

Size (bp)	Area	Size (bp)	Area
54.7	2447	182	373
56.9	292	186.2	1326
62.9	1977	189.1	110
65.1	259	192.1	107
66.4	216	193	245
68.3	999	195.4	376
69.4	650	197.3	1020
70.6	2238	203.2	134
72.2	1054	207.6	642
73.4	1593	214.1	206
74.4	1188	216.3	1319
75.7	430	222.9	435
77.4	549	224.1	496
81.3	3395	241.1	104
87.4	2839	268.7	420
89.2	2275	272.1	52
91.6	3638	274.2	441
93.5	1061	293.6	2231
99.9	2104	296.2	2987
104.4	287	301.7	183
107.9	419	306.3	398
109.4	1491	314.4	473
118.1	779	318.2	1821
121.7	891	320.6	4158
124.5	1077	322.4	5903
127.7	3075	325	623
132.8	228	327.9	752
134.6	1912	331.8	58
136.3	6296	333.6	268
138.2	4115	335.3	236
145.8	2769	376.3	66
148	8321	381.5	179
149.8	4483	397.3	774
153	280	465.4	1773
158	1494	467	6051
158.9	771	483.7	5456
160	1570	486.2	5935
167.9	947		

MspI digested TRF profiles. S-6 genotype.

Size (bp)	Area	Size (bp)	Area
54.4	3312	156	580
56.8	568	157.8	1154
62.7	1679	159.9	1277
64.4	192	167.7	572
68.2	1007	182	153
70.5	1340	185.8	326
71.9	1115	188.9	129
73.2	1419	192.3	57
74.2	1209	193.1	109
75.4	374	207.7	346
77.2	843	217.8	42
81	1358	224.2	294
82.7	1159	240.2	83
83.9	492	261.7	50
87.2	2343	264.1	192
89.1	2063	288	283
91.6	3909	293.3	1359
93.3	1068	294.4	554
94.9	312	296.1	2659
96.3	959	297.9	387
99.9	3005	306.3	278
104.3	561	312.2	70
106.2	530	314.2	319
108.1	672	318.1	1212
109.2	639	320.6	2479
109.9	498	322.2	3597
118	902	327.8	860
121.7	1084	333.4	165
123.8	914	341.4	332
127.6	2873	384.3	82
130.7	259	393	39
132.7	639	397.3	437
134.5	1494	400.5	1404
136.2	6976	433.9	1341
138.3	4253	436.2	2233
145.6	2172	465.4	1315
147.9	6016	466.9	5222
149.6	3341	483.6	2319
152.9	875	486.1	2344

MspI digested TRF profiles. Wild genotype.

Size (bp)	Area	Size (bp)	Area
52.4	4534	156.1	2268
54.5	9453	162.3	1113
56.9	2248	167.8	2076
62.7	4611	169.7	1615
67.9	5144	181.9	924
70.4	4906	189	1060
72	3593	191	992
73.1	3640	192.2	900
74.3	3615	193	898
77.3	3514	195.7	1316
81	9799	216.3	971
84	3391	219	340
87.3	7326	224.1	840
89	4593	236.4	283
91.5	6836	240.9	145
93.5	3748	245.3	724
98.5	2487	246.7	181
99.9	5097	264.4	227
102.5	3324	293.5	1203
104.3	2264	296.2	2775
108.2	3211	297.8	643
109.3	4088	301.9	391
118.1	3489	314.3	457
121.7	3556	318.2	1633
123.8	2417	320.8	2760
127.5	4248	322.4	1699
128.7	2105	323.4	1393
132.6	3477	324.9	552
134.6	3250	327.7	480
136.2	13987	335.3	191
138.4	6501	341	332
140.7	2847	397.4	252
143.4	8468	465.5	398
145.7	5771	467.2	1769
147.9	8615	483.8	1364
149.8	6581	486.1	889
153	1685		

MspI digested TRF profiles. Bulk soil.

Size (bp)	Area	Size (bp)	Area
54.4	9186	191.1	2384
55.6	2767	193.3	10124
56.3	2977	195.5	1767
58	2422	196.6	3391
59.9	10100	198.2	2356
61.1	2280	199.8	1955
62.3	2177	202.7	4324
63.5	5797	207.7	3687
64.7	1998	214.3	2777
67.6	1882	219.4	5452
68.6	2428	220.6	4940
70.5	22189	222.5	3140
71.7	3521	224.1	3134
72.8	3501	225	2719
74.8	2193	232.1	9724
75.7	2258	236	3280
82	2353	236.7	3908
83.8	2170	241	2221
91.5	3500	242.7	5043
93.9	1824	247	655
95.3	1125	255	1553
96.7	1134	257.9	2686
99.9	4859	258.6	1811
101.7	1101	260.4	2306
104.1	1847	262.1	5353
106.3	1531	264.4	2739
108.2	4213	271	1433
109.3	1262	291.7	5345
111	3118	293.3	4325
118.3	828	296.3	5662
121.4	1611	298.8	1013
130.6	6108	301.7	788
134.2	1390	306.3	394
137.6	2549	313.1	1876
147.6	1566	321.1	1068
153.1	1283	322.4	524
168.5	1896	329.6	1864
182.3	1908	333.5	606
187.5	1815	341.3	828
188.2	2162	410	3121