2019

FIELD EVALUATION OF TOBACCO ENGINEERED FOR HIGH LEAF-OIL ACCUMULATION

James Perry
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FIELD EVALUATION OF TOBACCO ENGINEERED FOR HIGH LEAF-OIL ACCUMULATION

THESIS

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in the College of Agriculture, Food and Environment at the University of Kentucky

By
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2019
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ABSTRACT

FIELD EVALUATION OF TOBACCO ENGINEERED FOR HIGH LEAF-OIL ACCUMULATION

The biofuel market is dominated by ethanol and biodiesel derived from cellulosic and lipid-based biomass crops. This is largely due to the relatively low costs and reliability of production. At present, production of non-food plant-derived oils for biofuel production in the U.S. is minimal. A research team from the Commonwealth Scientific and Industrial Research Organization (CSIRO), an independent Australian federal government research institution, has developed an efficient transgenic system to engineer oil production in tobacco leaves. This novel system is comprised of multiple transgenes that direct the endogenous metabolic flux of oil precursors towards triacylglycerol (TAG) production. Additional genes were incorporated to store and protect the accumulated oil in vegetative tissues. Preliminary greenhouse tests by the CSIRO research group indicated an oil content of >30% by dry weight (DW) in tobacco leaf lamina. Here we evaluated two transgenic lines against a non-transgenic control in 2017 and 2018 in greenhouse and field production systems. The 2017 pilot study showed that the high leaf-oil tobacco line was viable and will grow in the field in Kentucky. Chemical analyses revealed significantly higher oil content compared to the non-transgenic control despite several logistical setbacks. These promising discoveries prompted the deployment of additional transgenic line assessments and further data validation in 2018. Line evaluations in 2018 revealed that the LEC2:WRI1:DGAT:OLE transgenic line had the highest leaf oil content (≥19.3% DW⁻¹) compared to both the WRI1:DGAT:OLE transgenic line (≤5.6% DW⁻¹) and non-transgenic control (≤2.1% DW⁻¹). The results of this research will contribute to the successful development of transgenic tobacco lines engineered to accumulate high concentrations of TAG in the leaves.

KEYWORDS: tobacco, Nicotiana tabacum, biofuel, leaf-oil, triacylglycerol (TAG), wrinkled-1 (WRI1), diacylglycerol acyltransferase 1 (DGAT1), oleosin (OLE), leafy cotyledon 2 (LEC2)

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I dedicate my thesis to my loving wife Ashley Nicole Perry, my beautiful daughter Emilia Jo Perry, my wonderful parents Patty Saunders Perry and the late George Robert Perry, my sisters Emily Perry Benedict and Rebecca Bucher Shryock, and all of my family and friends who have supported me in my endeavors and pursuit of my dreams.
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# TABLE OF CONTENTS

ACKNOWLEDGEMENTS ........................................................................................................ iii

LIST OF TABLES ................................................................................................................... vi

LIST OF FIGURES ................................................................................................................ vii

Chapter 1: Literature Review
1.1 Introduction to Biofuels ........................................................................................................ 1
1.2 Sugar versus Lipid-Based Biofuels ..................................................................................... 1
1.3 Biofuel and the Economy .................................................................................................... 3
  1.3.1 Global Perspective ........................................................................................................ 3
  1.3.2 Consumer Perspective ................................................................................................ 4
1.4 Plant-Derived Lipids and Biosynthesis .............................................................................. 4
1.5 Metabolic Engineering of Vegetative/leaf lipids ................................................................. 6
  1.5.1 Wrinkled-1 (WRI1) ..................................................................................................... 6
  1.5.2 Leafy cotyledon 2 (LEC2) ........................................................................................... 7
  1.5.3 Diacylglycerol acyltransferase 1 (DGAT1) ................................................................. 7
  1.5.3 Oleosin (OLE) ............................................................................................................ 8
1.6 Tobacco as a biofuel feedstock .......................................................................................... 8
1.7 Objectives .......................................................................................................................... 9

Chapter 2: Field Evaluation of Tobaccos Engineered for High-Leaf Oil Accumulation
2.1 Introduction ......................................................................................................................... 11
2.2 Methods ............................................................................................................................. 12
  2.2.1 Experiment design ...................................................................................................... 12
  2.2.2 Experimental construct design .................................................................................... 13
  2.2.3 Transplant propagation .............................................................................................. 13
  2.2.4 Field conditions and preparation .............................................................................. 14
  2.2.5 Greenhouse transplanting & management (2017 only) .............................................. 14
LIST OF TABLES

Table 1  Recent reports on metabolic engineering of vegetative biomass for lipid-based biofuel production ................................................................. 28

Table 2  Major experiment event dates ................................................................................. 32

Table 3  Plant height and leaf number data 2017 .................................................................... 34

Table 4  Model statistics of harvest weight data from 2017 study ........................................... 37

Table 5  Harvest weight data from 2017 .................................................................................. 38

Table 6  Model statistics of harvest weight data from 2018 study ........................................... 39

Table 7  Harvest weight data from 2018 .................................................................................. 40

Table 8  Model statistics for 2017 monitor study (field only) .................................................. 41

Table 9  Model statistics of oil content by variety and leaf position for 2017 ........... 44

Table 10  Oil content by variety and leaf position data 2017 ............................................... 45

Table 11  Model statistics of oil content by variety and leaf position for 2018 ........... 46

Table 12  Oil content by variety and leaf position data 2018 ............................................... 47
**LIST OF FIGURES**

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Figure 1</td>
<td>Triacylglycerol (TAG) chemical structure example</td>
<td>29</td>
</tr>
<tr>
<td>Figure 2</td>
<td>Abbreviated plant lipid/TAG Biosynthesis metabolic pathway</td>
<td>30</td>
</tr>
<tr>
<td>Figure 3</td>
<td>Engineered constructs generated by CSIRO</td>
<td>31</td>
</tr>
<tr>
<td>Figure 4</td>
<td>Plant phenotypes seven days before harvest from 2017</td>
<td>33</td>
</tr>
<tr>
<td>Figure 5</td>
<td>Plant phenotypes 12 days before harvest from 2018</td>
<td>35</td>
</tr>
<tr>
<td>Figure 6</td>
<td>Larva of tobacco hornworm (<em>Manduca sexta</em>)</td>
<td>36</td>
</tr>
<tr>
<td>Figure 7</td>
<td>Average %TFA DW(^{-1}) of field samples at each sample point 2017</td>
<td>42</td>
</tr>
<tr>
<td>Figure 8</td>
<td>Average %TFA DW(^{-1}) of greenhouse samples at each sample point 2017</td>
<td>43</td>
</tr>
<tr>
<td>Figure 9</td>
<td>Field grown non-transgenic control change in FA profile during 2017</td>
<td>48</td>
</tr>
<tr>
<td>Figure 10</td>
<td>Field grown <em>LEC2:WR1I:DGAT:OLE</em> change in FA profile during 2017</td>
<td>49</td>
</tr>
<tr>
<td>Figure 11</td>
<td>Greenhouse grown non-transgenic control change in FA profile during 2017</td>
<td>50</td>
</tr>
<tr>
<td>Figure 12</td>
<td>Greenhouse grown <em>LEC2:WR1I:DGAT:OLE</em> change in FA profile during 2017</td>
<td>51</td>
</tr>
<tr>
<td>Figure 13</td>
<td>Field grown non-transgenic control final FA profile 2018</td>
<td>52</td>
</tr>
</tbody>
</table>
Figure 14  Field grown WR1I:DGAT:OLE final FA profile 2018,..........................53

Figure 15  Field grown LEC2:WR1I:DGAT:OLE final FA profile 2018,...............54
Chapter 1

Literature Review

1.1 Introduction to Biofuels

Research and development into plant-based energy sources offers a sustainable alternative to finite fossil fuel reserves. The concept of ‘biofuels,’ or liquid fuels composed of, or produced from, biological raw materials, has been a large area of study for several decades. Biofuels can be divided into two main categories: first- and second-generation. First-generation biofuels are produced from sugars, starch, or vegetable oils that originate from biological feedstocks derived from food crops and that are not generally considered sustainable (Robertson et al., 2017). This category includes ethanol, biodiesels, biogas, and solid fuels made from natural sources that may have negative ecological impacts. Biofuels derived from sustainable biological feedstocks that are not based on food crops or that are non-edible are considered to be second-generation. These fuel sources are still in the early stages of development and include cellulosic ethanol, algal-based production systems, and others such as waste vegetable oils. The actual sustainability of second generation biofuels, however, is still debated (Granda et al., 2007). Recently, a third generation of biofuels has been described that includes oil-producing microorganisms, such as microalgae, that are grown in biorefineries (Leong et al., 2018). Here we will focus on first- and second-generation biofuels produced from common agricultural crops.

1.2 Sugar-based versus lipid-based biofuels

Differences between the efficiencies of sugar-based feedstocks and lipid-based feedstocks in biofuel production have debated (Demirel, 2018). The most common sugar-based
biofuel is ethanol, which can be produced from cellulosic (cellulose) or lignocellulosic (lignocellulose) biomass by bioactive fermentation. Types of crops used for sugar-based biofuel production include maize (Zea mays), sugarcane (Saccharum spp.), switchgrass (Panicum virgatum), silvergrass (Miscanthus spp.), and others (Kikas et al., 2016). The energy and cost efficiency of ethanol production from green biomass is unclear and is still being studied (Bansal et al., 2016). The potential negative impacts of increased bioethanol production are also being addressed (Robertson et al., 2017). Expansion of the bioethanol industry coupled with the diversion of cellulosic materials from established markets, such as products produced for human or animal consumption, could impact domestic and global food security and prices (Han et al., 2017; Hao et al., 2017; Makenete et al., 2008). The large amounts of arable land required to satisfy the alternative energy market, also referred to as ‘energy sprawl’ (Trainor et al., 2016), can have indirect negative effects such as additional greenhouse gas emissions from industrialized crop production (Hertel et al., 2009). Research efforts in agronomy, plant breeding, and genetic engineering are underway to improve yields and overall energy efficiency to offset negative effects.

Lipid-based biofuels are typically produced by processing oilseeds, palm oil, engineered algae, or vegetable oils (Sawangkeaw and Ngamprasertsith, 2013). Currently, biodiesel is the dominant biofuel derived from lipid-based feedstocks. The sustainability, performance, and economic viability of other fuel types such as bio-jet fuel are also being studied (Tao et al., 2017). The primary lipid substrate extracted from plant biomass is triacylglycerol (TAG). Staple crops typically grown for lipid-based biofuels include rapeseed (Brassica napus), oil palm (Elaeis ssp.), and soybean (Glycine max), as well as others which are largely integrated into food production systems. Additional resources, including land, that
are required to meet the increased demand for lipid-based biofuels can also have negative impacts on the environment (Granda et al., 2007). As with sugar-based biofuels, efforts to develop lipid-based biofuel production systems face many challenges. Genetic engineering strategies have demonstrated that it is possible to increase yield efficiency, lipid quality, and sustainability of lipid-based biofuel crops (Vanhercke et al., 2013b).

1.3 Biofuel and the Economy

1.3.1 Global Perspective

Since 2000, the United States, Brazil, and the European Union have drastically increased biofuel production and use with a nearly 6-fold increase in production in the ten years from 2000-2010 (Moschini et al., 2012). The United States and Brazil have dedicated the majority of their biofuel production into ethanol from maize (United States) and sugarcane (Brazil). As of 2010, ethanol accounted for 75% of global biofuel production with the United States producing over 57% of the world’s bioethanol reserves (Appendix 2). In contrast, the European Union has primarily focused on the production of biodiesel from rapeseed (Appendix 3). A large driver for the production of biofuels has been regulatory policies mandating the use of biofuels for environmental and ecological reasons (Guo and Song, 2019; Moschini et al., 2012). The economic impacts of increased biofuel production are largely speculated to be the influence on commodity supply and demand, and thus ultimately, domestic food prices. Recent studies that have evaluated long-term correlations between biofuel production and domestic food prices over time in the United States have determined that it is difficult to measure without the use of economic models (Shrestha et al., 2019). This study determined that increasing food prices correlated with general inflation and crop production optimization, with no significant changes in food prices.
before or after the “biofuel boom” of the 2000s. These findings were inconsistent with several economic models which had determined that continued increases in biofuel production have prompted increases in food prices

1.3.2 Consumer Perspective

To utilize biofuel derived from agricultural biomass, U.S. consumers generally require access to vehicles or other equipment that have been specifically designed or modified to use them. For example, gasoline-powered passenger vehicles produced in the U.S. on or after 2001 were required by law to be able to burn E15 blended fuels (U.S. Department of Energy). Later, the introduction of E85 blended gasoline (51-83% ethanol) and “flex-fuel” equipped vehicles granted further regular consumer access to biofuels. As of July 2018, consumers were paying on average $3.05/gal for E85 fuel compared to $2.76/gal for regular gasoline (an 11% increase) (U.S. Department of Energy, see Appendix 4). Along with increased costs per gallon, flex-fuel vehicles running on E85 fuel travel 15-27% fewer miles per gallon (U.S. Department of Energy) making the cost justification difficult. Only a limited amount of passenger vehicles designed and approved to consume biodiesel and blended biodiesel products are available to U.S. consumers. However, the standardized biodiesel blends B20 (20% biodiesel) and B99 (99% biodiesel) are available in limited markets. As of July 2018, biodiesel consumers paid on average $2.52/gal for B20 and $2.59/gal for B99 compared to $2.75/gal for regular diesel (8% and 6% reduction, respectively) (U.S. Department of Energy, see Appendix 5) with little to no loss of engine performance.
1.4 Plant-derived lipids & biosynthesis

Plants use lipids as a carbonaceous energy source for several critical metabolic processes. For example, lipids are the main component in plasma membrane organization and function (Mamode Cassim et al., 2019) and are integral in the plant stress response and signaling (Dar et al., 2015; Turnbull and Hemsley, 2017). Accumulated plant lipids are generally stored in packaged lipid bodies (or oil droplets) within seeds to serve as a high energy source for germination and emergence (Laibach et al., 2015). Plant lipids can also accumulate in vegetative tissues (Xu and Shanklin, 2016) but usually at lower levels than in seeds due to the relatively high availability of stored carbohydrates.

The main component of plant oils is triacylglycerol (TAG) which is comprised of three fatty acids (FAs) esterified to glycerol (Figure 1) and is considered among the most energy-dense of natural compounds (Bates, 2016). The types of FAs linked to the glycerol in TAG can differ widely in terms of their chain length and degree of saturation, thus TAG refers to an entire class of diverse macromolecules. The high energy content of TAG can be considered favorable for lipid-based biofuel applications. The biosynthesis of plant oils is complex and involves the integration of several metabolic pathways.

Biosynthesis of all plant oil starts with the common substrate acetyl-CoA, which is converted to malonyl-CoA by the highly regulated enzyme acyl-CoA carboxylase (ACCase) in the chloroplast. ACCase serves as a major regulatory step for carbon flux into FAs. Malonyl-CoA is the main substrate for FA esterification by acyl carrier protein (ACP) to form chains 16 or 18 carbons long. A transport carrier protein (such as FAX1) transports elongated FAs out of the plastid into the cytosol where they serve as a pool for one of several modification pathways. TAGs are synthesized by the Kennedy Pathway in
which a single FA is acylated to glycerol-3-phosphate (G3P) by *acyl-CoA:glycerol-3-phosphate acyltransferase* (GPAT) to produce lysophosphatidic acid. A second FA is linked to glycerol by *acyl-CoA:lysophosphatidic acid acyltransferase* (LPAAT) to give phosphatidic acid. *Phosphatidic acid phosphatase* (PAP) then removes the sn-3 phosphate to form diacylglycerol (DAG). The pool of DAG serves as the main substrate for biosynthesis of TAG and several other integral membrane lipids such as phophatidylglycerol (PG), phosphatidylinositol (PI), phosphatidylcholine (PC), and phosphatidylethanolamine (PE). DAG is transformed into TAG by the final acylation of a third FA by *diacylglycerol acyltransferase* (DGAT) (Figure 2).

### 1.5 Metabolic Engineering of Vegetative/Leaf Lipids

Techniques in genetic engineering have been recently used to address plant lipid/oil yields and quality in several different plant species (Table 1). The use of green plant biomass (e.g. leaves and stalks), as opposed to oilseeds, to serve as a sustainable feedstock for lipid-based biofuels will require considerable research and development. A 2013 review discussed the limitations of oilseed crops compared to the potential of green biomass by metabolic engineering methods, citing limited success and understanding of lipid biosynthesis (Vanhercke et al., 2013b). These authors suggested, however, that efforts should shift from the engineering of oil seeds to vegetative tissues. The biomass potential of high-yielding vegetative crops coupled with metabolic engineering to direct the synthesis of oil in green tissues, specifically in the leaves, make a strong case for lipid-based biofuel applications. Research efforts have identified a few key regulatory factors including transcription factors, metabolic enzymes, and functional proteins that contribute
to elevated leaf oil accumulation. These identified enzymes and regulatory proteins are described below.

1.5.1 Wrinkled 1 (WRI1)

*Wrinkled 1* (WRI1) was first identified over 20 years ago while screening mutant *Arabidopsis thaliana* populations for seed oil accumulation (Focks and Benning, 1998). Mutations at the *wri1* locus cause a reduction in seed-specific oil content as well as a wrinkled leaf phenotype. Mutants have impaired incorporation of sucrose and glucose into TAG, which contrasts with their increased rate of acetate incorporation. WRI1 was later found to be an AP2/EREBP transcription factor reported to be important in the regulation of seed storage metabolism. Overexpression of WRI1 in *Arabidopsis thaliana* leads to a high-seed oil phenotype (Cernac and Benning, 2004). Other studies overexpressing WRI1 resulted in significantly increased vegetative oil content in *Sorghum bicolor, Nicotiana benthamiana,* and tobacco (*Nicotiana tabacum*) (Reynolds et al., 2015; Vanhercke et al., 2019; Vanhercke et al., 2017; Vanhercke et al., 2014; Vanhercke et al., 2013a).

1.5.2 Leafy cotyledon 2 (LEC2)

The *Leafy cotyledon 2* (LEC2) gene was initially shown to function in several embryo-specific pathways in *Arabidopsis thaliana*. The LEC2 protein was later determined to be a B3 domain-type transcription factor that is involved in the complex regulation of seed embryogenesis and maturation, and expression of the gene was found to be highest during seed development (Stone et al., 2001). LEC2 is tightly integrated with lipid biosynthesis and storage during seed production, and overexpression of the gene also leads to increased oil storage in the leaves (Santos Mendoza et al., 2005). LEC2 regulation of FA biosynthesis during seed development has been shown to be synergistic with WRI1 (Baud et al., 2007).
1.5.3 Diacylglycerol acyltransferase 1 (DGAT1)

*Diacylglycerol acyltransferase 1* (DGAT1) is a core metabolic enzyme that catalyzes the acylation and transfer of the third FA onto DAG to give TAG. Overexpression of DGAT1 in transgenic rapeseed/canola plants resulted in a significant increase in seed oil content, specifically TAG (Sharma et al., 2008; Taylor et al., 2009). Overexpression of DGAT1 in tobacco using a ribulose-bisphosphate carboxylase small subunit (*RbcS*) promoter led to a 20-fold increase in leaf oil content (Andrianov et al., 2010). Other recent studies using multi-transgene constructs, including *WRI1*, *LEC2*, and *DGAT* have also shown significant increases in tobacco leaf TAG content (Vanhercke et al., 2017; Vanhercke et al., 2014; Vanhercke et al., 2013a). Localized *DGAT1* and *LEC2* co-overexpression driven by xylem-specific promoters resulted in an increase in total stalk/stem oil and TAG content in tobacco (Nookaraju et al., 2014).

1.5.4 Oleosin (OLE)

Plant oils are generally stored in intracellular compartments and are coated with plant-specific oleosin (OLE) proteins, which are typically localized to oil bodies (also known as lipid droplets or oleosomes) and to the endoplasmic reticulum (Huang, 1992). Overexpression of *OLE*-type proteins greatly increased the accumulation of DAG and TAG within oil bodies of *Saccharomyces cerevisiae* (Parthibane et al., 2012) and *Arabidopsis* leaves when co-expressed with *phospholipid:diacylglycerol acyltransferase-1* (PDAT1; Fan et al. (2013)). Another study evaluated co-expression of *WRI1*, *DGAT1*, and *OLE* in *Zea mays* which showed increased leaf oil/TAG content in vegetative biomass/stover (Alameldin et al., 2017). Similar engineered configurations, including those co-expressed with a *LEC2* transgene, have shown dramatic increases in leaf oil in tobacco (Vanhercke
et al., 2017). Regulation of OLE has also been shown to be influenced by LEC2 in Arabidopsis (Kim et al., 2013).

### 1.6 Tobacco as a biofuel feedstock

The interest in vegetative crops grown specifically for use in biofuels has been met with some challenges. The main obstacles include cost, energy efficiency, food scarcity, energy capture, and sustainability. Several solutions have been proposed to address one or more of these problems including the deployment of multiple harvest cropping systems (Na et al., 2016; Shooshtarian et al., 2018), perennial crops (Miao and Khanna, 2017), and non-food/feed crops such as tobacco. Early exploration of tobacco biomass for biofuel production primarily focused on fermentation of tobacco stalks, a byproduct of the tobacco industry, to produce hydrolysates and ethanol (Martin et al., 2002). Tobacco seeds were also studied as a potential source of FAs, but modest seed yields limited success (Grisan et al., 2016). Leaf oil content among Nicotiana subspecies varies (Koiwai et al., 1983) and is generally <4% oil per dry weight (DW) in N. tabacum (tobacco).

Early published discussions of the use of tobacco leaves as a biofuel feedstock proposed utilizing tobacco’s ease of transformation to deploy molecular engineering techniques resulting in improved oil accumulation in leaves (Andrianov et al. 2010). Their report of overexpression of DGAT1 and LEC2 transgenes using RbcS promoters in Wisconsin 38 and NC-55 tobacco varieties showed a minimum 2-fold increase in leaf oil content and a dramatic shift towards TAG accumulation. Large reductions in linolenic acid and increases in oleic acid were also observed in the isolated TAG fractions. Another group from the Commonwealth Scientific and Industrial Research Organization (CSIRO) in Australia studied the synergistic influence of transiently co-expressed WRII and DGAT1 transgenes
in *N. benthamiana* and saw a 22-fold increase in TAG content compared to the non-transgenic control (Vanhercke et al., 2013a). Further development by the same group added a third transgene, *OLE*, in addition to *WR11* and *DGAT1* in transformed tobacco (Wisconsin 38). Results showed more than 15% TAG DW$^{-1}$ in the leaf tissues with no change in plant phenotype (Vanhercke et al., 2014). This tripartite system was also evaluated with the addition of a *LEC2* transgene, which resulted in >30% TAG DW$^{-1}$ in tobacco leaves (Vanhercke et al., 2017). These experiments studying novel transgenic tobacco lines for high-leaf oil accumulation were all performed in controlled environments such as a greenhouse. There is no published literature available on outdoor field studies of tobacco engineered for high leaf oil accumulation.

### 1.7 Objectives

The goal of the research presented here is to evaluate plant viability and leaf oil content of previously described tobacco lines engineered for high leaf-oil accumulation ("*WR11:DGAT:OLE*" and "*LEC2:WR11:DGAT:OLE*", both developed by CSIRO) compared to a non-transgenic control grown in an outdoor field system using commercial tobacco production practices. We hypothesize that the CSIRO-developed transgenic tobacco lines have higher leaf oil accumulation than the non-transgenic control. The specific objectives of this study are to (1) successfully propagate transgenic seedlings in the greenhouse and transplant seedlings into pots in the greenhouse or conventionally prepared field using an industry standard burley tobacco production model, (2) quantify differences in phenotype between the experimental lines, (3) evaluate average leaf oil content and FA profiles among the experimental lines, and (4) evaluate oil content and FA profile by leaf position within each experimental line.
Chapter 2

Field Evaluation of Tobacco Engineered for High Leaf-oil Accumulation

2.1 Introduction

Research on sustainable biofuel production from plant biomass has been ongoing for several decades. The broad spectrum of research being conducted includes industrial engineering (Huang et al., 2008), sustainable production (López-Bellido et al., 2014), molecular biology (Vanhercke et al., 2014), and alternative feedstock sourcing such as algae and microalgae (Khan et al., 2017). Currently, the sustainability and socioeconomic implications of traditional cellulosic staple crop production for use in biofuels, specifically ethanol, is still under debate (Granda et al., 2007). There are continued disputes about the viability of sugar-based biofuels vs. lipid-based biofuels (Demirel, 2018). At present, the lack of cost-competitive feedstock production models establishes a reliance on the sustainable cultivation of renewable, energy-dense plant biomass for biofuels.

To improve the performance of existing lipid-based bioenergy crops, researchers have developed novel metabolic systems capable of increased medium-chain and long-chain fatty acid accumulation in leaf tissues (as opposed to seeds) in multiple plant species (Vanhercke et al., 2019; Vanhercke et al., 2013a; Vanhercke et al., 2013b). Scientists at CSIRO have refined these engineered systems and deployed them in several high biomass crops including tobacco (Vanhercke et al., 2017). Using the tobacco plant model, researchers achieved >30% TAG accumulation on a dry weight (DW) basis in tobacco leaves grown under greenhouse conditions (Appendix 1). This high level of oil accumulation, coupled with the high biomass potential of domesticated tobacco, presents
an interesting case for renewable lipid-based biofuel production. As such, previous findings in the greenhouse needed to be further studied outdoors in a commercial tobacco field production system.

In this study, the performance of two genetically transgenic tobacco lines were evaluated compared to a non-transgenic control in an outdoor commercial tobacco production system. We hypothesize that the CSIRO-developed transgenic tobacco lines will have higher leaf oil accumulation than the non-transgenic control. The specific objectives of this study are to (1) successfully propagate transgenic seedlings in the greenhouse and transplant seedlings to pots in the greenhouse or conventionally prepared field using an industry-standard burley tobacco production model, (2) quantify differences in phenotype between the experimental lines, (3) evaluate average leaf oil contents and FA profiles among the experimental lines, and (4) evaluate oil contents and FA profiles by leaf position within the experimental lines.

2.2 Methods

2.2.1 Experimental Design

The field experiment was conducted during the 2017 and 2018 growing seasons at the University of Kentucky North Agriculture Experiment Station at Spindletop Farm in Lexington, Kentucky, USA (38.1234°, -84.0506°). Studies were permitted under USDA-APHIS-BRS Permits # 17-087-101r and 18-0025-101r-a1, and institutional permits UKIBC Protocol # B17-2964 and B17-2964-v2. The experiment utilized a randomized complete block (RCBD) design with experimental line as the only treatment. A leaf position (top, middle, bottom stalk position) split treatment and sample time split-split
treatment was added to evaluate oil accumulation and FA profile by leaf position and sample time in a separate analysis.

### 2.2.2 Experimental Lines & DNA Construct Design

All experimental lines were developed and generously provided by CSIRO scientists. The non-transgenic control was *N. tabacum* cv. Wisconsin 38. The first transgenic high-oil line, referred to herein as “*WR1I:DGAT:OLE*” because it was developed with the *WR1I:DGAT:OLE* construct (Figure 3A) included an intron-interrupted *Sesamum indicum Oleosin* (OLE) gene (Dr. Nick Roberts, AgResearch, Palmerston North, New Zealand) with coding regions flanked by *NotI* sites inserted into a pORE04-based binary expression vector. The vector contained a double enhancer region 35S promoter expressing the *NPTII* kanamycin resistance gene. A DNA fragment containing the *A. thaliana* WRII gene was cloned as an *EcoRI* fragment into the binary expression vector. The *A. thaliana* DGAT1 gene was then inserted into the *AsiSI* site, generating pJP3502 (Vanhercke et al., 2014).

A second high-oil line that included the LEC2 transgene in the construct (Figure 3B), referred to herein as “*LEC2:WR1I:DGAT:OLE*,” was transformed into the previously-described *WR1I:DGAT:OLE* line (T3 generation). A synthesized 3.6 kb DNA fragment containing *A. thaliana* LEC2 flanked by the *A. thaliana Senescence Associated Gene-12* (SAG-12) senescence-specific promoter and the *Glycine max* lectin polyadenylation terminator was inserted between the *SacI* and *NotI* restriction sites of a pORE04-based binary expression vector resulting in pOIL049. The vector contained a hygromycin resistance selectable marker gene (Vanhercke et al., 2017).
2.2.3 Transplant Propagation

On June 7 (2017) and March 27 (2018), tobacco seeds were pneumatically sown into 288-cell Styrofoam trays [34.3cm x 67.31cm x 5.1cm (Speedling®)] filled with Carolina Choice™ peat-based medium (Carolina Soil Co.). Seeded trays were floated in ponds filled with tap water in the greenhouse. Greenhouse conditions were set for 27°C/20°C day/night temperatures with no artificial light. After 14 days, 0.5 kg 1000 L⁻¹ of Peter’s® soluble fertilizer (20-10-20) was added to the pond water and mixed thoroughly with a circulation pump. Two weeks after seeding, Terramaster™ 4EC (Ettridazole, 37.4 mg L⁻¹ AI) was added to the float pond water to prevent growth of waterborne fungi, and the emulsion was suspended using a circulatory pump for 4 hours. Fungicide application was repeated every two weeks. Orthene® (Acephate, 0.84 kg ha⁻¹) and Manzate (Mancozeb, 0.23 g L⁻¹ AI) were applied via foliar spray for insect and foliar fungal prevention, respectively, every week until transplant. When subjectively determined, seedlings were intermittently clipped in position using a suspended electric mower to remove excess foliage and even plant competition. Greenhouse management of tobacco followed protocols outlined in the Burley and Dark Tobacco Production Guide published by the University of Kentucky Cooperative Extension Service (2017).

2.2.4 Field Conditions and Preparation

Soil for both the 2017 and 2018 trials was a Huntington Silt Loam (0-4% slope) type conventionally prepared by moldboard plow tillage and subsequent disking. Fertility regimen included 224 kg ha⁻¹ of soil-incorporated granulated urea (46-0-0) and 280 kg ha⁻¹ sulfate of potash (0-0-50). The herbicides Spartan® Charge™ (Carfentrazone-
ethyl/Sulfentrazone, 0.46kg ha\(^{-1}\) AI) and Command® 3ME (1.12kg ha\(^{-1}\) AI) were applied via broadcast surface application two days prior to transplant.

2.2.5 Greenhouse Transplanting & Management (2017 Only)

Non-transgenic control and \(LEC2:WRJ1:DGAT:OLE\) transplants were transferred to five-gallon plastic pots filled with ProMix® peat-based potting media and placed in the greenhouse. Greenhouse conditions were the same used for transplant propagation. Pots were outfitted with an automatic drip irrigation system supplying a 75 ppm solution of Peter’s® soluble 20-20-20 fertilizer. Plants were irrigated with solution daily and rinsed with unfertilized tap water every sixth and seventh day to flush out accumulated salts until final harvest.

2.2.6 Field Transplanting & Management

On July 25 (2017) and June 4 (2018), seedlings were transplanted into prepared soil using a carousel-type tobacco setter (RJ Equipment) calibrated for 23,919 plants ha\(^{-1}\) (91.5cm x 45.75cm spacing). In-furrow drench water was supplemented with the insecticides Orthene® (Acephate, 0.84kg ha\(^{-1}\)), and Coragen® SC (Chlorantraniliprole, 87.8g ha\(^{-1}\) AI), and the fungicide Ridomil® Gold (Mefenoxam, 113.4g ha\(^{-1}\) AI). Plants were irrigated as needed and no additional fertilizer was used. Weeds were manually removed as needed. Once individual experimental line populations demonstrated >80% elongated bud (CORESTA Stage 55.5) plants were topped (apical bud removed by hand) at the first leaf below the “flag” (highest positioned elongated axillary bud). All plants were maintained free of axillary shoots (“suckers”) by use of chemical suckercides and by manual removal until harvest 21 days after topping. In 2018 a severe storm occurred on July 20, resulting
in slight plant lodging and moderate lacerations to lower leaves in all tested lines. Plants were re-positioned vertically and subjective damage was assessed as minimal.

2.2.7 Monitoring Samples (2017 Only)

Oil monitoring samples were collected from random plants within each plot in the field and greenhouse intermittently until harvest. Samples were taken from random leaves positioned within the top third, middle third, or bottom third of the plant. Leaf discs (14 mm diameter) were cut from the middle position of the leaf lamina, avoiding the midrib and pronounced veins. Harvested discs were immediately placed in 1.5 mL microcentrifuge tubes and frozen on dry ice. The collected samples were subsequently lyophilized, ground to a fine powder using liquid N\textsubscript{2} and plastic pestles, and chemically analyzed (see Chemical Analysis [2.2.9]). Monitor samples were harvested every 7 days until final harvest starting on September 11.

2.2.8 Harvesting

One week prior to the estimated harvest time plant phenotypes were documented. Harvest occurred 21 days after topping respective lines. Samples of leaves were taken from random plants within each plot (n=15) at each leaf position (top, middle, or bottom third), weighed, and placed in paper bags. All harvested samples were dried in forced-air dryers set to 150°C to reduce likelihood of enzymatic degradation of lipids. Dried leaf samples were ground using a Wiley Mill (1mm screen), thoroughly homogenized, subsampled, and chemically analyzed. Immediately after initial leaf removal, whole plants selected at random (n=15) from within each plot were cut at the base and weighed for ‘whole plant biomass’ fresh weight (FW). All stalks were then removed, and material re-weighed to calculate ‘biomass
without stalk FW’ and ‘stalk FW.’ Stalks were collected into paper refuse bags and dried. Whole plant biomass, biomass without stalk, and stalk dry weights (DWs) were calculated using DW values obtained after drying process. Major experimental dates for each respective study listed in Table 2.

2.2.9 Chemical Analysis

The following protocol is adapted from Li et al. (2006) with modifications from Zhang et al. (2009), and also from Dr. David Hildebrand and Ms. Huihua Ji (University of Kentucky). Glass tubes (1 cm × 10 cm) with Teflon-lined screw caps were pre-rinsed thoroughly with chloroform and dried at 103°C (≥12 h) to remove any contaminating lipid residues and water, and they were precisely weighed. Samples of ~10 mg of ground tobacco were added to each tube. Tri-17:0 was added in toluene to tobacco at 20 µg/mg. Two mL of freshly prepared 0.001% BHT and 2.5% (v/v) H₂SO₄ in CH₃OH was added to each tube, which were tightly capped. Samples were vortex mixed for 30 seconds, heated to 90°C, vortexed again after ~30 minutes, reheated to 90°C for an additional hour and then cooled to room temperature. Isooctane (IO) + 0.001% BHT (1 mL) was added to each tube and the samples were vortexed. Approximately 200 µL of the upper layers after separation were transferred to GC vials. Another milliliter of IO was added, the solution was mixed, and 1 mL 0.9% KCl (or NaCl) was added.

The GC parameters were as follows: Injection volume 1µL; helium (He) carrier flow 1mL min⁻¹; split ratio 50:1; injector temperature 260°C; detector temperature 260°C; gradient program = 150°C for 3 minutes, ramp up 10°C min⁻¹ to 240°C and hold for 5 minutes. Calibration curves were generated (Appendix 6), detection limits established (Appendix 7), and inter/intra-day repeatability measured (Appendix 8). Palmitic (16:0), stearic (18:0),
oleic (18:1 n9), linoleic (18:2 n6), α-linolenic (18:3 n3) acids were targeted for quantification. Peaks determined to be trans-vaccenic acid (18:1 n7) were ignored due to the difficulty of differentiation from oleic acid (18:1 n9) in GC chromatograms (Appendix 9) and its extremely low natural incidence in plants.

2.2.10 Data Analysis

Collected biomass data was analyzed as an RCBD using the GLIMMIX procedure of SAS 9.4 (SAS Institute, Cary, NC). The 2017 oil monitoring study was analyzed as an RCBD split-split plot using the univariate GLIMMIX procedure with all data log-transformed as needed after validation tests of homogeneity (Bartlett’s) and normality (Shapiro-Wilks). Final oil content by leaf position data was analyzed as an RCBD split-plot also using the univariate GLIMMIX procedure with data log-transformed as needed after validation tests of homogeneity and normality. All final average total oil data were analyzed as an RCBD using the GLIMMIX procedure with all data log-transformed as needed after validation tests of homogeneity and normality. All reported values are in the original units.

2.3 Results

2.3.1 Phenotyping

Greenhouse-grown plants of both the non-transgenic control and LEC2:WR1I:DGAT:OLE lines grown in 2017 were smaller in stature compared to the field-grown plants grown in the same year. There was no subjective difference in overall size between either line grown in the greenhouse. Greenhouse-grown LEC2:WR1I:DGAT:OLE transgenic plants had a noticeably horizontal leaf angle compared to an elevated leaf angle (∼45°) on the non-transgenic control plants (Figure 4A, B). Plants grown in the field in 2017 were relatively
small in stature due to delayed seeding and transplanting. The 2017 field non-transgenic control plants had robust, upright, wide leaves (also at ≈45° upward angle) compared to \textit{LEC2:WR1I:DGAT:OLE} plants which were smaller in stature and had petite horizontally-oriented leaves (Figure 4C, D). Field-grown plants of both lines were comparatively darker green in color than those grown in the greenhouse. In the field, plants of the non-transgenic control lines were significantly taller (Table 3). Plant heights were similar among the lines grown in the greenhouse. Leaf number was significantly higher for \textit{LEC2:WR1I:DGAT:OLE} lines in the field compared to the non-transgenic control and was indifferent in greenhouse plants. Non-transgenic control and \textit{LEC2:WR1I:DGAT:OLE} lines grown in the 2018 field trial were larger than those grown in 2017 due to favorable seeding and transplanting dates. Non-transgenic control plants had large, robust leaves positioned at a 45° angle and had a closed canopy by harvest (Figure 5). \textit{LEC2:WR1I:DGAT:OLE} plants were slightly smaller in stature with smaller, thicker leaves positioned horizontally. The \textit{LEC2:WR1I:DGAT:OLE} plants showed a characteristic downward cupping (“tipping”) of the peripheral leaf lamina which was reminiscent to the coined “cobra” morphology often used in calcium deficiency diagnoses. The \textit{WR1I:DGAT:OLE} plants were truncated, with little to no internodal spacing, small, petite leaves, and a high tendency for axillary bud formation. It should be noted that ‘moderate’ to ‘severe’ tobacco hornworm (\textit{Manduca sexta} L. [Figure 6]) pressure was observed in the \textit{LEC2:WR1I:DGAT:OLE} plots (data not quantified).

\textbf{2.3.2 Harvested Biomass}

The 2017 trial yielded relatively low amounts of biomass for both the non-transgenic control and \textit{LEC2:WR1I:DGAT:OLE} lines in the greenhouse and field. ANOVA of 2017
harvest weight data (Table 4) showed that the non-transgenic control had higher fresh and dry leaf biomass in both growing conditions, with the non-transgenic control line significantly out-yielding \textit{LEC2:WR1I:DGAT:OLE} in terms of dry leaf weight by 64.8% in the field and 28.6% in the greenhouse (unanalyzed) (Table 5). Partitioning of harvested plant dry biomass into a leaf-to-stalk ratio (LSR) from the field yielded an LSR of 2.4 for the non-transgenic control and 2.6 for \textit{LEC2:WR1I:DGAT:OLE} (data not shown). An LSR of 2.0 for the non-transgenic control and 2.0 for \textit{LEC2:WR1I:DGAT:OLE} was calculated for the greenhouse grown plants.

Biomass recovery was higher in 2018 due to timely seeding and planting dates. ANOVA of the 2018 harvest weight data (Table 6) showed that total plant FW was significantly higher in the non-transgenic control (22,457 lb./A) compared to the \textit{LEC2:WR1I:DGAT:OLE} and \textit{WR1I:DGAT:OLE} lines (14,044, and 12,471 lbs. Ac\textsuperscript{1}, respectively) (Table 7). After drying, calculated total plant DW was insignificant among the tested lines. With the stalks removed, total FW biomass was again highest in the non-transgenic control (15,399 lbs. Ac\textsuperscript{1}) compared to \textit{WR1I:DGAT:OLE} and \textit{LEC2:WR1I:DGAT:OLE} (8,510 and 7,114 lbs. Ac\textsuperscript{1}, respectively). Biomass without stalk DW was highest in the \textit{WR1I:DGAT:OLE} (4,243 lbs. Ac\textsuperscript{1}) and lowest in \textit{LEC2:WR1I:DGAT:OLE} (2,807 lbs. Ac\textsuperscript{1}), although the difference was not significant. The LSR was higher in the \textit{WR1I:DGAT:OLE} (7.8) compared to the non-transgenic control and LEC (4.2 and 3.6, respectively, data not shown). Overall total plant moisture content (\%MC) was highest in non-transgenic control at 77\%, followed by \textit{LEC2:WR1I:DGAT:OLE} at 71\% and \textit{WR1I:DGAT:OLE} at 65\%.

2.3.3 2017 Monitoring Sampling
2.3.3.1 Average Oil Content

ANOVA of the 2017 %TFA DW\(^{-1}\) monitor data (Table 8) showed significant interactions between variety and sampling time as well as variety and leaf position. \(LEC2:WR1I:DGAT:OLE\) plants had significantly higher total average leaf oil accumulation at every sampled time point in the field (Figure 7) with a seasonal average 316% increase over non-transgenic control plants and a 368% increase at final harvest. The average oil accumulation in the non-transgenic control line did not surpass 1.25% throughout the duration of the season. In the greenhouse, \(LEC2:WR1I:DGAT:OLE\) plant oil accumulation was higher than the non-transgenic control and followed a positive quadratic trend peaking at 6.4% (Figure 8). Oil accumulation did not change throughout the season in greenhouse-grown non-transgenic control plants, having an average of 0.4% TFA DW\(^{-1}\). \(LEC2:WR1I:DGAT:OLE\) plants showed an accumulation increase over the non-transgenic control of 1720% at the final sampling time point in the greenhouse.

2.3.3.2 2017 Fatty Acid Profile

A significant interaction between variety and sampling time was detected for most analyzed FAs except for oleic acid and linoleic acid. The FA profile of non-transgenic control plants grown in the field remained steady between samples 1 and 4 (Figure 9). Notable differences in the final 2017 profiles between non-transgenic control and \(LEC2:WR1I:DGAT:OLE\) plants included a 34% reduction in α-linolenic acid and a 22% increase in linoleic acid. There was a shift in the \(LEC2:WR1I:DGAT:OLE\) profile from sample 1 to 4 with a displacement of oleic acid (30% to 2%) with linoleic acid (29% to 41%) and palmitic acid (26% to 36%) (Figure 10).
Plants grown in the greenhouse did not have the same changes in their FA profiles as did field grown tobacco. Non-transgenic control plants had a decrease in palmitic acid (42% to 32%), stearic acid (15% to 9%), and oleic acid (9% to 0%), and increases in linoleic (20% to 21%) and α-linolenic acid (14% to 38%) over the course of sampling (Figure 1). LEC2:WR1I:DGAT:OLE plants had an alternate shift in FA profile with a decrease in palmitic (50% to 49%), oleic (30% to 2%) and stearic (9% to 8%) acids and a nearly 4-fold increase in both linoleic acid (9% to 34%) and α-linolenic acid (2% to 7%) over the course of sampling (Figure 12).

2.3.4 Total oil accumulation by leaf position

ANOVA of the 2017 total oil accumulation by leaf position in LEC2:WR1I:DGAT:OLE plants showed significant differences in oil accumulation between leaf positions in the field (Table 9). There were no significant differences in oil accumulation between leaf positions in the non-transgenic control line with all mean values significantly less than LEC2:WR1I:DGAT:OLE at all leaf positions. The oil content gradient from the top to the bottom leaves in the LEC2:WR1I:DGAT:OLE line ranged from 7.31-2.32 %TFA DW⁻¹ in field-grown tobacco (Table 10). Oil accumulation levels in the LEC2:WR1I:DGAT:OLE line were evenly distributed in greenhouse plants with mean oil accumulation of 5.18 %TFA DW⁻¹ and 0.38 %TFA DW⁻¹ in the non-transgenic control line.

Variety-by-leaf position oil content ANOVA for 2018 showed that LEC2:WR1I:DGAT:OLE plants had the highest average oil content with 15.4% TFA DW⁻¹ ranging from 19.3% TFA DW⁻¹ to 9.2% TFA DW⁻¹ from top to bottom leaf positions (Table 12). The WR1I:DGAT:OLE plants had an average TFA DW⁻¹ ranging from 5.6% in the top leaves to 3.1% in the bottom leaves. Non-transgenic control plants had the lowest
average oil content of 2% TFA DW\(^{-1}\) and leaf position content ranging from 2.1-1.7% TFA DW\(^{-1}\) from the top to bottom positions.

2.3.5 2018 Final harvested oil profiles

Analysis of the final oil FA profile showed a significant variety-by-position interaction for all targeted constituents. The non-transgenic control oil profile consisted of a large proportion of \(\alpha\)-linolenic acid (18:3 n-3, 60%) compared to other target constituents and a small linoleic acid content (18:2 n-6, 16%) (Figure 13). The \(WR1:DGAT:OLE\) plants showed a reduction in \(\alpha\)-linolenic acid (21%) that was seemingly displaced by linoleic acid (37%) (Figure 14) when compared to the non-transgenic control. The \(WR1:DGAT:OLE\) FA profile also showed an increase in palmitic (26%) (16:0) and oleic acids (12%) (18:1 n-9) with little change in the stearic acid (18:0) content. The \(LEC2:WR1:DGAT:OLE\) line showed a further reduction in \(\alpha\)-linolenic acid to 7%, displaced by linoleic acid (45%) and a higher oleic acid content (19%) compared to \(WR1:DGAT:OLE\) (Figure 15).

2.4 Discussion

The 2017 study was seeded and transplanted relatively late due to seed acquisition logistical problems (Table 2). As a result, seedlings were 46 days old the day of transplant on July 25. The standard seedling transplant age in common commercial production is >60 days. Transplanting younger seedlings has been shown to result in poor a plant phenotype and yields in commercially grown tobacco (Miner, 1978). Additionally, late transplanting dates have also been shown to decrease tobacco leaf yield and quality (Shicheng et al., 2016; Someswara Rao and Patel, 1978; Wilkinson, 2005). Overall, the effects of seedling
age and transplanting date may have contributed to the poor leaf yield and oil contents in the tobacco plants grown in the 2017 study.

The phenotypic response of *Nicotiana* species engineered for increased leaf oil accumulation can vary. In both studies, all genetically engineered lines had altered plant phenotypes (Figure 4). The generalized negative phenotypes and yields resulting from this base construct were not reported in developmental greenhouse studies. The non-transgenic control cultivar, untransformed Wisconsin 38, is not widely grown as a commercial variety due to poor yields and leaf quality. The original use of Wisconsin 38 for high-oil line development at CSIRO was because it is a good laboratory line and is particularly amenable to genetic transformation. The negative plant phenotype observed in the *WR1I:DGAT:OLE* line grown outdoors is not entirely understood. Environmental impacts including large fluctuations in air and soil temperatures, transplant acclimation, solar radiation, and nutrient or water availability could have been contributing factors. *LEC2:WR1I:DGAT:OLE* plants were rather small when grown in the field in 2017, presumably due to an extremely late planting date which has been shown to decrease yields and quality in other tobacco production systems, most recently discussed by Shicheng et al (2016). Despite the yield loss, *LEC2:WR1I:DGAT:OLE* plants remained viable in the field with no visible signs of stress such as leaf discoloration or necrosis. In the 2018 field, *LEC2:WR1I:DGAT:OLE* plants had a characteristically unique phenotype with lateral leaves and peripheral lamina cupping, and we recovered comparatively low DW yields. Excessive leaf cupping, or tipping, in tobacco can be a characteristic symptom of severe tobacco calcium deficiency (McMurtrey, 1932). However, excessive cupping was not observed in the field in 2017 or in other tobacco plants in adjacent plots, which may
indicate possible unintended adverse effects of genetic engineering using the \textit{LEC2:WR1I:DGAT:OLE} DNA construct, although this is just speculation at present.

Furthermore, the leaf-to-stalk dry biomass ratio remained similar among both the non-transgenic control and \textit{LEC2:WR1I:DGAT:OLE} lines at \(\approx2.2\) (data not shown). In 2018, the total biomass FW of both engineered lines was less than in the non-transgenic control (Table 7). With the stalks removed, the biomass-without-stalk FW correlated with total biomass FW, while biomass-without-stalk DW did not. The line with the highest biomass-without-stalk DW in the 2018 trial was \textit{WR1I:DGAT:OLE}. However, this data can be misleading because this value includes all biomass stripped from the stalk which can include suckers, and suckers were noted to be very prevalent on \textit{WR1I:DGAT:OLE} plants. \textit{LEC2:WR1I:DGAT:OLE} plants did yield the lowest biomass-without-stalk DW in 2018, but the amount was higher than that generated in 2017.

The average leaf oil accumulation was higher in both experimental lines compared to the non-transgenic control in both studies (Table 12). The effect was reduced in 2017 compared to 2018, presumably due to the late planting date. \textit{WR1I:DGAT:OLE} plants grown in 2018 and \textit{LEC2:WR1I:DGAT:OLE} plants grown in 2017 and 2018 all had lower total oil content compared to greenhouse data reported by the CSIRO scientists who developed the technology (Appendix 1, Vanhercke et al. (2017)). Non-transgenic control and \textit{LEC2:WR1I:DGAT:OLE} plants grown in the greenhouse in 2017 had marginal oil accumulation compared to developer greenhouse reports, suggesting that the inferred differences in the growing environments greatly impacts line performance.

All tested lines in this study demonstrated an increasing leaf oil accumulation gradient from lower leaves to upper leaves (Table 12). The most pronounced of these was in
LEC2:WR1I:DGAT:OLE, and ranged from 9.2-19.3% oil DW$^{-1}$ in 2018 from lower to upper leaves. This phenomenon may be due to the decrease in leaf age from lower to upper leaves, with older leaves experiencing a higher rate of oil turnover. This trend could ultimately benefit project goals for sustainable biofuel production if deployed in a multiple harvest system such as tobacco that can regenerate new, young vegetative tissues after removal of the apical bud. Multiple harvest strategies have been successful in tobacco for biomass recovery in high-input systems (unpublished). Intermittent harvesting of younger foliage from regrowth of tobacco plants engineered for high leaf oil could help increase oil recovery over the growing season.

The leaf oil compositions for N. tabacum varieties are not well characterized in the literature. However, in this study, the Wisconsin-38 non-transgenic control had a background $\alpha$-linolenic acid content of approximately 50% when grown in the field. The structure of $\alpha$-linolenic acid (18:3 n3) is unique among the targeted quantified lipids in that it has three unsaturated bonds. $\alpha$-linolenic acid content was drastically reduced in both the WR1I:DGAT:OLE and LEC2:WR1I:DGAT:OLE lines, and appeared to be replaced by linoleic acid (18:2 n6). This could be caused by increased transport of 18:2 FAs away from the modification pathway or possible regulatory suppression of FAD7-like desaturase activity caused by the WR1I:DGAT:OLE or LEC2:WR1I:DGAT:OLE constructs (including the WR1I and LEC2 transcription factors) resulting in a reduction in the third desaturation event (Kusumi and Iba, 1998) and pooling of linoleic acid.

The reason behind the observed tobacco hornworm infestation in field-grown LEC2:WR1I:DGAT:OLE lines, as opposed to the other experimental lines, is not clear. There are few, if any, published studies describing defined dietary preferences of the
tobacco hornworm. However, because all lines are from the same genetic background (Wisconsin 38), it is suspected that the preference towards the \textit{LEC2:WRII:DGAT:OLE} line may be due to the sizeable reduction in 18:3 fatty acid content due to the addition of the \textit{LEC2} transgene. This would have to be validated by further entomologic study.

2.5 Conclusion

The measure of success of this study is limited. Genetically engineered lines which perform well in controlled conditions do not always show the same performance in outdoor systems. Here, significantly increased oil content was indeed observed for both the \textit{WR1I:DGAT:OLE} and \textit{LEC2:WR1I:DGAT:OLE} lines when compared to the non-transgenic control, but at a cost of poor phenotype and biomass yield. The engineered transgenic line with the highest leaf oil accumulation, \textit{LEC2:WR1I:DGAT:OLE}, could serve as a favorable construct configuration for future deployment in ‘elite’ tobacco varieties if the underlying negative phenotypes can be resolved. Observed oil profiles, largely octadecanoic acids (18:n), should serve as a favorable input for lipid-based biofuel production. Additionally, the measured oil accumulation gradient could be harnessed for a multiple harvest production system once a high-biomass/high-leaf oil line is developed. The results generated from these field trials will hopefully be used for advancement towards an economical and sustainable means of biofuel production.

2.6 Future Directions

This research requires further validation with additional field trials. Optimization of the transgenic tobacco transplanting date, fertility, plant spacing, and other agronomic practices will be necessary to accommodate the scalability required to fulfill potential
biofuel demands. The use of a multiple-harvest system is also proposed, as many tobacco varieties have the ability to regenerate shoots (ratoon) after intermittent harvests. Additionally, the transgenic constructs used in this study should also be evaluated in other tobacco cultivars, as opposed to the non-commercial variety Wisconsin-38, in an effort to improve harvestable DW leaf yields. The selection of appropriate cultivars to be used in a proposed multi-harvest system should also consider disease resistance, because tobacco plants grown at high population densities are prone to increased incidences of pathogen pressure. Another proposed option would be the use of other Nicotiana species and hybrids which may serve as improved platforms for multiple-harvest biomass production.

Materials in the leaf oil extraction by-products could also serve as a potential source of value-added products. Examples include cellulosic by-product materials which could be used for secondary biofuel production or could be reduced to activated carbon for other commercial uses. The effects of metabolic engineering of tobacco for high leaf oil accumulation on total plant/leaf alkaloids has not yet been evaluated. Value-added products include tobacco-specific alkaloids, sugar-esters, and other endogenous constituents that do have value in both the pharmaceutical and alternative-nicotine product markets.
### Tables and Figures

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**Table 1.** Genes used, and their sources, in recent reports describing the metabolic engineering of vegetative biomass for lipid-based biofuel production.
**Figure 1.** Triacylglycerol (TAG) chemical structure. Three fatty acids (FAs) are esterified to a glycerol backbone. The example shown is glycerol tristearate (stearin) a TAG that consists of three stearic acid (18:0) molecules. Graphic by J. Patrick Perry.
Figure 2. Abbreviated plant lipid/TAG Biosynthesis metabolic pathway. Gene abbreviations shown: acyl-CoA carboxylase (ACCase), acyl carrier proteins (ACPs), acyl-CoA:glycerol-3-phosphate acyltransferase (GPAT), acyl-CoA:lysophosphatidic acid acyltransferase (LPAAT), phosphatidic acid phosphatase (PAP), diacylglycerol acyltransferase (DGAT), and transcription factors wrinkled-1 (WRI1) and leafy cotyledon-2 (LEC2). Graphic by J. Patrick Perry.
Figure 3. Engineered DNA constructs generated by CSIRO scientists for high leaf oil production in tobacco. (A) Construct in the jP3502 binary expression vector used to produce the “WR11:DGAT:OLE” transgenic line. Construct elements: LB=left border; RB=right border; WRII=Wrinkled 1 (gene); Lectin= (gene, marker) (gene, marker) *Glycine max* Lectin terminator; OLE=Oleosin (gene); DGAT1=Diacylglycerol acyltransferase (gene); NPTII=Neomycin phosphotransferase II (gene, marker). (B) LEC2 construct that was transformed into a T3 generation “WR11:DGAT:OLE” transgenic plant using the pORE049-based binary expression vector, resulting in pOIL049. Construct elements: SAG12=Senescence activated gene-12 (promoter); LEC2=Leafy cotelydon-2 (gene); HPT=hygromycin B phosphotransferase (gene, marker).

Graphic by J. Patrick Perry. Adapted from Vanhercke et al. (2017).
### 2017 Study Major Experiment Events

<table>
<thead>
<tr>
<th>Event</th>
<th>Date</th>
<th>Days After Seeding (DAS)</th>
<th>Days After Transplant (DAT)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Seeding</td>
<td>9-Jun</td>
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<td>-</td>
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<tr>
<td>Transplant</td>
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<td>-</td>
</tr>
<tr>
<td>Topping</td>
<td>8-Sep</td>
<td>45</td>
<td>45</td>
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<tr>
<td>Monitor Sample 1</td>
<td>15-Sep</td>
<td>98</td>
<td>52</td>
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<tr>
<td>Monitor Sample 2</td>
<td>22-Sep</td>
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<td>Monitor Sample 3</td>
<td>29-Sep</td>
<td>112</td>
<td>66</td>
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<tr>
<td>Monitor Sample 4</td>
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<tr>
<td>Harvest</td>
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<td>119</td>
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### 2018 Study Major Experiment Events

<table>
<thead>
<tr>
<th>Event</th>
<th>Date</th>
<th>Days After Seeding (DAS)</th>
<th>Days After Transplant (DAT)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Seeding</td>
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<td>-</td>
<td>-</td>
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<tr>
<td>Transplant</td>
<td>4-Jun</td>
<td>69</td>
<td>-</td>
</tr>
<tr>
<td>Topping WT</td>
<td>16-Jul</td>
<td>111</td>
<td>42</td>
</tr>
<tr>
<td>Topping LEC2</td>
<td>25-Jul</td>
<td>120</td>
<td>51</td>
</tr>
<tr>
<td>Topping Parental</td>
<td>1-Aug</td>
<td>127</td>
<td>58</td>
</tr>
<tr>
<td>Harvest WT</td>
<td>6-Aug</td>
<td>132</td>
<td>63</td>
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<tr>
<td>Harvest LEC2</td>
<td>15-Aug</td>
<td>141</td>
<td>72</td>
</tr>
<tr>
<td>Harvest Parental</td>
<td>22-Aug</td>
<td>148</td>
<td>79</td>
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</table>

*Table 2. Major experiment event dates for the (A) 2017 and (B) 2018 field studies.*
Figure 4. 2017 tobacco plant phenotypes 7 days before harvest. (A) Greenhouse-grown non-transgenic control (Wisconsin 38), (B) greenhouse-grown LEC2, (C) field-grown non-transgenic control, and (D) field-grown LEC2.
2017 Plant Height Data

<table>
<thead>
<tr>
<th>Grow Condition</th>
<th>Line</th>
<th>Height (cm)</th>
<th>LSD</th>
<th>Leaf Number</th>
<th>LSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Greenhouse</td>
<td>Control</td>
<td>68.9</td>
<td>-</td>
<td>17.8</td>
<td>-</td>
</tr>
<tr>
<td>Greenhouse</td>
<td>LEC2:WRI1:DGAT:OLE</td>
<td>61.1</td>
<td>-</td>
<td>18.6</td>
<td>-</td>
</tr>
<tr>
<td>Field</td>
<td>Control</td>
<td>70.1</td>
<td>a</td>
<td>13.0</td>
<td>b</td>
</tr>
<tr>
<td>Field</td>
<td>LEC2:WRI1:DGAT:OLE</td>
<td>61.0</td>
<td>b</td>
<td>19.6</td>
<td>a</td>
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</tbody>
</table>

Table 3. Plant height and leaf number data from the 2017 experiment. Greenhouse data were not analyzed. The field data was analyzed using PROC GLIMMIX (SAS 9.4) (n=15) with means of field data separated by a Fisher’s protected LSD (α=0.05).
Figure 5. 2018 plant phenotypes in the field 12 days before harvest. (A) Non-transgenic control (B) *LEC2:WR11:DGAT:OLE*, and (C) *WR11:DGAT:OLE*. 
Figure 6. Larva of the tobacco hornworm (*Manduca sexta*). Photograph credit: James Castner, University of Florida
### 2017 Harvest Weight Model Statistics

<table>
<thead>
<tr>
<th>Effect</th>
<th>Total Plant DW (lb./A)</th>
<th>Stalk DW (lb./A)</th>
<th>Biomass w/o Stalk FW (lb./A)</th>
<th>Biomass w/o Stalk DW (lb./A)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Variety</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
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</tbody>
</table>

**Table 4.** Model statistics of harvest weight data from the 2017 study. Data were analyzed as a RCBD using the univariate GLIMMIX procedure in SAS 9.4 (SAS Institute, Cary, NC) (n=20) (α=0.05).
<table>
<thead>
<tr>
<th>Grow Condition</th>
<th>Line</th>
<th>Total Plant DW (lb./A)</th>
<th>LSD</th>
<th>Stalk DW (kg/ha)</th>
<th>LSD</th>
<th>Biomass w/o Stalk FW (kg/ha)</th>
<th>LSD</th>
<th>Biomass w/o Stalk DW (kg/ha)</th>
<th>LSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Greenhouse</td>
<td>Control</td>
<td>730</td>
<td>NA</td>
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<td>NA</td>
<td>2283</td>
<td>NA</td>
<td>486</td>
<td>NA</td>
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<tr>
<td>Greenhouse</td>
<td>LEC2:WRI1:DGAT:OLE</td>
<td>840</td>
<td>NA</td>
<td>280</td>
<td>NA</td>
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<td>NA</td>
<td>350</td>
<td>NA</td>
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<tr>
<td>Field</td>
<td>Control</td>
<td>2250</td>
<td>a</td>
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<td>a</td>
<td>13551</td>
<td>a</td>
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<td>a</td>
</tr>
<tr>
<td>Field</td>
<td>LEC2:WRI1:DGAT:OLE</td>
<td>780</td>
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<td>b</td>
<td>4315</td>
<td>b</td>
<td>564</td>
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</table>

**Table 5.** Harvest weight data for the 2017 study. Greenhouse data was not analyzed. Field data means separated by Fisher’s protected LSD ($\alpha=0.05$). NA=Not Analyzed.
## 2018 Harvest Weight Model Statistics

<table>
<thead>
<tr>
<th>Effect</th>
<th>Total Plant FW (lb./A)</th>
<th>Total Plant DW (lb./A)</th>
<th>Stalk FW (lb./A)</th>
<th>Stalk DW (lb./A)</th>
<th>Biomass w/o Stalk FW (lb./A)</th>
<th>Biomass w/o Stalk DW (lb./A)</th>
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</thead>
<tbody>
<tr>
<td>Variety</td>
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<td>NS</td>
<td>NS</td>
<td>0.0177</td>
<td>0.0095</td>
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</table>

Table 6. Model statistics of harvest weight data from 2018 the study. Data were analyzed as a RCBD using the univariate GLIMMIX procedure of SAS 9.4 (SAS Institute, Cary, NC) (n=30) (α=0.05). NS=Not Significant.
### 2018 Harvest Weight Data

<table>
<thead>
<tr>
<th>Grow Condition</th>
<th>Line</th>
<th>Total Plant FW (kg/ha)</th>
<th>Total Plant DW (kg/ha)</th>
<th>Stalk FW (kg/ha)</th>
<th>Stalk DW (kg/ha)</th>
<th>Biomass w/o Stalk FW (kg/ha)</th>
<th>Biomass w/o Stalk DW (kg/ha)</th>
<th>LSD</th>
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<tbody>
<tr>
<td>Field</td>
<td>Control</td>
<td>25171</td>
<td>a</td>
<td>5566</td>
<td>-</td>
<td>7911</td>
<td>1067</td>
<td>4449</td>
</tr>
<tr>
<td>Field</td>
<td>LEC2:WRI1:DGAT:OLE</td>
<td>13978</td>
<td>b</td>
<td>4091</td>
<td>-</td>
<td>6003</td>
<td>859</td>
<td>3146</td>
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<tr>
<td>Field</td>
<td>WRI1:DGAT:OLE</td>
<td>15741</td>
<td>b</td>
<td>5427</td>
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<td>6203</td>
<td>615</td>
<td>4756</td>
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</table>

**Table 7.** Harvest weight data for the 2018 study. Means separated by Fisher’s protected LSD (α=0.05).
### 2017 Field Oil Monitor Model Statistics

<table>
<thead>
<tr>
<th>Effect</th>
<th>Total Fatty Acid (TFA) (%DW)</th>
<th>Palmitic Acid (16:0)</th>
<th>Stearic Acid (18:0)</th>
<th>Oleic Acid (18:1 n9)</th>
<th>Linoleic Acid (18:2 n6)</th>
<th>α-Linolenic Acid (18:3 n3)</th>
</tr>
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<tbody>
<tr>
<td>Variety</td>
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<tr>
<td>Time</td>
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<td>NS</td>
<td>NS</td>
<td>&lt;.0001</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Variety*Time</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>&lt;.0001</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Position*Time</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>&lt;.0001</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Variety<em>Position</em>Time</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>&lt;.0001</td>
<td>NS</td>
<td>NS</td>
</tr>
</tbody>
</table>

Table 8. ANOVA for the 2017 trial oil monitoring data (Field Only). Analyzed as an RCBD split-split-plot with variety as the main treatment, leaf position as the split treatment, and sample time as the split-split treatment using the univariate PROC GLIMMIX procedure in SAS 9.4 (n=30).
Figure 7. Average %TFA DW\(^{-1}\) of 2017 field grown plants at each sample point. Data was analyzed using PROC GLIMMIX in SAS 9.4. Sample time effect was not significant; no supported regressions were detected.
Figure 8. Average %TFA DW$^{-1}$ of 2017 greenhouse-grown plants at each sample point. Data were not statistically analyzed.
### 2017 Variety by Leaf Position Model Statistics

<table>
<thead>
<tr>
<th>Effect</th>
<th>Total Fatty Acid (TFA) (%DW)</th>
<th>Palmitic Acid (16:0)</th>
<th>Stearic Acid (18:0)</th>
<th>Oleic Acid (18:1 n9)</th>
<th>Linoleic Acid (18:2 n6)</th>
<th>α-Linolenic Acid (18:3 n3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Variety</td>
<td>0.0039 NS</td>
<td>NS</td>
<td>NS</td>
<td>0.0045</td>
<td>0.0011</td>
<td>0.0010</td>
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<tr>
<td>Position</td>
<td>0.0007 &lt;.0001</td>
<td>&lt;.0001</td>
<td>NS</td>
<td>NS</td>
<td>0.0356</td>
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<tr>
<td>Variety*Position</td>
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<td>0.0004</td>
<td>NS</td>
<td>NS</td>
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</table>

**Table 9.** ANOVA for the 2017 leaf oil data (Field Only). Analyzed as an RCBD split-plot with variety as the main treatment and leaf position as the split treatment using the univariate PROC GLIMMIX procedure in SAS 9.4 (n=30).
### 2017 Variety by Position Values and Mean Separations

<table>
<thead>
<tr>
<th>Variety</th>
<th>Position</th>
<th>Total Fatty</th>
<th>Palmitic LSD</th>
<th>Stearic LSD</th>
<th>Oleic LSD</th>
<th>Linoleic LSD</th>
<th>α- LS</th>
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</thead>
<tbody>
<tr>
<td>WT</td>
<td>Top</td>
<td>1.12 D</td>
<td>3.19 B</td>
<td>1.56 BC</td>
<td>0.41 -</td>
<td>2.86 D</td>
<td>3.82 -</td>
</tr>
<tr>
<td>WT</td>
<td>Middle</td>
<td>0.91 D</td>
<td>3.19 B</td>
<td>1.46 C</td>
<td>0.27 -</td>
<td>2.85 D</td>
<td>3.89 -</td>
</tr>
<tr>
<td>WT</td>
<td>Bottom</td>
<td>0.64 D</td>
<td>3.38 A</td>
<td>1.82 A</td>
<td>0.16 -</td>
<td>2.75 D</td>
<td>3.83 -</td>
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<td>3.04 C</td>
<td>1.58 BC</td>
<td>1.23 -</td>
<td>3.17 C</td>
<td>2.04 -</td>
</tr>
<tr>
<td>LEC2:WRI1:DGAT:OLE</td>
<td>Middle</td>
<td>3.38 B</td>
<td>3.34 A</td>
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<td>1.47 -</td>
<td>3.43 B</td>
<td>2.60 -</td>
</tr>
<tr>
<td>LEC2:WRI1:DGAT:OLE</td>
<td>Bottom</td>
<td>2.36 C</td>
<td>3.40 A</td>
<td>1.78 A</td>
<td>1.35 -</td>
<td>3.61 A</td>
<td>2.25 -</td>
</tr>
</tbody>
</table>

Table 10. 2017 oil content values by variety and leaf position. Means separated by a Fisher’s protected LSD (α=0.05).
### Table 11. ANOVA for the 2018 leaf oil content by variety and leaf position. Analyzed as an RCBD split-plot with variety as the main treatment and leaf position as the split treatment using the univariate PROC GLIMMIX procedure in SAS 9.4 (n=30)

<table>
<thead>
<tr>
<th>Effect</th>
<th>Total Fatty Acid (TFA) (%DW)</th>
<th>Palmitic Acid (16:0)</th>
<th>Stearic Acid (18:0)</th>
<th>Oleic Acid (18:1 n9)</th>
<th>Linoleic Acid (18:2 n6)</th>
<th>α-Linolenic Acid (18:3 n3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Variety</td>
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<td>&lt;.0001</td>
<td>&lt;.0001</td>
<td>&lt;.0001</td>
<td>&lt;.0001</td>
<td>NS</td>
</tr>
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<td>&lt;.0001</td>
<td>&lt;.0001</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>Variety*Position</td>
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<td>&lt;.0001</td>
<td>0.0005</td>
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</table>
### 2018 Variety by Position Values and Mean Separations

<table>
<thead>
<tr>
<th>Variety</th>
<th>Position</th>
<th>Total Fatty Acid (TFA) (%DW)</th>
<th>Palmitic Acid (16:0) LSD</th>
<th>Stearic Acid (18:0) LSD</th>
<th>Oleic Acid (18:1 n9) LSD</th>
<th>Linoleic Acid (18:2 n6) LSD</th>
<th>α-Linolenic Acid (18:3 n3) LSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>Top</td>
<td>2.13</td>
<td>E</td>
<td>0.38</td>
<td>E</td>
<td>0.06</td>
<td>E</td>
</tr>
<tr>
<td>WT</td>
<td>Middle</td>
<td>2.14</td>
<td>E</td>
<td>0.37</td>
<td>E</td>
<td>0.06</td>
<td>E</td>
</tr>
<tr>
<td>WT</td>
<td>Bottom</td>
<td>1.72</td>
<td>E</td>
<td>0.31</td>
<td>E</td>
<td>0.06</td>
<td>E</td>
</tr>
<tr>
<td>WRI1:DGAT:OLE</td>
<td>Top</td>
<td>5.59</td>
<td>D</td>
<td>1.46</td>
<td>C</td>
<td>0.24</td>
<td>C</td>
</tr>
<tr>
<td>WRI1:DGAT:OLE</td>
<td>Middle</td>
<td>5.35</td>
<td>D</td>
<td>1.39</td>
<td>C</td>
<td>0.23</td>
<td>C</td>
</tr>
<tr>
<td>WRI1:DGAT:OLE</td>
<td>Bottom</td>
<td>3.08</td>
<td>E</td>
<td>0.81</td>
<td>D</td>
<td>0.14</td>
<td>D</td>
</tr>
<tr>
<td>LEC2:WRI1:DGAT:OLE</td>
<td>Top</td>
<td>19.29</td>
<td>A</td>
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<td>A</td>
<td>0.54</td>
<td>A</td>
</tr>
<tr>
<td>LEC2:WRI1:DGAT:OLE</td>
<td>Middle</td>
<td>17.82</td>
<td>B</td>
<td>4.41</td>
<td>A</td>
<td>0.52</td>
<td>A</td>
</tr>
<tr>
<td>LEC2:WRI1:DGAT:OLE</td>
<td>Bottom</td>
<td>9.22</td>
<td>C</td>
<td>2.98</td>
<td>B</td>
<td>0.41</td>
<td>B</td>
</tr>
</tbody>
</table>

Table 12. Oil content data from 2018 study by variety and leaf position. Means separated by a Fisher’s protected LSD ($\alpha=0.05$)
Figure 9. Changes in leaf oil FA profiles in field-grown non-transgenic control plants during the 2017 monitoring period. (A) Sample time 1 (Sept. 15) and (B) sample time 4 (Oct. 6).
Figure 10. Changes in leaf oil FA profiles in field grown *LEC2*:*WRI1*:*DGAT*:*OLE* plants during the 2017 monitoring period. (A) Sample time 1 (Sept. 15) and (B) sample time 4 (Oct. 6).
Figure 11. Changes in leaf oil FA profile in greenhouse-grown non-transgenic control plants during the 2017 monitoring period. (A) Sample time 1 (Sept. 15) and (B) sample time 4 (Oct. 6).
Figure 12. Changes in leaf oil FA profile in greenhouse-grown *LEC2:WRI1:DGAT:OLE* plants during the 2017 monitoring period. (A) Sample time 1 (Sept. 15) and (B) sample time 4 (Oct. 6).
Figure 13. Average leaf oil FA profile in field-grown non-transgenic control plants from the 2018 study.
Figure 14. Average leaf oil FA profile in field-grown WR11:DGAT:OLE plants from the 2018 field study.
Figure 15. Final average leaf oil FA profile in field-grown *LEC2:WRI1:DGAT:OLE* plants from the 2018 study.
Appendix 1. Triacylglycerol (TAG) content on a dry weight (DW) basis in senescing leaves of wildtype and transgenic (T1) *N. tabacum* plants sampled at seed setting. WT, wildtype *N. tabacum*; Parent, high oil *N. tabacum* line expressing 3 transgenes involved in lipid biosynthesis (WRI1, DGAT1, OLEOSIN); SDP1, silencing of the SDP1 TAG lipase in the high oil background; LEC2, overexpression of the *A. thaliana* LEC2 gene in the high oil background. Error bars represent standard deviations of triplicate analyses on three individual plants for each genotype. Adapted from Vanhercke et al. (2017).
Appendix 2. World production of biofuels (thousands of barrels per day) for ethanol. Adapted from Moschini et al. (2012).
Appendix 3. World production of biofuels (thousands of barrels per day) for biodiesel. Adapted from Moschini et al. (2012).
Appendix 4. Average retail fuel prices in the United States for gasoline and E85 ethanol from April 2000 – October 2018. Figure by U.S. Department of Energy.
Appendix 5. Average retail fuel prices in the United States for diesel and B99 biodiesel from April 2005 – October 2018. Figure by U.S. Department of Energy.
<table>
<thead>
<tr>
<th></th>
<th>Regression equation</th>
<th>Correlation coefficient</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oleic acid (18:1)</td>
<td>$y=1.2250x-0.0136$</td>
<td>0.9967</td>
</tr>
<tr>
<td>Vaccenic acid (18:1)</td>
<td>$y=1.2998x-0.0108$</td>
<td>0.9996</td>
</tr>
<tr>
<td>Linoleic Acid (18:2)</td>
<td>$y=1.2240x-0.0119$</td>
<td>0.9988</td>
</tr>
<tr>
<td>α-Linolenic Acid (18:3)</td>
<td>$y=1.1643x-0.0161$</td>
<td>0.9991</td>
</tr>
<tr>
<td>Palmitic Acid (16:0)</td>
<td>$y=1.1605x-0.0124$</td>
<td>0.9986</td>
</tr>
<tr>
<td>Stearic Acid (18:0)</td>
<td>$y=1.2386x-0.0042$</td>
<td>0.9988</td>
</tr>
</tbody>
</table>

**Appendix 6.** Calibration equations and correlation coefficients for 2018 oil analysis.
### Established detection limits for each analyzed FA.

<table>
<thead>
<tr>
<th>Fatty Acid</th>
<th>Calibration Curve Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oleic acid (18:1)</td>
<td>10 – 200 ug/ml</td>
</tr>
<tr>
<td>Vaccenic acid (18:1)</td>
<td>10 – 150 ug/ml</td>
</tr>
<tr>
<td>Linoleic Acid (18:2)</td>
<td>20 – 400 ug/ml</td>
</tr>
<tr>
<td>α-Linolenic Acid (18:3)</td>
<td>20 – 400 ug/ml</td>
</tr>
<tr>
<td>Palmitic Acid (16:0)</td>
<td>20 – 400 ug/ml</td>
</tr>
<tr>
<td>Stearic Acid (18:0)</td>
<td>10 – 200 ug/ml</td>
</tr>
</tbody>
</table>

**Appendix 7.** Established detection limits for each analyzed FA.
### Appendix 8

Tested intra and inter-day repeatability using same GC-FID.

<table>
<thead>
<tr>
<th></th>
<th>Inter days (n = 9)</th>
<th></th>
<th>Intra Day (n = 3)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>tobacco sample</td>
<td>%CV</td>
<td>Tobacco sample</td>
<td>%CV</td>
</tr>
<tr>
<td></td>
<td>average (µg mL⁻¹)</td>
<td></td>
<td>average (µg mL⁻¹)</td>
<td></td>
</tr>
<tr>
<td>Oleic acid (18:1)</td>
<td>130.6</td>
<td>1.9</td>
<td>132.6</td>
<td>3.2</td>
</tr>
<tr>
<td>Linoleic Acid (18:2)</td>
<td>322.9</td>
<td>1.5</td>
<td>327.2</td>
<td>1.9</td>
</tr>
<tr>
<td>α-Linolenic Acid (18:3)</td>
<td>106.9</td>
<td>1.3</td>
<td>107.5</td>
<td>2.6</td>
</tr>
<tr>
<td>Palmitic Acid (16:0)</td>
<td>257.4</td>
<td>2.9</td>
<td>263.0</td>
<td>1.6</td>
</tr>
<tr>
<td>Stearic Acid (18:0)</td>
<td>42.1</td>
<td>1.8</td>
<td>42.7</td>
<td>3.0</td>
</tr>
</tbody>
</table>
Appendix 9. Example chromatograms of A. non-transgenic control (low oil) or B. LEC2 (high oil) leaf oil.
References


66


VITA

Name: James Patrick Perry

Education
B.Sc., Agriculture Biotechnology, University of Kentucky, Completed December 2014

Positions Held

Research Coordinator
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May 2016 – December 2016

Graduate Research Assistant
University of Kentucky, College of Agriculture, Kentucky Tobacco R&D Center
January 2015 – May 2016

Student Research Assistant
University of Kentucky, College of Agriculture, Kentucky Tobacco R&D Center
May 2012 – January 2015

Northern Business Unit Intern
Helena Chemical Company
May 2014 – August 2014

Scholastic and Professional Honors
Seath Man of the Year Award, FarmHouse Fraternity May 2014
EPOSCoR Research Assistantship Recipient, January 2015
Helena NBU Intern of the Year (3rd Place), August 2014
Vice President, FarmHouse Fraternity 2012-2013
Kentucky Farm Bureau Scholarship Recipient, August 2010

Presentations
Donald Sparks IPSS Graduate Symposium, April 2019
Title: Field Evaluation of Tobacco Grown for High Leaf oil Accumulation
Shelby County Middle School Field Trip, November 2018
Title: Kentucky Tobacco Research and Development Center: Beyond Tobacco

Kentucky Burley Co-Op, November 2018
Title: Exploration of Production Practices for the Medicinal Plant Artemisia annua L.

2018 CORESTA Congress (Kunming, China), October 2018
Title: Field Evaluation of Tobacco Engineered for High Leaf Oil Accumulation

Industry Presentation, Chinese Pharmaceutical Company, Luizhou China, October 2018
Title: Development of Scalable Production Practices for Artemisia annua L.

Kentucky Joint Legislative Agriculture Committee, September 2018
Title: Exploration of Production Practices for the Medicinal Plant Artemisia annua L.

Kentucky Tobacco Research & Development Center Board Meeting, September 2018
Title: Exploration of Production Practices for the Medicinal Plant Artemisia annua L.

Artemiflow & Kentucky Economic Development Board Visit, August 2018
Title: Production of the Antimalarial Drug Artemisinin from the Medicinal Plant Artemisia annua L. (Annual Wormwood)

University Tobacco Field Day, August 2018
Title: Low Conversion Screening of LA Burley and Flue-cured Tobacco Varieties

Kentucky Hemp Industries Association Meeting, April 2018
Title: University of Kentucky Industrial Hemp Agronomic Research Updates

University of Kentucky Hemp Field Day, August 2017
Title: Industrial Hemp Grain Variety Trial

University of Kentucky Field Day, August 2017
Title: Optimization of Harvest Practices for Nicotine Production for Alternative Tobacco Products

University of Kentucky Hemp Field Day, August 2016
Title: Industrial Hemp Variety Trial Updates

**KTRDC Annual Reports**

Harvest Determination by intermittent Testing of Leaf Tissue in Field Grown Artemisia annua. 2018

Preliminary Research: Field Trial of Genetically Engineered Tobacco Lines Capable of High Oil Accumulation for Biofuel Applications. 2018

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Differences in Germination Evaluation Methods for Industrial Hemp Varieties: A Mini-Experiment. 2018

Evaluation of Seeding Method Effects on Fiber Yield in Industrial Hemp. 2017
Effects of Seed Size and Seed Coating on Germination, Emergence, Establishment, and Seedling Vigor in Industrial Hemp. 2017

Evaluation of Heliae Bio stimulant Products. 2017

Varieties and Production Practices to Maximize nicotine to be used in emerging Tobacco Products. 2016

Effects of Seeding Rate on Harvestable Components of Industrial Hemp Fiber Varieties. 2017

Determination of Optimal Industrial Hemp Varieties for Kentucky Farmers. 2017

Preliminary Research: Field Trial of Genetically Engineered Tobacco Lines Capable of High Leaf Oil accumulation. 2017

Evaluation of Proprietary Biostimulant Ag-1000 on Stalk and Grain Yield on Industrial Hemp 2017.

Dual-purpose Industrial Hemp Nitrogen Trial. 2016


Evaluation of the Use of Chemical Desiccants to Improve Mechanical Hemp Grain Harvesting. 2016

Evaluation of Seven Hemp Varieties of Hemp for Grain Production. 2016