ABSTRACT

SMITH, WILLIAM A. Utilizing Genome Editing Technologies to Develop Ultra-low Nicotine Tobacco Lines. (Under the direction of Dr. Ralph Dewey).

The alkaloid nicotine is the most distinguishing feature of tobacco plants and comprises approximately 90% of total alkaloids found in most tobacco plants. Due to the addictiveness of nicotine, future regulations may be placed on combustible tobacco products that would require the nicotine content to be much lower than what is currently on the market. The level of nicotine being considered by the FDA for a proposed future mandate is 0.4 mg/g within the tobacco filler, an amount that is approximately 95% lower than exists in cigarettes currently on the market. Although naturally occurring genetic mutations exist that can reduce the nicotine content of tobacco plants, it will be difficult to develop ultra-low nicotine varieties through traditional breeding that meet this stringent standard. In this thesis, genome editing technologies were applied to mutate key steps in the nicotine biosynthetic pathway to explore new alternatives for generating ultra-low nicotine tobaccos.

Berberine Bridge Enzyme-Like (*BBL*) genes encodes enzymes that catalyze one of the final steps in alkaloid biosynthesis. The *BBL* gene family is comprised of six closely related genes, three of which are expressed at moderate to high levels (*BBLa*, *BBLb* and *BBLc*) and three at low levels, *BBLd-1, BBLd-2*, and *BBLe*. Previous research has shown that mutating the three "major" *BBL* genes can dramatically reduce nicotine content, but not as low as 0.4 mg/g. In Chapter 2, custom-designed meganuclease constructs encoding enzymes directed to bind and cut the "minor" *BBL* genes, *BBLd-1, BBLd-2* and *BBLe* were introduced into two popular commercial varieties (K326 and TN90) that already possessed mutations in the major *BBL* genes. The addition of mutations in the minor *BBL* genes gave inconsistent results with respect to both nicotine reduction and yield.

The enzyme quinolate phosphoribosyltransferase (QPT) serves as an entry point into the pyridine nucleotide pathway that is responsible for the production of both the essential cellular co-factor NAD, and well as the nonessential pyridine alkaloids, including nicotine. Prior research has suggested that the tobacco genome encodes two closely related *QPT* genes: *QPT1* which is expressed at a basal level throughout the tobacco plant, and *QPT2* which is expressed at very high levels in the nicotine producing root tissues and under conditions where nicotine production has been stimulated by wounding or hormone treatment. In Chapter 3, the CRISPR/Cas9 technology was used to test whether the specific knockout of the *QPT2* genes represents a viable strategy for developing high quality ultra-low nicotine tobaccos. Greenhouse-grown *qpt2* plants accumulated dramatically reduced nicotine in comparison to the wild-type control parent, while displaying only modest growth differences. In contrast, when the same *qpt2* lines were grown in a field environment, plant growth was severely inhibited. Due to the poor performance of these field-tested tobacco lines, we conclude that the knockout of *QPT2* function is not a viable strategy for producing commercially useful, low nicotine tobaccos.

Two additional steps of the alkaloid pathway that were investigated in this thesis are those catalyzed by the enzyme methylputrescine oxidase (MPO) and an isoflavone reductase-like enzyme referred to as A622. Chapter 4 reports the results of using genome editing technologies to investigate the effects of knocking out *A622* and *MPO* gene function in both wild-type backgrounds as well as tobaccos possessing mutations in the three major *BBL* genes. In addition to alkaloid content, several growth measurements were recorded on greenhouse-grown lines that carried *A622* and *MPO* mutations, both with and without additional *BBL* mutations. Of the various nicotine reducing strategies tested in this thesis, combining *mpo* with *bbl* mutations holds

the greatest promise as means for obtaining ultra-low nicotine tobacco cultivars that retain acceptable agronomic and quality standards.

Utilizing Genome Editing Technologies to Develop Ultra-low Nicotine Tobaccos

by
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A thesis submitted to the Graduate Faculty of
North Carolina State University
in partial fulfillment of the
requirements for the degree of
Master of Science

Crop Science

Raleigh, North Carolina
2022

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BIOGRAPHY

William Smith was born in 1990 to parents Jeff and Sheila in Winston-Salem, NC. Even though he grew up near tobacco fields in a city that is strongly associated with tobacco he never imagined he would ever have anything to do with the plant. He attended North Carolina State University where he double majored in Biology and History earning a BS. Before graduating in 2012 he started working in Dr. Imara Perera's lab where he quickly discovered his passion for research related to plants. Afterwards he accepted a full time Research Technician position in Dr. Ralph Dewey's lab to manage the greenhouse space, high-throughput genotyping, and pursue projects related to the development of low nicotine. In 2015 he decided to become a part-time MS student with a focus towards expanding his knowledge of genome editing and new molecular biology techniques, while continuing to work as a full-time in Dr. Dewey's lab. After completing his degree, he plans to continue working in Dr. Dewey's lab.

ACKNOWLEDGMENTS

First, I would like to thank my advisor and Principal Investigator Dr. Ralph Dewey for being willing to take me as a student while continuing to work in his lab. This has required a large degree of flexibility and willingness to accommodate my schedule, including the many complications caused by Covid-19. His continued guidance and support shows me what kind of research scientist I want to be as I continue my career. I would also like to thank Dr. Ramsey Lewis and Dr. De-Yu Xie for being a part of my committee.

Next, I would like to thank our lab manager Carol Griffin who has been a consistent presence that ensures our lab stays functioning. Since I have been a part-time student there have been many post-docs that have come through the lab and given me guidance. Specifically, I would like to acknowledge Dr. Yuki Matsuba, Dr. Devarshi Selote, Dr. Yihai Wang, Dr. Jianli Lu, and Dr. Hong Ma. I would also like to thank Kevin Stroup and every member of the Dewey Lab that has helped me and just been amazing people to work with throughout the years.

Thank you to 22nd Century Inc. for the partial financial support for this research.

Finally, I would like to thank my girlfriend Ash, my friends, and family for their continued support and encouragement throughout my MS work.

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CHAPTER 1

Literature Review

Introduction

Tobacco (*N. tabacum*) has been a key agricultural product in America since the time of its early settlement. Of the various forms of tobacco use, it is the combustible products, especially cigarettes, that impose the greatest negative health consequences on society. Public smoking bans, high taxation rates, concerted public educational efforts, and alternatives such as e-cigarettes and vaping products have resulted in substantial reductions in cigarette smoking in the US. Nevertheless, according to the Centers of Disease Control (CDC), cigarette smoking still accounts for over 480,000 US deaths annually, reducing life expectancy by over 10 years (https://www.cdc.gov/tobacco/data_statistics/fact_sheets/health_effects/tobacco_related_mortality/index.htm). Thus, there is a great need to implement additional strategies to reduce the prevalence of smoking in society and mitigate the harm caused by these products.

The alkaloid nicotine is arguably the single most distinguishing feature of tobacco plants and products derived therefrom. In nature, nicotine serves as a defensive compound against insect herbivory. Nicotine functions as an insecticide by causing a continual excitation of acetylcholine receptors in neurons leading to paralysis and even death (Baldwin et al., 2001). In humans, nicotine-mediated acetylcholine receptor stimulation leads to a strong addiction response for those who consume tobacco products. While nicotine is not carcinogenic, the need to reinforce the nicotine stimulation causes the addict who consumes cigarettes to expose themselves to an array of harmful combustion products responsible for the majority of the negative health consequences associated with smoking.

As early as the 1990s, public health advocates have been promoting the idea of regulating the levels of nicotine in cigarettes to levels below that which could sustain an addiction response as a strategy for reducing cigarette consumption (Benewitz and Henningfield, 1994). This concept has gained momentum in recent years as numerous studies funded by the National Institute of Health (NIH) and the US Food and Drug Administration (FDA) have demonstrated that smokers provided with very low nicotine cigarettes will smoke less, and for those who are attempting to quit, switching to low nicotine cigarettes for a period of time can help wean them from their habit (reviewed in Donny and White, 2022). These findings have prompted the FDA to consider the possibility of imposing a mandate that would require cigarettes sold in the US to contain ultra-low levels of nicotine (United States Food and Drug Administration, 2018). The nicotine threshold currently being promoted for cigarettes under this proposed mandate is 0.4 mg/g in the cigarette filler. This is a level that has been recommended by the World Health Organization (2015) and represents an approximate 95% reduction in comparison to a typical commercial cigarette. In keeping with the FDA's interest in promoting the use of low nicotine cigarettes, this agency recently approved the commercialization of very low nicotine cigarettes as a Modified Risk Tobacco Product (MRTP) that can be marketed as a product that "Helps You Smoke Less" (https://www.fda.gov/news-events/press-announcements/fda-authorizes-marketing-tobacco-products-help-reduce-exposure-and-consumption-nicotine-smokers-who).

To date, there is only one tobacco variety, Vector 21-41, that has been approved by the FDA for use under the MRTP designation. Vector 21-41 is a burley market type that possesses an antisense construct directed against a quinolinate phosphoribosyltransferase gene using a root specific promoter (Xie et al., 2004). Given that: (1) the typical "American Blend" cigarette consists of a blend of three distinct market types (flue-cured, burley and oriental); (2) the average

nicotine content of Vector 21-41 was reported to be 1.44 mg/g (which is higher than the recommended 0.4 mg/g target); and (3) many countries prohibit the growth and/or sales of genetically modified (GM) tobacco, there is a need to develop new strategies for the production of ultra-low nicotine tobaccos in order to meet the growing demand as an MRTP-designated product as well to accommodate possible future mandates from the FDA.

There are a variety of factors that can influence the nicotine content of tobacco plants grown that are grown commercially. Agronomic practices such as reduced nitrogen fertilization and omitting the standard practice of topping the plants prior to flowering can lower the alkaloid yield, but not nearly to the extent achievable through genetic modification (Lewis et al., 2020; Cheek et al., 2021). Genetic variability for nicotine content exists naturally. Nicotine content in tobacco plants is largely controlled by two distinct loci *Nic1* and *Nic2* (originally named *A* and *B*, respectively), and their mutant alleles *nic1* and *nic2* have been used to breed low-nicotine tobacco cultivars. Although *nic1nic1/nic2nic2* double homozygous mutants can reduce alkaloid levels from an average of 15 – 45 mg/g to approximately 2.0 – 4.5 mg/g (Lewis 2019) this still well exceeds the 0.4 mg/g target of the proposed low nicotine mandate. Furthermore, the use of *nic1* and *nic2* to reduce alkaloid levels in tobacco is associated with negative effects on yield and/or cured leaf quality (Chaplin et al., 1976; Legg et al., 1970; Legg and Collins, 1971; Chaplin et al., 1983; Wang et al., 2008). Another naturally occurring genetic means that can greatly reduce the nicotine content of the tobacco plant is a phenomenon referred to as “nicotine conversion”, where a quiescent nicotine demethylase gene (*CYP82E4*) becomes activated and metabolizes the majority of the leaf nicotine to nornicotine (Siminszky et al., 2005; Lewis et al. 2008). Ultimately, the exploitation of nicotine conversion is not an attractive alternative for

developing now nicotine tobaccos as nornicotine can also be addictive as well as be readily nitrosated to produce the potent carcinogen *N'*-nitrosonornicotine (Hecht, 2008).

Advances in the fields of molecular biology, plant transformation, and genome editing have not only led to the elucidation of the genes involved in the production of nicotine, but they have also created tools that can be applied toward genetically modify the alkaloid content in ways not found in nature. The two most effective biotechnology-based approaches that have resulted in reducing the nicotine content of the tobacco plant involve either: (1) targeting individual steps of the alkaloid biosynthetic pathway; or (2) targeting transcription factors (TFs) that regulate that pathway. Highlights of the knowledge that has been gained in both of these areas is presented below.

Alteration of Genes in the Nicotine Biosynthetic Pathway

In commercial tobacco cultivars, nicotine represents 90–95% of the total alkaloid (Dewey and Xie, 2013). Nicotine is synthesized in the root, transported to leaves, and stored in leaf cell vacuoles (Saunders 1979; Baldwin, 1999; Morita et al., 2009). Throughout the last thirty years, a great deal of knowledge has been gained in understanding the molecular genetics underlying the biosynthesis of tobacco alkaloids. Figure 1 shows a simplified depiction of the alkaloid biosynthetic pathway as it is understood today. Nicotine is comprised of a pyrrolidine and a pyridine ring, each of which are produced by independent metabolic pathways. The pyrrolidine ring can originate from either arginine or ornithine, resulting in the production of putrescine which is methylated by a putrescine methyltransferase (PMT) enzyme to produce *N*-methylputrescine. The oxidative deamination of *N*-methylputrescine by the enzyme methylputrescine oxidase (MPO) produces 4-methylaminobutanal compound which

spontaneously cyclizes to form the *N*-methyl-D^1-pyrrolium ring that feeds into nicotine biosynthesis. The pyridine ring originates from nicotinic acid, a product of the pyridine nucleotide cycle whose primary function is to supply the cell with the essential co-factor NAD (Fig. 1.1). The enzyme that directly mediates the entry of metabolites into the pyridine nucleotide cycle is quinolinic acid phosphoribosyl transferase (QPT) (Wagner et al., 1985). Although the steps in between nicotinic acid and the final coupling of the resultant pyridine ring to the *N*-methyl-D^1-pyrrolium ring are still unclear, convincing evidence has been presented that demonstrate the involvement of enzymes referred to as A662 and a berberine bridge-like (BBL) enzyme (Dewey and Xie, 2013). The specific steps of the nicotine biosynthetic pathway that have been investigated the most thoroughly at the molecular genetic level, and whose expression has been modified to alter alkaloid content are briefly described below.

I. ODC and ADC

Both ornithine decarboxylase (ODC) and arginine decarboxylase (ADC) are capable of producing putrescine for alkaloid synthesis. ODC utilizes ornithine as the substrate while ADC uses arginine in a three-step process involving agmatine and *N*-carbamoyl putrescine as intermediates (Malmberg, et al., 1998). *ODC* transcripts are almost exclusively expressed in roots, while *ADC* transcript levels are very low in roots but high in flowers (Wang, et al., 2000). *ADC* and *ODC* transcripts are both induced by topping (Wang, et al., 2000). Southern blot analysis showed that both enzymes are controlled by small gene families with multiple isoforms in the tobacco genome (Xu, et al., 2004). Down regulation of *ADC* in transgenic tobacco did not lead to a decrease in nicotine levels in an early study (Chintapakorn and Hamill, 2007), but result in a moderate decrease in a more recent examination (Martinez et al., 2020). In contrast, the suppression of *ODC* resulted in significant reductions in nicotine content and substantial

increases in anatabine (DeBoer, et al., 2011; Martinez et al., 2020). Therefore, it has been speculated that the ODC route is more preferred than ADC for producing the putrescine intermediate that is utilized for alkaloid biosynthesis.

II. PMT

PMT represents the first committed step in the synthesis of the pyrrolidine ring. *NtPMT1a* was first isolated by subtractive hybridization using cultured roots of tobacco plants possessing the naturally occurring low alkaloid mutants *nic1* and *nic2* (Hibi, et al., 1994). Subsequently, four additional *PMT* isoforms *NtPMT1b, NtPMT2, NtPMT3*, and *NtPMT4*, have been found in tobacco genome (Hashimoto, et al., 1998; Riechers and Timko, 1999). All five *NtPMT* genes are specifically expressed in the root (Riechers and Timko, 1999; Sierro et al., 2014). PMT gene expression is upregulated by wounding, topping, and jasmonate (JA) treatment, but downregulated by auxin (Hibi, et al., 1994; Winz and Baldwin, 2001).

Previous studies demonstrated that the PMT-catalyzed step is rate limiting and is an important regulatory point in alkaloid production (Saunders and Bush, 1979; Wagner and Wagner, 1985; Feth et al., 1986). The reduction of *PMT* expression via a co-suppression mechanism in the wild species *N. sylvestris* resulted in nicotine levels that were only 2% of that observed in the wild type control (Sato et al. 2001). RNA interference (RNAi)-mediated inhibition of *PMT* expression also significantly lowered nicotine accumulation in conventional tobacco plants (Chintapakorn and Hamill, 2003; Wang, et al., 2008). Similar to the results observed with *ODC* suppression, nicotine reduction was accompanied by substantial increases in anatabine. The prevailing theory for this phenomenon lies in the fact that unlike the other tobacco alkaloids, anatabine is derived solely from the pyridine ring pathway (see Fig. 1.1). It has thus been hypothesized that the down regulation of genes in the pyrrolidine ring pathway

leads to enhanced anatabine production owing to the lack of pyrrolidine ring substrate that normally diverts the majority of the pyridine ring metabolite flow toward nicotine (Dewey and Xie, 2013).

III. MPO

The oxidative deamination of N-methylputrescine is catalyzed by MPO enzymes. The *MPO1* gene was identified by two separate research groups, one using reverse transcription PCR with degenerate oligonucleotides corresponding to conserved amino acid sequences found in plant copper-dependent amine oxidases (Heim, et al., 2007) and the other using differential expression profiling of *nic1nic1/nic2nic2* versus wild-type tobacco materials (Katoh, et al., 2007). *MPO1* genes are highly root specific (Sierro et al., 2014; Kajikawa et al., 2017, and are induced by treatment with JA and repressed by auxin (Heim, et al., 2007). Similar to downregulation of *ODC* and *PMT, MPO* suppression resulted in a decreased nicotine phenotype accompanied by substantial increases in the anatabine content in tobacco hairy root cultures and BY-2 culture cells (Shoji and Hashimoto, 2008). Southern blot analysis predicted multiple *MPO* genes within the tobacco genome (Heim et al., 2007). A gene designated *MPO2* encodes a protein that is 88% identical to MPO1 in amino acid sequence (Katoh et al., 2007). MPOs are a copper-containing amino oxidases that have the potential of oxidizing multiple diamines and the MPO1 enzyme was shown to accept N-methylputrescine as the most preferred substrate (Heim et al., 2007; Katoh et al., 2007). Later studies by Naconsie et al. (2014), however, showed that MPO2 enzymes preferentially accept non-methylated diamines (such as putrescine) over methylated diamines (like *N*-methylputrescine) prompting the authors to suggest renaming *MPO2* genes using the more generic nomenclature diamine oxidase 1 (*DAO1*). A very recent study has shown that RNAi-mediated inhibition of *MPO* expression can lead to dramatic

reductions in nicotine in field grown plants, particularly when combined with mutations in *BBL* genes (Kernodle et al., 2022).

IV. AO

Aspartate oxidase (AO) catalyzes the oxidation of aspartic acid to produce a-iminosuccinate, which become further metabolized to quinolinic acid, the substrate of the QPT enzymes (Fig. 1.1). RNAi suppression of *AO* gene expression resulted in dramatic reductions in nicotine content, but the leaves were discolored and the plants displayed an early senescence leaf phenotype (Martinez et al., 2020).

V. QPT2

QPT2 was isolated and characterized by two research groups independently (Song, 1997; Sinclair, et al., 2000). *QPT2* transcripts are greatly enhanced in roots, especially in the root cortex of the fully differentiated region as opposed to the root tip (Song, 1997). *QPT2* was strongly induced 4 hours post-topping and continued to be elicited after 24 hours, and was inhibited by auxin treatment (Song, 1997). The study by Sinclair et al. (2000) showed both leaf wounding and JA treatment stimulated *QPT2* expression. The closely related *QPT1* genes share 94% nucleotide identity to *QPT2* in the coding region. *QPT1* is expressed throughout all tissues at a basal level and is not responsive to topping, wounding or plant hormone signaling (Shoji and Hashimoto, 2011a). Therefore, it has been speculated that *QPT1* may simply be a housekeeping gene dedicated to the production of the cellular NAD required for primary metabolism and may contribute toward nicotine biosynthesis. As mentioned above, the suppression of *NtQPT2* mediated by an antisense construct driven by its own root specific *QPT2* promoter resulted in a greatly reduced nicotine phenotype as compared to Burley 21 parental plants, leading to the

development of the low nicotine line Vector 21-41, which yielded an average nicotine content of 1.44 mg/g dry weight (Xie et al. 2004).

VI. A622

A622 was first identified in the same study where the *NtPMT1a* gene was discovered (Hibi et al., 1994). *A622* displays an expression pattern very similar to *PMT* (Shoji et al., 2002; Cane et al., 2005; Kidd et al., 2006) which lead to the speculation that it may play a role in alkaloid production. The A622 protein is an NADPH-dependent isoflavone reductase-like homolog (Hibi et al., 1994; Shoji et al., 2002). Attempts to define the specific reaction catalyzed by A622 enzymes using recombinant A622 protein and *in vitro* enzymatic assays provided with NADPH and potential substrates, however, failed to shed light on its function (Shoji et al., 2002).

RNAi mediated suppression of *A622* caused a substantial reduction of anabasine, nicotine and anatabine in the wild species *N. glauca* (DeBoer et al., 2009). In *N. tabacum,* in addition to nicotine reduction, the compound nicotinic acid β-N-glucoside (NaNG) was found to be enhanced in *A622* suppressed hairy roots and BY-2 cell cultures. Instead of representing an intermediate in the nicotine biosynthetic pathway, NaNG was proposed to be a detoxification product of nicotinic acid (Kajikawa et al., 2009). Interestingly, no intact transgenic plants with effectively suppressed *A622* expression were recovered in that study, suggesting that severe inhibition of *A622* may be inhibit growth in *N. tabacum.* A major conclusion by the researchers conducting the study was that because NaNG was the primary pyridine compound that accumulated in A622 knockdown hairy root and cell cultures, A622 likely acts upon a NA derivative but prior to ring condensation as depicted in Fig. 1. A recent study using CRISPR/Cas9 mutagenesis of *A622* genes confirmed that their loss of function is associated with

aberrant growth and development, particularly when grown in a field environment (Burner et al. 2022a).

VII. BBL

A group of four berberine enzyme-like (BBL) genes were identified that were posited to play a role in the final stages of pyridine alkaloid ring condensation (Kajikawa et al., 2011). Similar to the strategies that proved to be successful for isolating *PMT1a, A622* and *MPO1*, the four *BBL* genes, designated *BBLa-d*, were identified through the characterization of genes controlled by the *Nic1* and *Nic2* loci based on differential expression between double homozygous mutant *nic1nic1/nic2nic2* materials and their corresponding wild-type lines. Further experimentation showed that *BBL* expression followed a similar pattern to that observed in other alkaloid biosynthetic genes that had been previously characterized (Kajikawa et al., 2011). Similar to the situation with A622, researchers have been unable to define the exact metabolic reaction(s) catalyzed by the BBL enzymes using *in vitro* enzyme assays. The conclusion that these enzymes catalyze a reaction near the end of the alkaloid pathway came from the observation that plants inhibited in *BBL* expression accumulate the novel alkaloid dihydrometanicotine (DMN). DMN consists of a pyridine ring with a straight chained *N*-(methylamino)-butyl moiety, a compound that could only have been produced during or after the condensation of the pyridine and pyrrolidine rings. DMN does not appear to serve as a substrate for BBL enzymes (Kajikawa et al. 2011), but its presence in the BBL knockdown lines provided strong evidence that BBL catalyzes one of the last, and possibly the very last, step in nicotine, anatabine and anabasine biosynthesis.

Of the numerous molecular genetic strategies that have been pursued in attempt to produce low nicotine tobaccos, mutagenesis of members of the *BBL* gene family has shown

particular promise. RNAi-mediated down regulation of the *BBL* family was shown to decrease nicotine levels to as low as 0.4% dry weight (4 mg/g), compared to 2.5% for the controls (Lewis et al. 2015). In this same study it was demonstrated that combining EMS-induced knockout mutations in the three most highly transcribed members of the *BBL* family, *BBLa*, *BBLb* and *BBLc*, resulted in a 13-fold decrease in the total alkaloid content of the tobacco leaf. Despite these notable decreases, the nicotine remaining in these plants still exceeded the 0.4 mg/g levels being recommended by the FDA and World Health Organization (WHO, 2015). Although *BBLa*, *BBLb* and *BBLc* are the most highly transcribed members of the tobacco *BBL* gene family (Kajkawa et al., 2011), the tobacco genome possess other sequences that share high DNA sequence homology with these genes (Sierro et al., 2014). Thus, it is possible that these additional *BBL* homologs could account for some, if not all of the alkaloid that is still present in plants possessing the nonfunctional *bbla*, *bblb* and *bblc* EMS mutant genes. A report has been published claiming that nicotine-free tobaccos can be produced by knocking out all six major and minor *BBL* genes (Schachtsiek and Stehle, 2019). As elaborated further below, that claim is contradicted by the results of this these that are presented in Chapter 2.

Alteration of Transcription Factors Involved in Nicotine Biosynthesis

Most of the major steps in the nicotine biosynthetic pathway have been elucidated in the past two to three decades. An understanding of the regulation of this pathway at the transcriptional level, however, has a more recent history. Transcription factors (TFs) are the key players in regulating the transcription of metabolic pathway genes. Because of their influence on pathway regulation, the suppression of one or more major TFs that regulate the alkaloid biosynthetic pathway would be considered a viable strategy for the development of low nicotine tobaccos. A tobacco TF database has been established based on *in silico* analysis of genome

sequence data. More than 2500 TFs representing 64 families were identified in this analysis (Rushton et al. 2008). Although the identification of the complete suite of TFs involved in nicotine biosynthesis must still be considered a work in progress, research efforts reported to date have revealed three TFs that appear to mediate exceptionally strong control over the production of alkaloids in tobacco: *ERF189, ERF199,* and *Myc2a*.

The ethylene response factor (ERF) gene called *ERF189* was first identified as one of seven *ERF* family TF genes located on the chromosomal deletion that defines the *nic2* locus (Shoji et al., 2010). Subsequent investigation revealed that *ERF189* was the most important, if not sole, *ERF* gene of this cluster contributing toward alkaloid regulation (Shoji and Hashimoto, 2015; Kajikawa et al., 2017). In addition to showing high homology to *ERF189* and a similar root specific expression pattern, *ERF199* was shown to map to the same region of the tobacco genome as the *Nic1* locus. In a recent study, Qin et al. (2021) demonstrated that the *nic1* low nicotine phenotype could be attributed to inactivation of *ERF199*. Interestingly, no differences in the *ERF199* primary DNA sequence were observed in *Nic1* versus *nic1* tobaccos, suggesting that the inactivation of *ERF199* in *nic1* lines is mediated through an epigenetic mechanism (Qin et al., 2021). CRISPR/Cas9-based mutagenesis of *ERF189* and *ERF199* decreased the alkaloid levels in the leaf to 2-4% of that observed in wild-type controls (Hayashi et al., 2020). Additional evidence supporting the concept that inactivation of *ERF189* and *ERF199* gene function is the basis of the low alkaloid phenotypes attributable to *nic2* and *nic1*, respectively, including the discovery and characterization of additional alleles at these loci, is found in the recently published report of Shoji et al. (2022).

Myc2a, the other TF that serves as a master regulator of alkaloid biosynthesis in *Nicotiana* is a member of the basic helix-loop-helix TF family. Originally identified in *N.*

benthamiana as a positive regulator of JA-inducible alkaloid biosynthesis (Todd et al. 2010), the *N. tabacum* ortholog was subsequently reported to function similarly (Shoji and Hashimoto, 2011b). Recently, CRISPR/Cas9 mutagenesis of *Myc2a* in wild-type tobacco plants was shown to lower leaf nicotine levels by approximately 80% (Sui et al., 2020). A naturally occurring 5 bp frame-shift mutation in *Myc2a* was also found during a screen of low alkaloid accessions from a tobacco germplasm collection (Burner et al., 2022). When the low alkaloid line TI313 was crossed to the commercial variety K326, a ~50% reduction in both nicotine and total alkaloids was observed in F_2 individuals that were homozygous for the 5 bp *myc2a* mutation.

Development of Commercially Acceptable Ultralow Nicotine Tobaccos

Despite the wealth of information that has been accrued with respect to how the genetics of the tobacco plant can be altered in a manner that reduces the alkaloid content of the plant, developing cultivars that are agronomically acceptable and can reliably meet the rigorous target of 0.4 mg/g is far from trivial. The great majority of the studies cited in sections II and III were conducted in lab or greenhouse environments using “lab accessions” of tobacco that are smaller, have a shorter generation time, and inherently accumulate less alkaloid than commercial varieties. Information from studies of this nature can reveal promising trends but are inadequate for assessing the viability of the given strategy when introduced into commercially acceptable varieties and grown under field conditions using standard practices such as topping. An excellent example of the dangers in making assumptions based only on non-topped greenhouse growth data can be seen from the recent report by Schachtsiek and Stehle (2019) who claimed to produce nicotine-free tobacco plants by using the CRISPR/Cas9 system to knock out all six members of the *BBL* gene family in some nondescript genetic background. As will be seen in

Chapter 2 of this thesis, not only is there considerable nicotine produced when all six *BBL* genes are knocked out in the popular commercial varieties K326 and TN90 when grown under standard field conditions, but the amounts of nicotine also produced were 3- to 10-fold higher than the 0.4 mg/g FDA target.

Another phenomenon frequently associated with perturbations of the alkaloid biosynthetic pathway in tobacco are negative associations with normal plant growth and development (including yield), cured leaf quality, and leaf chemistry. This is well exemplified by the early senescence phenotype association with RNAi-mediated suppression of *AO* expression (Martinez et al., 2020). Also, as described in section II, down regulation of genes encoding enzymes of the pyrrolidine ring pathway result in plants having anatabine as the most abundant alkaloid, in quantities several-fold higher than exist in wild-type tobacco plants. Because the pharmacological effects of anatabine and/or its derivatives have been minimally investigated in comparison to nicotine, the high anatabine trait could prove detrimental to regulatory approval by the FDA, or even consumer acceptance. Another potential consequence associated with metabolic pathway disruption is the possibility that toxic intermediates or byproducts normally found in low amounts may build up to levels where normal plant growth and development is impacted.

Finally, the majority of the studies where tobacco alkaloid levels have been altered using the tools of molecular biology have relied on the permanent incorporation of a transgene (most often RNAi or antisense constructs) to generate the low nicotine phenotypes. Not only do GM tobaccos face additional regulatory hurdles in order to be deployed, but many countries prohibit their use and they risk public rejection by segments of the population even in countries where they are allowed. Genome editing technologies differ from traditional transgene-based

approaches in that after the genes of interest are precisely mutated, the genome editing transgene can be segregated away leaving an end product possessing no foreign DNA. This eliminates regulatory hurdles in many countries in the world (including the US) and would likely receive greater public acceptance.

This thesis is centered on the use of genome editing technologies to explore new avenues for generating ultralow nicotine tobacco cultivars that contain no foreign DNA sequences. Chapter 2 describes the results of knocking out the minor *BBL* genes *BBLd-1, BBLd-2* and *BBLe* in genetic backgrounds that possessed mutations in the major genes *BBLa, BBLb* and *BBLc,* using custom designed meganuclease constructs. In Chapter 3, the CRISPR/Cas9 technology is used to test whether the specific knockout of the *QPT2* genes that have been proposed to be dedicated to alkaloid biosynthesis (while leaving the presumed 'housekeeping' *QPT1* isoforms intact) represents a viable strategy for developing high quality ultra-low nicotine tobaccos. Finally, Chapter 4 reports the results of using CRISPR/Cas9 and designer meganucleases to investigate the effects of knocking out *A622* and *MPO* gene functions, respectively, both in wild-type backgrounds as well as tobaccos possessing mutations in the three major *BBL* genes.

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Tables and Figures

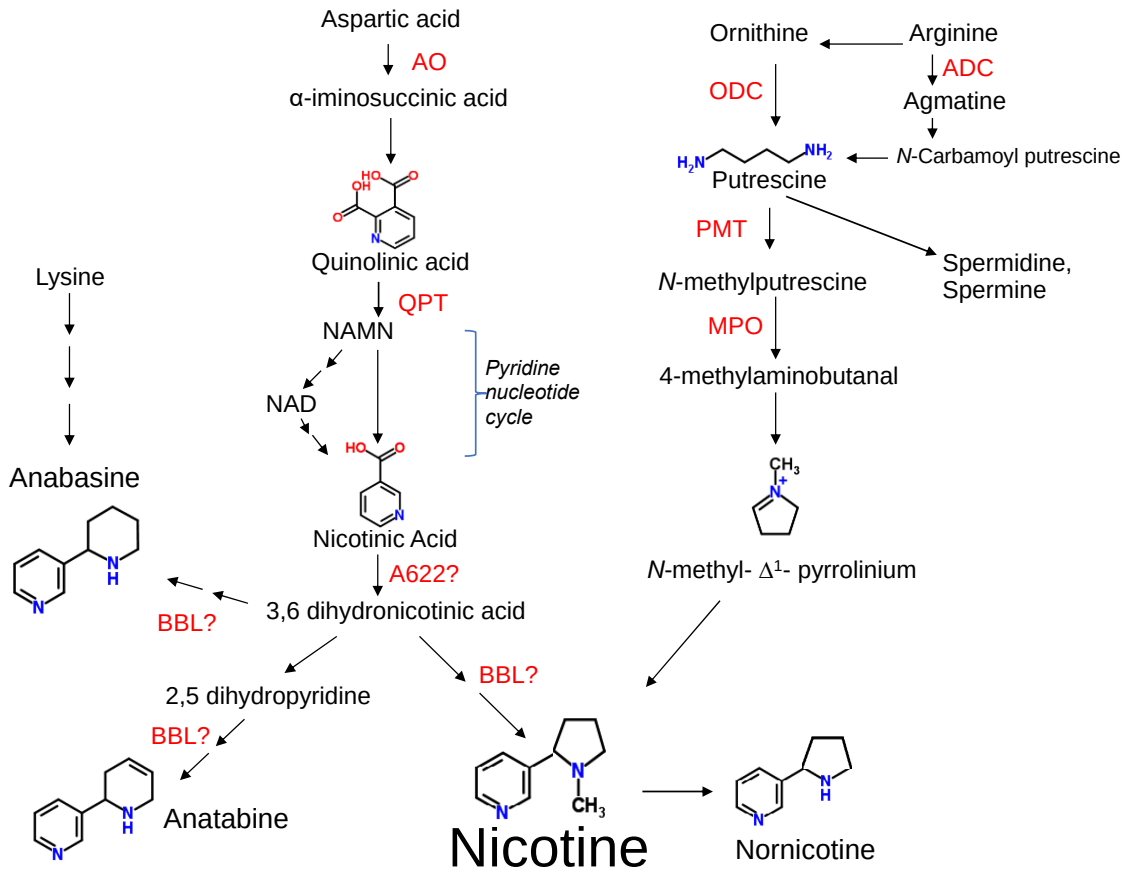

Figure 1.1 Simplified schematic of alkaloid biosynthesis in N. tabacum (modified from Dewey and Xie, 2013). Steps of the pathway that are discussed in the text are shown in orange: A622 (isoflavone reductase-like protein); ADC (arginine decarboxylase); AO (aspartate oxidase); BBL (berberine bridge-like); MPO (N-methylputrescine oxidase); ODC (ornithine decarboxylase); PMT (putrescine methyltransferase) QPT (quinolinate phosphoribosyltransferase).

CHAPTER 2

Targeted Mutagenesis of Minor *Berberine Bridge Enzyme-Like (BBL)* Genes in Tobacco

(some of the results presented in this chapter were published in: *Lewis, R.S., Drake-Stowe, K.E., Heim, C., Steede, T., Smith, W. and Dewey, R.E. 2020. Genetic and agronomic analysis of tobacco genotypes exhibiting reduced nicotine accumulation due to induced mutations in Berberine Bridge Like (BBL) Genes. Frontiers Plant Sci. 11:368*. A converted PDF of this manuscript is found in Appendix A).

Introduction

As described in Chapter 1, there is great interest in developing tobacco varieties that produce ultra-low levels of the addictive compound nicotine. In terms of harm reduction, the most compelling argument for producing tobacco products with minimal amounts of nicotine stems from reports demonstrating that cigarettes containing nicotine levels below a critical threshold can no longer initiate or maintain an addiction response (Benowitz and Henningfield, 1994; Benowitz et al., 2007). Indeed, numerous clinical trials that have been funded by the NIH and FDA have shown that for smokers who want to quit, transitioning to low-nicotine cigarettes can help them achieve this goal (reviewed in Donny and White, 2022). These studies have recently prompted the FDA to approve the commercialization of very low nicotine-containing cigarettes as modified risk products that can be marketed with the label "Helps You Smoke Less" (https://www.fda.gov/news-events/press-announcements/fda-authorizes-marketing-tobacco-products-help-reduce-exposure-and-consumption-nicotine-smokers-who). Furthermore, in June of 2022, the FDA announced renewed interest in the possibility of mandating that future combustible tobacco products sold in the US contains tobacco with non-addictive levels of

nicotine (https://www.fda.gov/news-events/press-announcements/fda-announces-plans-proposed-rule-reduce-addictiveness-cigarettes-and-other-combusted-tobacco), an idea that was initially proposed several years ago (United States Food and Drug Administration, 2018).

Of the numerous genetic strategies that have been pursued in attempt to produce low nicotine tobaccos, mutagenesis of members of the *Berberine Bridge Like (BBL)* gene family has shown particular promise. Tobacco *BBL* genes encode enzymes that function near the very end of pyridine alkaloid pathways, although the specific reaction that they catalyze has yet to be defined (Dewey and Xie, 2013). RNA inhibition (RNAi)-mediated down regulation of the *BBL* gene family was shown to decrease nicotine levels to as low as 0.4% dry weight, compared to 2.5% for the controls (Lewis et al. 2015). In this same study it was demonstrated that combining EMS-induced knockout mutations in the three most highly transcribed members of the *BBL* family, *BBLa, BBLb* and *BBLc*, resulted in a 13-fold decrease in the total alkaloid content of the tobacco leaf. Despite these notable decreases, the nicotine remaining in these plants still exceeds the 0.4 mg/g, or 0.04% dry weight, levels being recommended by the FDA and World Health Organization (WHO, 2015). Although *BBLa, BBLb* and *BBLc* are the most highly transcribed members of the tobacco *BBL* gene family (Kajkawa et al., 2011), the tobacco genome possesses other sequences that share high DNA sequence homology with these genes. Thus, it is possible that these additional *BBL* homologs could account for some, if not all of the alkaloid that is still present in plants possessing the nonfunctional *bbla, bblb* and *bblc* EMS mutant genes. The objective of this study was to identify the complete complement of *BBL* family genes in tobacco and determine whether further decreases in alkaloid could be attained by knocking out their function in tobacco genetic backgrounds that already carry the *bbla, bblb* and *bblc* mutations.

Materials and Methods

Targeted mutagenesis of BBLd-1, BBLd-2 and BBle

Plasmids encoding meganucleases designed to cleave specific targets in the *BBLd-1, BBLd-2* and *BBLe* genes were produced by the company Precision Biosciences (precisionbiosciences.com). Construct BBL1-2x.81 encodes a nuclease that was engineered to recognize a unique 22 bp sequence within *BBLe*. Due to the high sequence identity shared between *BBLd-1* and *BBLd-2*, it was possible to design a single nuclease (BBL7-8x.90) capable of targeting both of these genes. In order to avoid unintended cleavage at nontarget sites, for each custom-designed nuclease, a 22 bp target site was chosen that failed to be present anywhere within the *Nicotiana tabacum* reference genomes that have been deposited in GenBank. The specific recognition sequences for BBL1-2x.81 and BBL7-8x.90 are 5-GAGGAGCTCGTGAGTACCATTC-3' and 5'- GTCATTATCGTACCAGAGAGCA, respectively. As shown in Figure 2.1, the selected target sites occur in the upper one third of the gene sequences. These sites are located within the sequences encoding the highly conserved FAD binding domain and upstream of the highly conserved berberine bridge motif. Therefore, mutation events leading to a frame shift at these locations would be predicted to produce completely nonfunctional protein products. Constructs BBL1-2x.81 and BBL7-8x.90 were cloned into the binary plant expression vector pCAMBIA2300 (www.cambia.org) and transformed into tobacco leaf discs of the following genotypes: K326 222, TN90 222, and TN90 SRC 222. The numbers following the cultivar name represent the *BBL* loci *BBLa*, *BBLb* and *BBLc*, respectively: 0 = wild type at both alleles, 1 = heterozygous for the mutant *bbl* allele, and 2 = homozygous mutant at the give *bbl* locus. In cases where mutations in minor *BBL* genes is also indicated, the gene positions are extended in the order *BBLd-1*, *BBLd-2* and *BBLe*. For

example, a K326 plant that is homozygous for mutant alleles in the three major *BBL* genes as well as *BBLe*, is homozygous wild type at *BBLd-1* and heterozygous for a mutation at *BBLd-2* would be designated K326 222012.

The custom designed meganuclease constructs in pCAMBIA2300 were introduced into tobacco using standard *Agrobacterium*-mediated transformation protocols (Horsch et al., 1985). Young kanamycin resistant T_0 plants were screened for mutations in the target gene(s) of interest by PCR amplification of the three minor *BBL* genes using primers designed to uniquely amplify *BBLd-1*, *BBLd-2* or *BBLe*. DNA sequencing of the amplification products was conducted by the NCSU Genomic Sciences Laboratory (https://research.ncsu.edu/gsl). T_1 generation progeny of T_0 individuals that possessed induced mutations of greatest interest were screened by PCR using primers specific to the selectable marker gene (*nptII*) to identify those individuals that lost the genome editing transgene via segregation. *NptII*-minus progeny were then genotyped for the *bbl* mutations observed in the T_0 generation. To combine mutations in the respect minor *BBL* genes, crosses were made amongst the mutant lines followed by allele-specific genotyping to fix the mutations in various combination.

Plant growth and evaluation

Field evaluation of minor *BBL* gene mutations in the TN90 SRC 222 background was conducted in 2017 at research stations in Waynesville and Laurel Springs, NC. Plantings were arranged with single 20 plant rows as the experimental units and managed according to standard practice for burley tobaccos. After air-curing the leaves were stripped, grouped into one of four stalk positions, weighed, and graded by a former USDA tobacco grader. Calculations for grade index were represented as an average across the four stalk positions. Data analysis was conducted using PROC MIXED of SAS9.3 (SAS Institute, Cary, NC).

A preliminary field evaluation for assessing the effects of mutations in minor *BBL* genes in the TN90 222 background was conducted in 2017 at a research station near Clayton, NC. In 2018, a more complete study in both the TN90 and K326 backgrounds was conducted at the Clayton station. In the 2018 studies, the tester and control lines were arranged in a randomized complete block design with 35 replications. Tobacco plants were grown in the field using standard agronomic practices, including topping and the application of suckercide. At harvest, each plant was cut at the base with a machete and weighed using a fish scale. The third leaf from the top of each plant was deveined, dried and analyzed for total alkaloid (primarily nicotine) and reducing sugar content by the NCSU Tobacco Analytical Services Lab. Comparisons among multiple means were conducted according to the Ryan-Einot-Gabriel-Welsch (REGWQ) multiple range test.

Results and Discussion

The BBL Gene Family

In the original manuscript by Kajikawa et al. (2011), the *BBL* gene family was described as being comprised of three major genes, *BBLa, BBLb* and *BBLc*, and one minor gene, *BBLd*, based on the degree to which the genes were transcribed. We conducted a BLAST-based analysis of the various databases found in GenBank to determine whether there was evidence for the existence of any other *BBL* family members within the tobacco genome. These results revealed the presence of two additional *BBL* family members. One of these shared 95% nucleotide identity and 94% predicted amino acid identity with *BBLd*. Given that *BBLd* and this new isoform are very closely related to each other and are distinct from the other *BBL* genes (sharing less than 80% identity with any other gene family member), we referred to these sequences as

BBLd-1 (the original *BBLd* reported by Kajikawa et al., 2011) and *BBLd-2* (the new isoform). The other novel *BBL* family member we identified shares 90 – 92% nucleotide identity with the previously characterized *BBLa*, *BBLb* and *BBLc* sequences. This new family member was designated *BBLe.* The nucleotide sequence and predicted protein sequence identities among the six members of the *BBL* gene family are shown in Table 2.1. The DNA and predicted protein sequences of the minor *BBL* genes *BBLd*-1, *BBLd-2* and *BBLe* are shown in Figure 2.1.

In silico analyses of EST databases using *BBL* gene sequences as the query can serve as a useful indicator to predict the relative contribution of individual members of a gene family toward the overall transcript pool. The results of an *in silico* analysis of the *BBL* family are shown in Table 2.2. Consistent with the characterization of the *BBL* gene family reported by Kajikawa et al. (2011), the *in silico* analysis suggests that *BBLa*, *BBLb* and *BBLc* are the most highly transcribed members of this gene family. This is also consistent with the dramatic reduction in alkaloid content observed in tobacco plants mutated at these three loci (Lewis et al., 2015). Although fewer ESTs were found corresponding to *BBLd-1* and *BBLe*, their presence in the EST database proves that they are in fact transcribed and are therefore likely to be contributing toward the overall BBL activity of the tobacco plant. This is particularly true for *BBLe* given its greater sequence similarity to *BBLa, BBLb* and *BBLc*, as well as its increased representation in the *in silico* analysis compared to *BBLd-1*.

An assessment of the relative levels of *BBL* gene expression can also be inferred from tobacco genomics studies. In Supplementary Table 6 of the manuscript by Sierro et al. (2014), both RNA-seq and Affymetrix-based transcriptome data was shown for the genes involved in the major steps of the tobacco alkaloid pathway. Although that table displayed the *BBL* gene family as being comprised of seven gene, close inspection of the sequence called BBL2-S-2 revealed

that this sequence was a pseudogene, given the presence of a frame-shift mutation within the coding region of the gene (data not shown). Overall, the transcriptomics data presented in Supplementary Table 6 of Sierro et al. (2014) suggested that *BBLa* and *BBLb* are transcribed at high levels; *BBLc* and *BBLe* are transcribed at low levels, and *BBLd-1* and *BBLd-2* are transcribed at very low levels.

Although the minor *BBL* genes, *BBLd-1, BBLd-2* and *BBLe* encode proteins with sequence homology to the major *BBL* gene products, this does not prove that they catalyze the same enzymatic reactions. A standard method for demonstrating enzyme function is to express the gene at high levels in a heterologous system such as *E. coli* or yeast, and assay for function *in vitro* using known or presumed substrates. In the case of tobacco BBL enzymes, however, the purification of recombinant protein from genes expressed in the yeast *Pichia pastoris* followed by incubation in the presence of putative substrates failed to define the specific reaction catalyzed by these enzymes (Kajikawa et al., 2011). To date, there have been no reports of the development of any *in vitro* assay that can be used to measure the enzymatic function of tobacco BBL enzymes.

Genome Editing-Mediated Knockout of Minor BBL Gene Function

Even if an assay existed that could establish whether one or more of the minor *BBL* gene products was capable of catalyzing a reaction involved in the alkaloid biosynthetic pathway, it still may or may not have a measurable impact on alkaloid accumulation, depending on whether it is expressed at sufficiently high levels within the alkaloid producing tissues within the root. Thus, the only way to definitively assess the influence of the minor *BBL* genes on alkaloid biosynthesis above and beyond that which can be attributed to *BBLa, BBLb,* and *BBLc* is to disrupt the function of these genes in a tobacco background that already is triple homozygous

mutant at the three major *BBL* loci. To do this, the custom-designed meganuclease constructs designated BBL7-8x.90 (targeting *BBLd-1* and *BBLd-2*) and BBL1-2x.81 (targeting *BBLe*) were introduced via *Agrobacterium*-mediated transformation into the triple mutant backgrounds K326 222, TN90 222 and TN90 SRC 222 (see Materials and Methods for mutant *bbl* nomenclature description). SRC stands for 'Stable Reduced Converter', a trait defined by mutations in three nicotine demethylase genes whose products are responsible for the enzymatic conversion of nicotine to nornicotine (Lewis et al., 2010).

Over 150 kanamycin resistant T_0 plants were screened for mutations in *BBLd-1* and *BBLd-2*, or *BBLe,* depending on which of the two genome editing constructs was used for transformation. Mutation screening was conducted by isolating genomic DNAs from the leaves of very young T_0 plants and amplifying the regions of interest through PCR using primers flanking the targeted cut sites. The resulting PCR products were subsequently analyzed by DNA sequence analysis. If a genome editing event occurred soon after transformation and prior to the first cell division, this can be readily detected on a sequence chromatogram as the sequence(s) will diverge from WT at the edited site. If there are two distinct sequencing patterns of equal intensity at the site of sequence divergence, one WT and the other edited, we classified this plant as being "monoallelic", as only one of the two alleles of the target gene would appear to have been mutated. Plants possessing a single edited sequence pattern that differed from WT were designated as "biallelic" events. Given the large number of cells that comprise a young tobacco leaf, editing events that may have occurred at a late-stage post-transformation and are only present in a small number of cells would not be detected using this screening method. Plants containing late event mutations would be considered "chimeric" and would be of lesser interest as those editing events would be much less likely to be present in the germline and inherited by

the progeny. A mutation event that occurred after the first cell division but still early in the regeneration process, however, could be represented in high enough copy number to appear on the chromatogram, but may or may not be present in the germline and inherited to the next generation. Genotyping T_1 generation individuals from a mutant T_0 plant is the only definitive way to determine whether a mutation observed in T_0 generation transformants was heritable.

T_0 plants containing genome editing events mediated by constructs BBL7-8x.90 and BBL1-2x.81, and contained a mutant allele that could be clearly defined upon examination of the chromatograms are shown in Figure 2.2. Nuclease-mediated editing is manifest as short deletions and/or insertions emanating from the predicted cut site of the enzyme. Overall, approximately 18% of the T_0 plants transformed with BBL1-2x.81 or BBL7-8x.90 screened positive for mutations at the intended *BBL* loci. Of particular interest were plants harboring deletion/insertion mutations in a *BBLd-1*, *BBLd-2* or *BBLe* gene whose net loss or gain of nucleotides is a number that is not divisible by three, as mutations of this nature would be predicted to lead to a completely nonfunctional protein product given that the reading frame of the gene sequences upstream of the mutation site would not be in frame with those downstream of the mutation. Also of high priority were plants transformed with the BBL7-8x.90 construct that possessed mutations in both *BBLd-1* and *BBLd-2* (such as TN90 SRC 222 plant #36 and K326 222 plant #7), as this would reduce the complexity of the subsequent crossing that would be necessary to combine all three mutations into a single plant.

To pyramid minor *BBL* gene mutations into the TN90 SRC 222 background, genome edited plants #36 and #115 (Fig. 2) were crossed. The progeny from this cross were originally screened with PCR primers specific for the *nptII* selectable marker gene to identify individuals that had lost the genome editing transgene. *NptII*- plants were subsequently screened by

molecular genotyping to identify every mutant gene combination in the TN90 SRC 222 background, namely, TN90 SRC 222200, TN90 SRC 222020, TN90 SRC 222002, TN90 SRC 222220, TN90 SRC 222202, TN90 SRC 222022, and TN90 SRC 222222. In a similar manner, crosses among the mutant T_0 individuals #29, #135 and #143, followed by molecular genotyping resulted in a similar series of mutant lines in the TN90 222 background: TN90 222200, TN90 222020, TN90 222002, TN90 222220, TN90 222202, TN90 222022, and TN90 222222. Finally, K326 222 mutant lines #7 and #42 were intercrossed and genotyped to generate the following lines: K326 222202 and K326 222212. Interestingly, we could never fix the *bbld-2* mutation originating from T_0 plant #7, as all progeny from plants possessing this 10 bp *bbld-2* deletion mutation only gave rise to homozygous WT and heterozygous WT/mutant progeny at a frequency of approximately 1:1.

The *BBL* minor gene mutation series was completed first in the TN90 SRC 222 background. In 2017, the minor *BBL* mutant lines were tested in two different field locations. Although the line TN90 SRC 222 parental line that is homozygous for EMS-induced mutations in the three major *BBL* genes showed dramatic reductions in the major alkaloid nicotine and the minor alkaloids nornicotine, anatabine and anabasine in comparison to WT TN90 SRC, no combination of minor *BBL* gene mutations resulted in further reductions (Fig. 2.3). Cured leaf yield was numerically lower in most lines containing one or more minor *BBL* gene mutations, but these reductions were not deemed statistically significant. Cured leaf quality, as assessed by grade index, was significantly lower in just two of the genotypes possessing at least on additional minor *BBL* mutation: TN90 SRC 222200 and TN90 222220 (Fig. 2.3).

By the 2017 field season, fixed lines representing the TN90 222 minor mutant *BBL* series had yet to be completed, but a small number of each genotype of interest could be identified by

marker screening of a segregating population derived from a self-pollinated TN90 222111 individual. To obtain a preliminary indication of the effects of the minor *BBL* mutations in this background, between 4 and 8 plants per genotype, together with 7 TN90 wild type controls, were grown at the research station in Clayton, NC in a randomized pattern (note: the combination TN90 222020 was omitted because only a single plant of this genotype was identified). At the time of harvest, alkaloid measurements were taken on similarly positioned leaves from each plant in the field. In all tobacco plants possessing three or more *BBL* mutations, anabasine and anatabine levels were extremely low, ranging in content between 0.21 – 0.26 mg/g, and 0.06 – 0.11 mg/g, respectively. Because of the inherent variability that occurs in nicotine to nornicotine conversion among burley plants that lack stabilizing mutations in nicotine demethylase genes (such as TN90 SRC), differences in the combined nicotine + nornicotine content is arguably the best measure for assessing the potential of minor *BBL* mutations to reduce nicotine content in the TN90 222 materials. The means of nicotine content alone, and nicotine + nornicotine are shown in Table 2.4.

Due to the small sample size and lack of a robust experimental design, a statistical analysis was not considered meaningful, and the results obtained from this study must be interpreted cautiously. Nevertheless, the overall trends were interesting. Consistent with our past observations, plants possessing only the triple major *BBL* mutations (TN90 222 000) averaged an 87% reduction in nicotine, and 76% reduction in nicotine + nornicotine in comparison to the TN90 WT control. Numerically, every genotype possessing additional mutations in one or more minor *BBL* gene averaged less alkaloid accumulation than the TN90 222 000 genotype, with the differences being most pronounced when the combined nicotine + nornicotine data are compared. Genotypes possessing additional mutations in minor *BBL* genes averaged between 16

to 25% less nicotine + nornicotine than TN90 222000 segregants from the same population that possessed no minor *BBL* mutations (Table 2.3).

In 2018, a field trial was conducted at the research station near Clayton, NC on what we considered to be the most informative mutant lines developed in the K326 222 and TN90 222 backgrounds. Plants possessing mutations in the three major BBL genes (K326 222000) displayed an 88% reduction in total alkaloid content (nicotine + nornicotine + anabasine + anatabine) compared to WT K326 (Table 2.4). The addition of mutations in the minor BBL genes reduced alkaloid content even further, ranging from 92 - 94% less than that observed in the WT control. Biomass measurements followed a similar trend. The biomass of the K326 triple mutant line averaged 72% of that observed in the WT, and the addition of mutations in the minor *BBL* genes negatively impacted plant growth even further, with the biomass averages ranging from 40 – 49% of the WT. For both traits, the additional reductions in alkaloid levels and biomass conferred by mutating the minor BBL genes was statistically significant.

For plant materials in the TN90 background, the TN90 222 line showed an 88% reduction in total alkaloid content compared to the WT control. The aerial biomass of this line was also significantly reduced, averaging 76% of TN90 WT, and the reducing sugars were modestly higher as well. Unlike the plants in the K326 background, however, the lines with additional mutations in the minor *BBL* genes were essentially indistinguishable from the TN90 222 line for all three traits.

Discussion

When genome editing-mediated knockout mutations in the minor *BBL* genes *BBLd-1*, *BBLd-2* and *BBLe* were combined with EMS-induced mutations in the major *BBL* genes (*BBLa*,

BBLb and *BBLc*) in three distinct genetic backgrounds, the results were mixed. In some genetic backgrounds and/or environments the addition of minor *BBL* mutations was correlated with an incremental decrease in alkaloid beyond that which could be attributed to the major *BBL* gene mutations, whereas in other cases further reductions were not observed. Likewise, in some experiments the minor *BBL* mutations were associated with additional decreases in yield, while no such decreases were observed in others. Consequently, one should proceed with caution when utilizing minor *BBL* mutations in a program of alkaloid reduction.

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Tables and Figures

Table 2.1 Percent nucleotide and predicted amino acid sequence identity among members of the *BBL* gene family. Amino acid identities are shown in parentheses.

	BBLa	***BBLb***	***BBLc***	***BBLd-1***	***BBLd-2***	***BBLe***
BBLa	100 (100)	93 (92.8)	87.7 (83.4)	75.4 (65.1)	75.8 (66.5)	89.5 (86.4)
BBLb		100 (100)	89.4 (84.1)	76.6 (66.7)	77.9 (68.0)	92.4 (89.9)
BBLc			100 (100)	76.8 (68.6)	79 (69.9)	95.6 (87.8)
BBLd-1				100 (100)	95.3 (93.8)	76.6 (67.5)
BBLd-2					100 (100)	78.2 (69.3)
BBLe						100 (100)

Table 2.2 *In silico* analysis of *BBL* gene expression.

BBL Isoform	EST Library Hits	Root-Specific ESTs[a]
BBLa	38	38
BBLb	11	10
BBLc	16	16
BBLd-1	2	1
BBLe	4	4

[a] In addition to cDNA from libraries generated solely from root tissue, cDNAs isolated from pooled leaf, flower, and root cDNA libraries were also included.

Table 2.3 Preliminary analysis of minor *BBL* gene mutations on alkaloid content in a TN90 222 background. Wild type TN90 plants are highlighted in beige; TN90 222 individuals are highlighted in yellow. For both nicotine (left) and nicotine + nornicotine (right), genotypes are listed from greatest to least according to their alkaloid content.

Genotype	# of plants	Nicotine (mg/g)
TN90 WT 000000	7	**37.2039**
TN90 222000	7	**4.7251**
TN90 222222	7	**4.2519**
TN90 222220	4	**3.9062**
TN90 222202	4	**3.8557**
TN90 222002	5	**3.6868**
TN90 222022	8	**3.5519**
TN90 222200	4	**2.8248**

Genotype	# of plants	Nicotine + Nornicotine (mg/g)
TN90 WT 000000	7	**40.1023**
TN90 222000	7	**9.7165**
TN90 222002	5	**8.1233**
TN90 222022	8	**7.8634**
TN90 222222	7	**7.5113**
TN90 222220	4	**7.3984**
TN90 222202	4	**7.3632**
TN90 222200	4	**7.3228**

Table 2.4 Traits of BBL mutant and control lines in K326 background grown at Clayton, NC in 2018.

Line[b]	*BBL* Genotype	Total Alkaloids[a] (% dry weight)	Above-ground Biomass (kg)	Reducing Sugars (%)
K326	WT	1.96 ^{A}	1.79 ^{A}	4.1 ^{B}
K326 222	222000	0.24 ^{B}	1.28 ^{B}	4.4 ^{AB}
K326 1S9	222212	0.16 ^{C}	0.88 ^{C}	5.0 ^{A}
K326 1B4	222202	0.14 ^{C}	0.75 ^{C}	4.2 ^{B}
K326 11F8	222202	0.13 ^{C}	0.72 ^{C}	4.5 ^{AB}

a. Number of plants for each line ranged from 33 to 35.

Table 2.5 Traits of BBL mutant and control lines in TN90 background grown at Clayton, NC in 2018.

Line[b]	*BBL* Genotype	Total Alkaloids[a] (% dry weight)	Above-ground Biomass (kg)	Reducing Sugars (%)
TN90	WT	3.21 ^{A}	0.992 ^{A}	2.1 ^{B}
TN90 222	222000	0.37 ^{B}	0.754 ^{B}	2.5 ^{A}
TN90 4K7	222222	0.44 ^{B}	0.761 ^{B}	2.4 ^{A}
TN90 2F1	222002	0.41 ^{B}	0.766 ^{B}	2.4 ^{A}
TN90 1M9	222220	0.40 ^{B}	0.768 ^{B}	2.4 ^{A}
TN90 1D9	222222	0.40 ^{B}	0.772 ^{B}	2.4 ^{A}

a. Number of plants for each line ranged from 32 to 35.

BBLd-1 (same as GenBank accession AB604221)

ATGAAACGAAATATATCCATGTTTCTTCAGCTTCTGCTCATTATTCTGATGATGATCA
GCTTCTTATTTACTTCTCTTCTTGTACCTTCGGTCTCTGCAACAACTCTCAATACCATT
TCCACCTGTTTAATCAATTACAAAGTCAGTAACTTCTCTGTTTACCCAACAAGGAAT
CATGCTGGTAATAGTTACTATAACTTGCTTGATTTCTCCATTCAGAATCTCCGATTCG
CAGCGTGCTCTAAACCAAAACCAACTGTCATTATCGTACCAGAGAGCAAGGAGCAG
CTGGTGAGCAGCGTTCTGTGTTGCAGACAAGGCTCGTATGAAATCAGAGTAAGGTG
CGGTGGACACAGTTATGAAGGGACTTCATCAGTTTCCTTTGATGGTTCCCCATTTGTG
GTCATTGATTTGATGAAATTAGACGGCGTTTCAGTGGATGTGGATTCAGAAACCGCG
TGGGTACAGGGCGGCGCTACACTTGGCCAGACTTATTATGCCATTTCCCGAGCCAGC
AACGTTCATGGATTTTCAGCTGGTTCTTGCCCAACAGTTGGGGTTGGCGGGCACATT
TCCGGGGGTGGTTACGGATTTTTATCCAGAAAATATGGACTTGCTGCTGATAACGTG
GTGGATGCTCTTCTTGTTGATGCGGAAGGACGGCTATTAGACCGCAAAGCCATGGGA
GAAGAAATCTTTTGGGCCATCAGAGGTGGAGGTGGAGGAATTTGGGGAATCATTTA
CGCCTGGAAAATCCGATTGCTCAAAGTGCCCAAGACCGTGACCAGTTTCATAATCCC
TAGGCCTGGCTCCAAACGATATGTGTCCCAACTAGTTCACAAATGGCAACTTGTTGC
ACCAAAGTTAGAGGATGAATTTTATCTATCGATCTCCATGAGCTCTCCTAGTAAAGG
AAACATTCCTATTGAAATAAATGCCCAATTCAGCGGATTTTACCTAGGTACAAAAAC

CGAAGCCATTTCCATCTTGAATGAGGCCTTTTCGGAGTTGGGAGTTCTGGAAGGTGA
CTGCAAAGAAATGAGTTGGATTGAATCAACACTTTTCTTCTCCGAATTAAATGACGT
TGCGAATTCCTCCGATGTCTCTCGTTTGAAAGAGCGTTACTTTGAAAACAAATCATA
CTTCAAAGCCAAATCAGACTATGTGAAGACCCCAATTTCAGTGGGTGGGATTATGAC
GGCTCTTAATGTTCTTGAGAAAGAACCCAACGGACATGTCATCTTGGACCCTTATGG
TGGAGCCATGCAAAGAATTAGTGAGGAAGCTATTGCTTTCCCTCATAGAAAGGGTA
ACCTTTTCGGAATTCAATATCTAGTAGTGTGGAAAGAAAAGGACAATAATAATATTG
TCAAGAGCAATATTGGGTACATAGAGTGGATAAGAGAGTTTTACAATACAATGGCA
CCCCATGTTTCAAGTTCACCTAGGGCAGCTTATGTCAACTACATGGATCTGGACCTT
GGAGTGATGGACGACTACTTATTGCCATGTACTAGTACTACTGCGTCTGCTAATCAT
GCCGTGGAGAGAGCAAGGGTCTGGGGTGAAAAGTATTTCTTGAATAACTATGATAG
ATTGGTCAAAGCTAAGACAAAAATTGACCCACTAAACGTTTTTCGACATCAACAGG
GCATCCCTCCTTTGTTCGCCTCAATGCAAGAGTATACCTATAGTAGTAAA**TGA**

Predicted amino acid sequence (GenBank Accession BAK18781)

MKRNISMFLQLLLIILMMISFLFTSLLVPSVSATTLNTISTCLINYKVSNFSVYPTRNHAGN
SYYNLLDFSIQNLRFAACSKPKPTVIIVPESKEQLVSSVLCCRQGSYEIRVRCGGHSYEGT
SSVSFDGSPFVVIDLMKLDGVSVDVDSETAWVQGGATLGQTYYAISRASNVHGFSAGS
CPTVGVGGHISGGGYGFLSRKYGLAADNVVDALLVDAEGRLLDRKAMGEEIFWAIRGG
GGGIWGIIYAWKIRLLKVPKTVTSFIIPRPGSKRYVSQLVHKWQLVAPKLEDEFYLSISMS
SPSKGNIPIEINAQFSGFYLGTKTEAISILNEAFSELGVLEGDCKEMSWIESTLFFSELDDV
ANSSDVSRLKERYFENKSYFKAKSDYVKTPISVGGIMTALNVLEKEPNGHVILDPYGGA
MQRISEEAIAFPHRKGNLFGIQYLVVWKEKDNNNIVKSNIGYIEWIREFYNTMAPHVSSS
PRAAYVNYMDLDLGVMDDYLLPCTSTTASANHAVERARVWGEKYFLNNYDRLVKAK
TKIDPLNVFRHQQGIPPLFASMQEYTYSSK

BBLd-2

ATGAAACGAAATATATCCATGTCTCTTCAGCGTTTGCTCATAATTCTGATGATGATC
AGCTTCTTATTTACTTCTCTTCTTGTACCTTCCGTCTCTGCTACAAATCTCAATACCAT
TTCCACCTGTTTGATCAATTACAAAGTCAGTAACTTCTCTGTTTATCCAACAAGGAAT
CATGCTGGTAATAGGTACTATAACTTGCTTGATTTCTCCATTCAGAATCTCCGATTCG
CAGCGTCCTCTAAACCAAAACCAACGGTCATTATCGTACCAGAGAGCAAGGAGCAG
CTGGTGAGCAGCGTTCTGTGTTGCAGACAAGGTTCTTATGAAATCAGAGTAAGGTGC
GGAGGACACAGTTATGAAGGGACTTCTTACGTTTCCTTTGATGGTTCCCCATTTGTG
GTCATTGATTTGATGAAATTAGATGATGTTTCGGTAGATTTGGATTCCGAAACCGCG
TGGGTACAAGGTGGCGCTACACTTGGCCAGACTTATTATGCCATTTCCCGGGCCAGT
GACGTTCATGGATTTTCAGCTGGTTCTTGCCCAACAGTTGGGGTTGGGGGCCACATT
TCCGGGGGTGGCTTTGGATTTTTATCAAGAAAATATGGACTTGCTGCTGATAACGTG
GTGGATGCTCTTCTTGTTGATGCGGAAGGACGGCTATTAGACCGCAAAGCCATGGGA
GAAGAAGTGTTTTGGGCCATCAGAGGTGGTGGTGGAGGAATTTGGGGAATCATTTA
CGCCTGGAAAATCCGATTGCTCAAAGTGCCCAAGACTGTGACTAGTTTCATAGTCCC
TAGGCCTGGCTCCAAACGATATGTGTCCCAACTAGTTCACAAATGGCAACTTGTTGC
ACCAAAGTTAGACGATGACTTTTATCTATCGATCTCCATGAGCTCTGCTAGTAAAGG
AAACATTCCTATTGAAATAAATGCCCAATTCAGCGGATTTTACCTAGGTACAAAAAC
CGAAGCCATTTCCATCTTGAATGAGGCCTTTCCGGAGTTGGGAGTTGTGGAAAGTGA

CTGCAAAGAAATGAGTTGGATTGAATCAACACTTTTCTTCTCCGAATTAGATAACGT
TGCGAACACCTCCGATGTCTCTCGTTTGAAAGAGCGTTACTTTGAAAACAAATCATA
CTTCAAAGCCAAATCAGACCATGTGAAGACCCCAATTTCAGTGGGAGGGATTATGA
CAGCTCTTGATGTTCTTGAGAAAGAACCAAATGGACATGTCATCTTTGACCCTTATG
GTGCAGCCATGCAGAGAATTAGCGAGGAAGCTATTGCTTTCCCTCATAGAAAGGGT
AACCTATTCAGAATTCAATATCTAGTAGTGTGGAAAGAAAAGGACAATAATAATAT
TGCCAAGAGCAATGGGTACATAGAGTGGATAAGAGAGTTTTACAATACAATGGCAC
CCCATGTTTCTAGTTCACCTAGGGCAGCTTATGTCAACTATATGGATCTGGACCTTGG
AGTGATGGACGACTACTTAATGCTAAATACTAGTATTACTGCCTCTGCTGATCATGC
CGTGGAGAGAGCAAGGGTCTGGGGTGAAAAGTATTTCTTGAATAACTATGATAGAT
TGGTCAAAGCTAAGACAAAAATTGACCCACTAAACGTTTTTCGACATCAACAGGGC
ATCCCTCCTATGTTCGCCTCAATGCCAGAGCATACCTATAGTAGTAAA**TGA**

Predicted amino acid sequence

MKRNISMSLQRLLIILMMISFLFTSLLVPSVSATNLNTISTCLINYKVSNFSVYPTRNHAG
NRYYNLLDFSIQNLRFAASSKPKPTVIIVPESKEQLVSSVLCCRQGSYEIRVRCGGHSYEG
TSYVSFDGSPFVVIDLMKLDDVSVDLDSETAWVQGGATLGQTYYAISRASDVHGFSAG
SCPTVGVGGHISGGGFGFLSRKYGLAADNVVDALLVDAEGRLLDRKAMGEEVFWAIRG
GGGGIWGIIYAWKIRLLKVPKTVTSFIVPRPGSKRYVSQLVHKWQLVAPKLDDDFYLSIS
MSSASKGNIPIEINAQFSGFYLGTKTEAISILNEAFPELGVVESDCKEMSWIESTLFFSELD
NVANTSDVSRLKERYFENKSYFKAKSDHVKTPISVGGIMTALDVLEKEPNGHVIFDPYG
AAMQRISEEAIAFPHRKGNLFRIQYLVVWKEKDNNNIAKSNGYIEWIREFYNTMAPHVS
SSPRAAYVNYMDLDLGVMDDYLMLNTSITASADHAVERARVWGEKYFLNNYDRLVK
AKTKIDPLNVFRHQQGIPPMFASMPEHTYSSK

BBLe

ATGTTTCCAATCATAATTCTGATCAGCTTTTCATTTACTTTCCTCTTTGCTAGTGTTAC
TAGTGGAGCAGGAGGAGTTACAAATCTTTCCACCTGTTTAATCAACCACAATGTCCA
TAACTTCTCTATTTACCCCACAAAGAATGATCAAAGTAGTAGTAATTACTTTAACTT
GCTCGATTTTTCCCTTCAGAATCTTCGATTTGCTGCATCTTACATGCCGAAACCAACG
GTCATTATCCTACCAAACAGCAAAGAGGAGCTCGTGAGTACCATTCTTTGTTGCAGA
CAAACATCTTATGAAATCAGAGTAAGGTGCGGAGGACACAGTTACGAGGGAACTTC
TTACGTTTCCTTTGACGGTTCCCCTTTCGTGATCGTTGACTTGATGAAATTAGACGAC
GTTTCAGTAGATTTGGATTCCGAAACAGCTTGGGCTCAGGGCGGCGCAACAATTGGC
CAAATTTATTACGCCATTTCCAGGGTTAGTGACGTTCATGCATTTTCAGCAGGTTCGG
GACCAACAGTAGGATCTGGAGGTCATATTTCAGGTGGCGGCTTTGGACTAATGTCCA
GAAAATTCGGACTCGCTGCTGATAGTGTCGTTGATGCTCTTCTAATTGATGCTGAAG
GACGGTTATTAGACCGGAAAGCCATGGGAGAAGACGTATTTTGGGCAATCAGAGGT
GGCGGCGGTGGAAATTGGGGAATTATTTATGCCTGGAAAATTCGATTACTCAAAGTG
CCTAAAATCGTAACAACTTGTATGATCTATAGGCCTGGATCCAAACAATACGTGGCT
CAACTACTTCAGAAATGGCAAATAGTTACTCCAAATTTGGCCGATGATTTTACTCTA
GGAGTACTCATGAGACCTATAGATCTGCGGGCGGATATGAATTACGGAAATACTAC
TCCTATTGAAACATTTCCCCAATTCAATGCACTTTATTTGGGTCCAAAAACTGAAGC
GGTTTCCATATTAAATGAGGCATTTCCAGAGCTGGACGCTAAGAATGATGACGCCAA
AGAAATGACTTGGATTGAGTCAGCACTTTTCTTTTCCGAATTAGATAACGTATTCGG

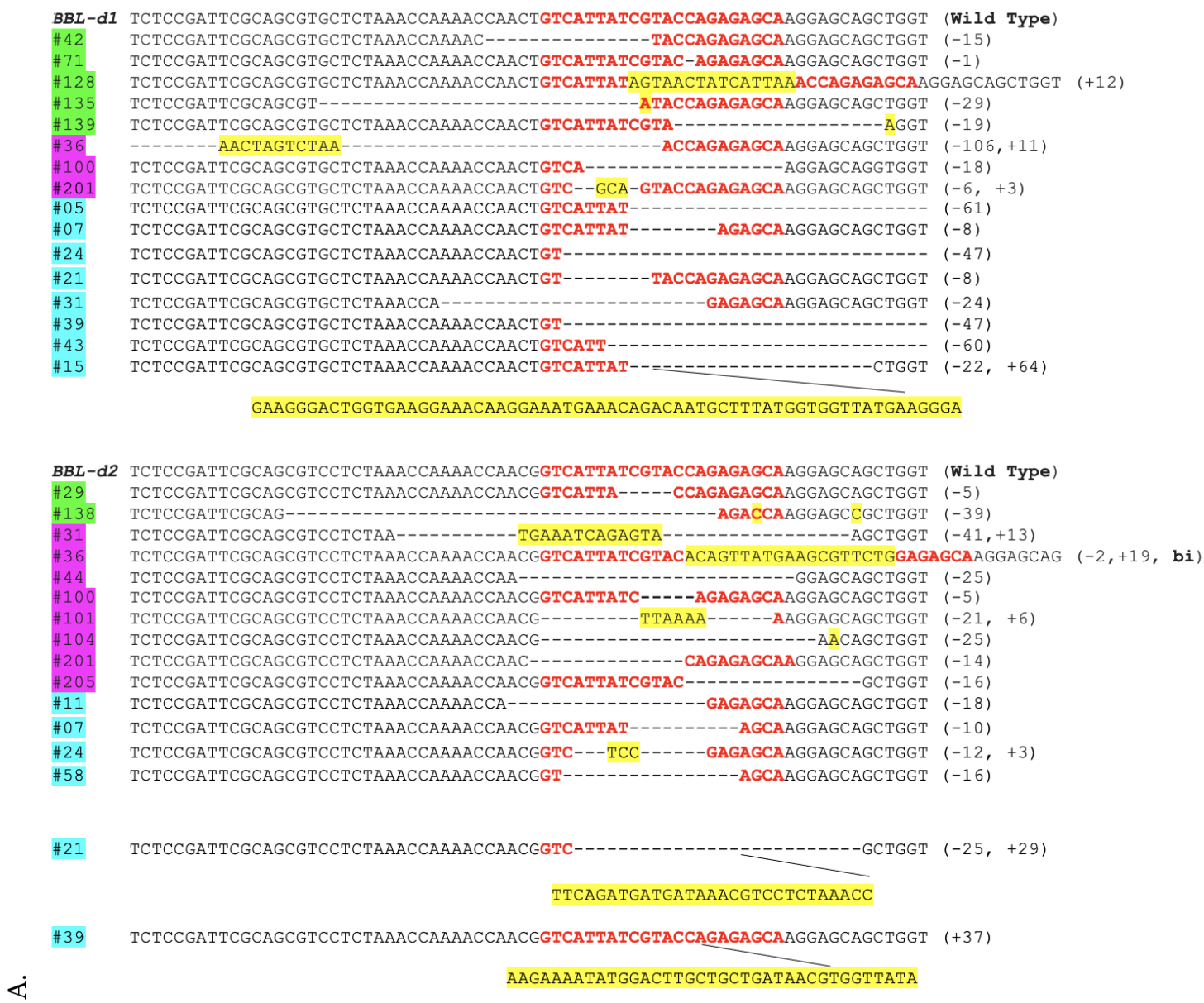
GAACTCCTCTGACGATATCTCCCGTTTGAAAGAACGCTACATGGACGCAAAAACTTT
CTTCAAAGGCAAATCAGATTTTGTGAAGACTCCATTTTCAATGGACGCGATGATGAC
AGCTCTTGTTGAACTCGAGAAAAACCCCAAGTCATTCCTTGTCTTCGATCCTTATGGC
GGAGTCATGGACAAGATTAGTGATCAAGCTATTGCTTTCCCTCATCGAAAGGGTAAC
CTTTTCGCGGTTCAATATTATGCATTTTGGAACGAAGAGGACGATGCCAAGAGCAAC
GAGTACATAGAGTGGACAAGGGGATTTTACAATAAAATGGCGCCTTTTGTTTCAAGC
TCGCCAAGGGGAGCTTATATCAACTACTTGGATATGGATCTTGGAGTGAATATGGAC
GACGACTACTTACTGCGAAATGCTAGTAGTCGTAGTTCTTCTTCCTCTGTTGATGCTG
TGGAGAGAGCTAGAGCGTGGGGTGAAATGTATTTCTTGCATAACTATGATAGGTTGG
TTAAAGCTAAGACACAAATTGATCCACTAAATGTTTTTCGACATGAACAGAGTATAC
CTCCTATGCTTGGTTCAACGCAAGAGCACAGTAGTGAA**TGA**

Predicted amino acid sequence

MFPIIILISFSFTFLFASVTSGAGGVTNLSTCLINHNVHNFSIYPTKNDQSSSNYFNL
LDFSLQNLRFAASYMPKPTVIILPNSKEELVSTILCCRQTSYEIRVRCGGHSYEGTS
YVSFDGSPFVIVDLMKLDDVSVDLDSETAWAQGGATIGQIYYAISRVSDVHAFS
AGSGPTVGSGGHISGGGFGLMSRKFGLAADSVVDALLIDAEGRLLDRKAMGED
VFWAIRGGGGGNWGIIYAWKIRLLKVPKIVTTCMIYRPGSKQYVAQLLQKWQIV
TPNLADDFTLGVLMRPIDLRADMNYGNTTPIETFPQFNALYLGPKTEAVSILNEA
FPELDAKNDDAKEMTWIESALFFSELDNVFGNSSDDISRLKERYMDAKTFFKGK
SDFVKTPFSMDAMMTALVELEKNPKSFLVFDPYGGVMDKISDQAIAFPHRKGNL
FAVQYYAFWNEEDDAKSNEYIEWTRGFYNKMAPFVSSSPRGAYINYLDMDLGV
NMDDDYLLRNASSRSSSSSVDAVERARAWGEMYFLHNYDRLVKAKTQIDPLNV
FRHEQSIPPMLGSTQEHSSE

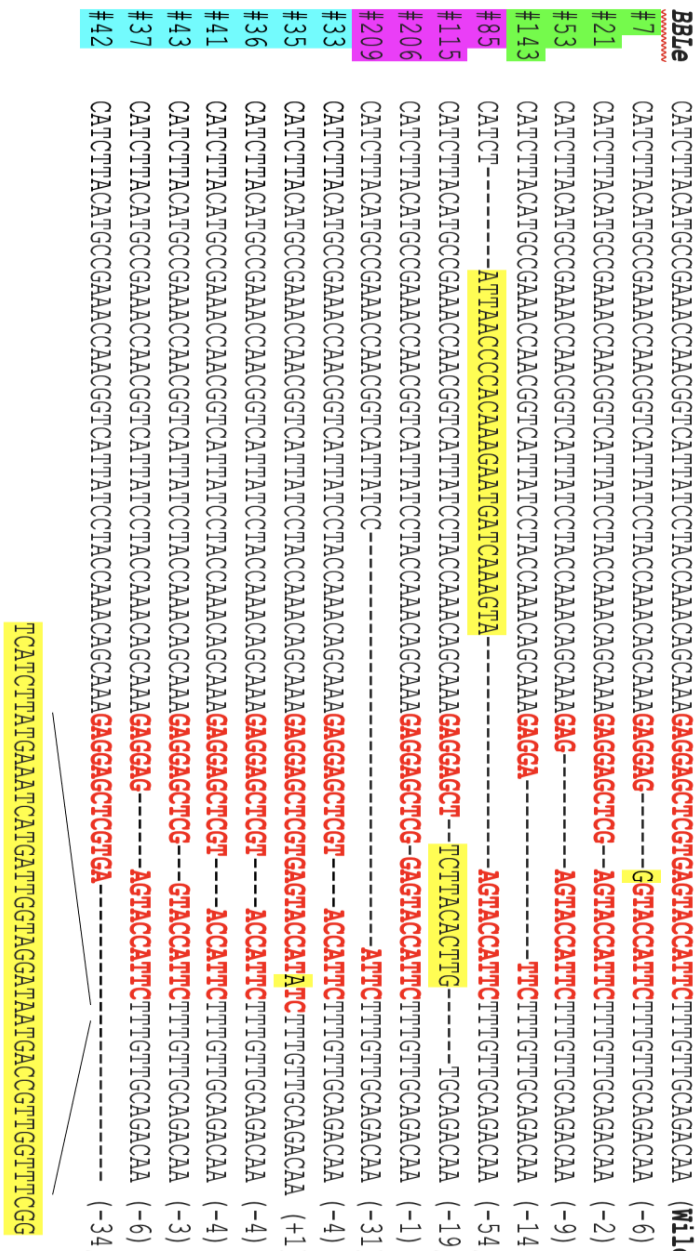
Figure 2.1. DNA and predicted protein sequences of *BBLd-1, BBLd-2* and *BBLe.* Start and stop codons are in bold, underlined type. Locations of the 22 bp target sites of the custom-designed meganuclease enzymes are highlighted in yellow. FAD-binding domain is highlighted green; berberine bridge motif is highlighted in magenta.

Figure 2.2 Genome editing events using custom-designed meganuclease constructs. Wild type sequences in the targeted region of BBLd-1, BBLd-2 (A) and BBLe (B) are shown above the various mutation events identified at that locus. Nucleotides in red represent the 22 bp target site of the designer nuclease. Dashes represent nucleotides that have been deleted, and nucleotides shaded yellow represent insertions. Numbers in parentheses at the right of each T0 plant indicate the size of the deletion and/or insertion event(s) in that plant. In T0 plants where a "bi" is shown in parentheses, the indicated mutation is predicted to be biallelic (i.e. homozygous). For all other T0 plants the mutation is predicted to be monoallelic (heterozygous) or chimeric (not present in all cells of the plant). T0 plants shaded green are in a TN90 222 background; plants shaded purple are in a TN90 SRC 222 background; T0 plants shaded blue are in a K326 222 background.


BBL-d1 TCTCCGATTCGCAGCGTGCTCTAAACCAAAACCAACTGTCATTATCGTACCAGAGAGCAAGGAGCAGCTGGT (Wild Type)
#42 TCTCCGATTCGCAGCGTGCTCTAAACCAAAAC----------TACCAGAGAGCAAGGAGCAGCTGGT (-15)
#71 TCTCCGATTCGCAGCGTGCTCTAAACCAAAACCAACTGTCATTATCGTAC-AGAGAGCAAGGAGCAGCTGGT (-1)
#128 TCTCCGATTCGCAGCGTGCTCTAAACCAAAACCAACTGTCATTATAGTAACTATCATTAAACCAGAGAGCAAGGAGCAGCTGGT (+12)
#135 TCTCCGATTCGCAGCGT----------ATACCAGAGAGCAAGGAGCAGCTGGT (-29)
#139 TCTCCGATTCGCAGCGTGCTCTAAACCAAAACCAACTGTCATTATCGTA----------AGGT (-19)
#36 --------AACTAGTCTAA----------ACCAGAGAGCAAGGAGCAGCTGGT (-106,+11)
#100 TCTCCGATTCGCAGCGTGCTCTAAACCAAAACCAACTGTCA----------AGGAGCAGGTGGT (-18)
#201 TCTCCGATTCGCAGCGTGCTCTAAACCAAAACCAACTGTC--GCA-GTACCAGAGAGCAAGGAGCAGCTGGT (-6, +3)
#05 TCTCCGATTCGCAGCGTGCTCTAAACCAAAACCAACTGTCATTAT---------- (-61)
#07 TCTCCGATTCGCAGCGTGCTCTAAACCAAAACCAACTGTCATTAT--------AGAGCAAGGAGCAGCTGGT (-8)
#24 TCTCCGATTCGCAGCGTGCTCTAAACCAAAACCAACTGT---------- (-47)
#21 TCTCCGATTCGCAGCGTGCTCTAAACCAAAACCAACTGT--------TACCAGAGAGCAAGGAGCAGCTGGT (-8)
#31 TCTCCGATTCGCAGCGTGCTCTAAACCA----------GAGAGCAAGGAGCAGCTGGT (-24)
#39 TCTCCGATTCGCAGCGTGCTCTAAACCAAAACCAACTGT---------- (-47)
#43 TCTCCGATTCGCAGCGTGCTCTAAACCAAAACCAACTGTCATT---------- (-60)
#15 TCTCCGATTCGCAGCGTGCTCTAAACCAAAACCAACTGTCATTAT----------CTGGT (-22, +64)
GAAGGGACTGGTGAAGGAAACAAGGAAATGAAACAGACAATGCTTTATGGTGGTTATGAAGGGA
BBL-d2 TCTCCGATTCGCAGCGTCCTCTAAACCAAAACCAACGGTCATTATCGTACCAGAGAGCAAGGAGCAGCTGGT (Wild Type)
#29 TCTCCGATTCGCAGCGTCCTCTAAACCAAAACCAACGGTCATTA-----CCAGAGAGCAAGGAGCAGCTGGT (-5)
#138 TCTCCGATTCGCAG----------AGACCAAGGAGCCGCTGGT (-39)
#31 TCTCCGATTCGCAGCGTCCTCTAA----------TGAAATCAGAGTA----------AGCTGGT (-41,+13)
#36 TCTCCGATTCGCAGCGTCCTCTAAACCAAAACCAACGGTCATTATCGTACACAGTTATGAAGCGTTCTGGAGAGCAAGGAGCAG (-2,+19, bi)
#44 TCTCCGATTCGCAGCGTCCTCTAAACCAAAACCAA----------GGAGCAGCTGGT (-25)
#100 TCTCCGATTCGCAGCGTCCTCTAAACCAAAACCAACGGTCATTATC-----AGAGAGCAAGGAGCAGCTGGT (-5)
#101 TCTCCGATTCGCAGCGTCCTCTAAACCAAAACCAACG---------TTAAAA------AAGGAGCAGCTGGT (-21, +6)
#104 TCTCCGATTCGCAGCGTCCTCTAAACCAAAACCAACG----------AACAGCTGGT (-25)
#201 TCTCCGATTCGCAGCGTCCTCTAAACCAAAACCAAC----------CAGAGAGCAAGGAGCAGCTGGT (-14)
#205 TCTCCGATTCGCAGCGTCCTCTAAACCAAAACCAACGGTCATTATCGTAC----------GCTGGT (-16)
#11 TCTCCGATTCGCAGCGTCCTCTAAACCAAAACCA----------GAGAGCAAGGAGCAGCTGGT (-18)
#07 TCTCCGATTCGCAGCGTCCTCTAAACCAAAACCAACGGTCATTAT----------AGCAAGGAGCAGCTGGT (-10)
#24 TCTCCGATTCGCAGCGTCCTCTAAACCAAAACCAACGGTC---TCC------GAGAGCAAGGAGCAGCTGGT (-12, +3)
#58 TCTCCGATTCGCAGCGTCCTCTAAACCAAAACCAACGGT----------AGCAAGGAGCAGCTGGT (-16)
#21 TCTCCGATTCGCAGCGTCCTCTAAACCAAAACCAACGGTC----------GCTGGT (-25, +29)
TTCAGATGATGATAAACGTCCTCTAAACC
#39 TCTCCGATTCGCAGCGTCCTCTAAACCAAAACCAACGGTCATTATCGTACCAGAGAGCAAGGAGCAGCTGGT (+37)
AAGAAAATATGGACTTGCTGCTGATAACGTGGTTATA

A.

B.


BBLe
#7
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#53
#143
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#35
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#43
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#42
CATCTTACATGCCGAAACCAACGGTCATTATCCTACCAAACAGCAAAGAGGAGCTCGTGAGTACCATTCTTTGTTGCAGACAA (Wild Type)
CATCTTACATGCCGAAACCAACGGTCATTATCCTACCAAACAGCAAAGAGGAG-----GGTACCATTCTTTGTTGCAGACAA (-6)
CATCTTACATGCCGAAACCAACGGTCATTATCCTACCAAACAGCAAAGAGGAGCTCG--AGTACCATTCTTTGTTGCAGACAA (-2)
CATCTTACATGCCGAAACCAACGGTCATTATCCTACCAAACAGCAAAGAG---------AGTACCATTCTTTGTTGCAGACAA (-9)
CATCTTACATGCCGAAACCAACGGTCATTATCCTACCAAACAGCAAAGAGGA----------TTCTTTGTTGCAGACAA (-14 bi)
CATCT-------ATTAACCCCACAAAGAATGATCAAAGTA----------AGTACCATTCTTTGTTGCAGACAA (-54, +28)
CATCTTACATGCCGAAACCAACGGTCATTATCCTACCAAACAGCAAAGAGGAGCT--TCTTACACTTG-----TGCAGACAA (-19, +11)
CATCTTACATGCCGAAACCAACGGTCATTATCCTACCAAACAGCAAAGAGGAGCTCG-GAGTACCATTCTTTGTTGCAGACAA (-1)
CATCTTACATGCCGAAACCAACGGTCATTATCC----------ATTCTTTGTTGCAGACAA (-31)
CATCTTACATGCCGAAACCAACGGTCATTATCCTACCAAACAGCAAAGAGGAGCTCGT----ACCATTCTTTGTTGCAGACAA (-4)
CATCTTACATGCCGAAACCAACGGTCATTATCCTACCAAACAGCAAAGAGGAGCTCGTGAGTACCATATCTTTGTTGCAGACAA (+1)
CATCTTACATGCCGAAACCAACGGTCATTATCCTACCAAACAGCAAAGAGGAGCTCGT----ACCATTCTTTGTTGCAGACAA (-4)
CATCTTACATGCCGAAACCAACGGTCATTATCCTACCAAACAGCAAAGAGGAGCTCGT----ACCATTCTTTGTTGCAGACAA (-4)
CATCTTACATGCCGAAACCAACGGTCATTATCCTACCAAACAGCAAAGAGGAGCTCG---GTACCATTCTTTGTTGCAGACAA (-3)
CATCTTACATGCCGAAACCAACGGTCATTATCCTACCAAACAGCAAAGAGGAG-----AGTACCATTCTTTGTTGCAGACAA (-6)
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(-34,+47 bi)

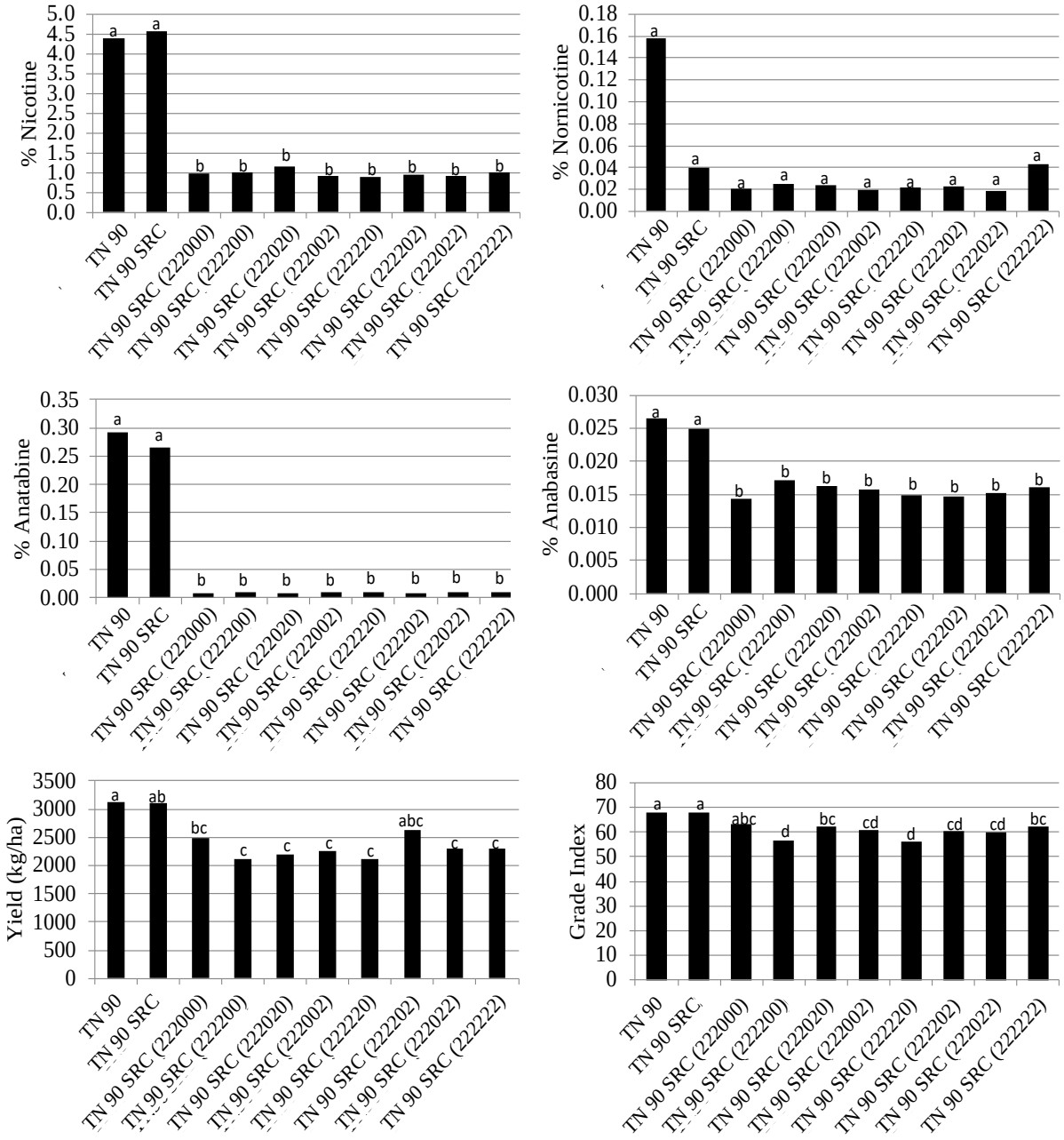

Figure 2.3. Field evaluation of minor *bbl* mutations. Tobacco plants were evaluated for alkaloid content, yield, and grade index. Means represent averages from two independent field environments. Means with different letters above the bar graphs are significantly different at the $P < 0.05$ significance level. Figure taken from Lewis et al. 2020.

CHAPTER 3

Knockout of a Key Gene of the Nicotine Biosynthetic Pathway Severely Affects Tobacco Growth under Field, but not Greenhouse Conditions

(manuscript submitted to BMC Research Notes)

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Abstract

Objective: There is great interest in developing tobacco plants containing minimal amounts of the addictive compound nicotine. Quinolate phosphoribosyltransferase (QPT) is an important enzyme both for primary (NAD production) and secondary (pyridine alkaloid biosynthesis) metabolism in tobacco. The duplication of an ancestral QPT gene in *Nicotiana* species has resulted in two closely related QPT gene isoforms: *QPT1* which is expressed at modest levels throughout the plant, and *QPT2* which is coordinately regulated with genes dedicated to alkaloid biosynthesis. This study evaluated the utility of knocking out *QPT2* function as a means for producing low alkaloid tobacco plants.

Results: CRISPR/Cas9 vectors were developed to specifically mutate the tobacco *QPT2* genes associated with alkaloid production. Greenhouse-grown *qpt2* plants accumulated dramatically less nicotine than controls, while displaying only modest growth differences. In contrast, when *qpt2* lines were transplanted to a field environment, plant growth and development was severely inhibited. Two conclusions can be inferred from this work: (1) *QPT1* gene function alone appears to be inadequate for meeting the QPT demands of the plant for primary metabolism when grown in a field environment; and (2) the complete knockout of *QPT2* function is not a viable strategy for producing agronomically useful, low nicotine tobaccos.

Keywords: quinolate phosphoribosyltransferase, CRISPR/Cas9, low nicotine, *Nicotiana tabacum*

Introduction

Nicotine is the most abundant alkaloid produced in tobacco plants, typically accounting for >90% of the total alkaloid pool, and 2 – 6% of the leaf biomass (dry weight) when grown commercially. In recent years there has been increased interest in developing tobacco varieties that possess very low levels of nicotine while retaining acceptable agronomic qualities. Two of the motivating factors behind this interest include: (1) evidence from numerous clinical studies that have shown that smokers who switch to cigarettes containing nicotine levels below that capable of sustaining an addiction response will smoke less and/or find it easier to quit (reviewed in [Donney et al., 2022]); and (2) the possibility that the US Food and Drug Association (FDA) may mandate such reductions in future cigarette products (USDA, 2018). From a plant genetic perspective, lowering the nicotine content of the leaf can be accomplished through the utilization of naturally occurring mutations, genetic engineering, and genome editing (Xie et al., 2004; Lewis and Martinez, 2020; Qi et al., 2021). In the majority of cases reported to date, however, the genetic alterations of nicotine content do not decrease the nicotine levels below FDA's target threshold to assure the failure to initiate or sustain an addiction response and/or are negatively associated with agronomic and quality traits (Lewis et al., 2018). Thus, there is a need to discover alternative genetic traits that can confer sufficiently low levels of nicotine while retaining agronomic acceptability.

Genes encoding the enzyme quinolate phosphoribosyltransferase (QPT) are intriguing targets for reducing nicotine in tobacco. Unlike most of the steps the nicotine biosynthetic pathway that are dedicated solely to alkaloid production, QPT plays a critical role in primary metabolism as well, serving as the entry point into the pyridine nucleotide pathway responsible for production of the ubiquitous cellular co-factor NAD (Dewey et al., 2013). Characterization of

the *QPT* gene family in *Nicotiana* revealed the presence of two closely-related isoforms: *QPT1* which is constitutively expressed at a relatively low level throughout the plant, and *QPT2* whose expression is induced at high levels in root tissue in response to stimuli known to activate alkaloid production (Shoji et al., 2011; Ryan et al., 2012). These distinct expression patterns led to the proposal that the ancient duplication of a housekeeping *QPT* gene resulted in the evolution of an isoform primarily dedicated to alkaloid biosynthesis (*QPT2*) while retaining a copy that could serve the needs for primary metabolism (*QPT1*) (Shoji et al., 2011).

Because *QPT1* and *QPT2* share 94% nucleotide sequence identity, it would be difficult if not impossible to use techniques such as RNA inhibition (RNAi) or antisense-suppression to down regulate one isoform without simultaneously inhibiting the other. Indeed, when *QPT* gene function was suppressed using an RNAi construct driven by the CaMV 35S promoter, total *QPT* transcript reduction led to severe stunting, abnormal leaf and flower morphologies, and photosynthetic deficiencies in lab and greenhouse grown plants (Khan et al., 2017). When an anti-sense suppression strategy was used to down regulate *QPT* activity specifically within the root, however, low nicotine lines that were otherwise phenotypically normal were reported (Xie et al., 2004). Because neither of these strategies would be predicted to uniquely repress a single *QPT* isoform, they could not address the issue of whether the targeted disruption of *QPT2* alone represents a viable means for generating low nicotine tobaccos. In this study, genome editing was used to introduce frame-shift mutations in the *QPT2* genes of two commercial tobacco varieties, and alkaloid profiles and growth characteristics were measured in plants grown both in a greenhouse and field environment.

Methods

Targeted mutagenesis of QPT2

The *QPT2*-specific target sequence 5'-AGCCACCAAGAATACAAGAG-3' was cloned into the sgRNA cassette of the CRISPR/Cas9 vector pGREB31 (Addgene) and transformed into tobacco varieties K326 and TN90 using *Agrobacterium* as previously described (Bovert et al., 2002). T_0 plants were screened for mutations in *QPT2_T*, *QPT2_S*, *QPT1_T* and *QPT1_S* by PCR amplification using primers specific for each gene that flank the target site, followed by DNA sequence analysis. The primers used in this study and PCR conditions are listed in Table 3.1; DNA sequencing was conducted at the NCSU Genomic Sciences Laboratory (https://research.ncsu.edu/gsl). T_1 generation lines were screened for the absence of the *hptII* selectable marker, as well as the absence of segregating WT *QPT2* loci.

Greenhouse and field growth and evaluation

Seeds were germinated and grown in a growth chamber for 48 days. Twelve plants per genotype (T_2 generation) were subsequently transplanted to 9" pots and transferred to a greenhouse. Each plant was topped upon the first appearance of bud formation and suckers were removed manually for the next 10 days. The mid-rib was removed from leaves selected for alkaloid analysis and the lamina was dried to completeness in a drying oven. Alkaloid analysis was conducted by the NCSU Tobacco Analytical Services Lab as described previously (Lewis et al., 2015). For field-grown plants, seeds were sown on float trays in a greenhouse for 73 days with occasional mowing to promote root growth. Transplants were transferred to the field and grown using standard agronomic production practices. Statistical analysis was performed on the various measurements by conducting individual t-tests between mutant *qpt2* lines and their relevant WT controls.

Results

To determine the effects of knocking out *QPT2* function into tobacco, two varieties representing each of the major tobacco market types, flue-cured (K326) and burley (TN90), were selected as the recipient backgrounds. Previous tobacco genome analyses revealed that TN90 possesses the *QPT2_T* and *QPT2_S* genes derived from the ancestral species *N. tomentosiformis* and *N. sylvestris*, respectively, while K326 only contains the *QPT2_T* copy (Sierro et al., 2014). The 20 bp CRISPR-Cas9 target sequence that was selected for *QPT2* gene disruption is shown in Fig. 3.1. This sequence was selected because: (1) no other sequence in any public draft of the tobacco genome perfectly matched this 20mer; and (2) there is a 3 bp indel and two single nucleotide polymorphisms between these 20 bp and the comparable region of *QPT1* which should restrict targeted mutagenesis to *QPT2* isoforms.

K326 and TN90 were transformed with the *QPT2*-targeting CRISPR/Cas9 construct, and dozens of T_0 individuals were screened for *QPT2* mutations. One K326 line (K19) and two TN90 lines (T8 and T21) were selected for further analysis. K19 was biallelic for a 1 bp insertion and 1 bp deletion at the *QPT1_T* locus (Fig. 3.1). T8 was homozygous for the same 1 bp insertion at *QPT1_T* and was monoallelic for a 2 bp deletion at *QPT2_S*. T21 was monoallelic for a 1 bp insertion at *QPT1_T* and was homozygous for a 1 bp insertion in *QPT2_S*. Each of these mutations caused frame shifts that would lead to premature stop codons anywhere from 12 to 76 bp downstream of the mutation. Given that only the first 33 aa of what is normally a 351 aa protein would be retained in each of these mutant loci, it was assumed that these mutations would result in the complete loss of gene function. PCR amplification and sequence analysis of the *QPT1* genes in these same individuals confirmed that no *QPT1* gene had been mutated. Plants K19, T8 and T21 were self-pollinated and numerous T_1 progeny were genotyped to

identify those that had lost the CRISPR/Cas9 vector and were homozygous mutant at all *QPT2* loci.

Twelve T_2 generation individuals for each of the *qpt2* mutant genotypes, along with their corresponding WT controls, were randomized within the same greenhouse and grown to maturity. Upon the first observation of bud formation, the date was recorded and the plant was topped by excising the floral meristem together with the first 6 – 8 immature leaves. The plants were grown an additional 10 days post-topping, at which time the following data were collected: plant height, leaf number and total leaf weight. In addition, an equivalently positioned leaf (5^{th} or 6^{th} leaf from the top) was selected for alkaloid analysis.

In both the TN90 and K326 backgrounds, plant height was reduced between 13 – 20%, and leaf number decreased by an average of 2 – 4 leaves per plant in the *qpt2* mutant lines (Fig. 3.2A and 3.2C). Total leaf weight was decreased approximately 13% and 17% in lines T8 and 19, respectively, in comparison to their WT controls; a more substantial decrease of 27% was observed in line T21 (Fig. 3.2D). Line T21 also displayed the greatest difference in flowering time, with buds appearing on average 11 days later than WT TN90 plants. T8 plants flowered an average of 6 days later than WT (Fig. 3.2B). Flue-cured line K19 flowered on average 4.5 days earlier than its WT control, but this difference was not considered statistically significant.

In contrast to the modest differences in overall growth phenotypes observed between WT tobaccos and their corresponding *qpt2* mutants, alkaloid profiles differed dramatically (Fig. 3.2E and 3.2F). Nicotine levels in the *qpt2* lines were reduced between 91 – 96%; similar reductions were observed in nornicotine content. Anatabine and anabasine comparisons were not included because their levels were below the level of quantification in the majority of the *qpt2* individuals.

Overall, the results from the greenhouse study suggested that knocking out *QPT2* function in tobacco may represent a viable means for producing low alkaloid tobacco and warranted further evaluation in a field environment. In keeping with traditional agronomic practice, seeds from each line were initially planted in float trays in a greenhouse prior to transplanting the young plantlets to the field. Within the greenhouse float trays there were no obvious phenotypic differences between the *qpt2* mutant and control lines (W. Smith, personal observation). Tobaccos in the burley (T8, T21 and TN90 WT) and flue-cured (K19, K326 WT and an unrelated low nicotine line in K326) backgrounds were transplanted to the field in separate experimental plots, comprised of 30 plants per line planted in a randomized complete block design. Surprisingly, the growth of all three *qpt2* lines was extraordinarily suppressed in the field. By 32 days post-transplant, *qpt2* individuals were just marginally larger than when transplanted from the float trays (Fig. 3.3). As the growing season continued and the control plants grew large, shading provided an additional impediment to their growth. As a result, none of the *qpt2* lines within the designed experiments grew taller than 30 cm, nor did they flower, which precluded the ability to obtain meaningful alkaloid data. By chance, however, extra K19 plants had been chosen to serve as border rows for an unrelated experiment within the same field. Despite remaining stunted throughout the entire growth season, in the absence of undue shading competition, most of the K19 border plants developed to the extent where they initiated flowering. The K19 border plants were topped, treated with suckercide and a subset assayed for alkaloid content. Fig. 3.4 shows representative K19 border plants on the day of harvest, and how the average nicotine content of the *qpt2* plants was 99% reduced in comparison to that observed in K326 WT plants grown in a different part of the same field.

Conclusions

Our results support the notion that *QPT1* isoforms alone can largely accommodate the plant's need for NAD, and other products of the pyridine nucleotide cycle, under conditions of minimal environmental stress, and that an additional contribution from *QPT2* genes is required when grown in the field. The physiological demands of outdoor growth that necessitate this additional contribution are unknown but may include: (1) stresses associated with transplant shock when transferred from the greenhouse to the field; (2) mechanical stresses caused by exposure to wind; and (3) increased temperature extremes and variable water availability. Although genome editing-mediated knockout of *QPT2* loci yielded tobaccos with dramatically reduced leaf nicotine content, the associated negative impacts on plant growth and development under standard field conditions precludes the use of lines possessing these mutations for the commercial production of low nicotine tobaccos.

Limitations

The main limitation was that this study was conducted during a single year in a single field environment. It is thus possible that the detrimental effects of knocking out *qpt2* function may not always be as extreme as documented in this report. Nevertheless, the fact that field growth can, if even only under certain environments, result in the type of severe growth reduction reported here would prevent consideration of mutations of this nature for commercial deployment.

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Tables And Figures

Table 3.1.
Primers and PCR conditions used in this study

Gene	Forward primer 5'-3'	Reverse primer 5'-3'	Denaturation Temp/time	Annealing Temp/time	Extension Temp/time
QPT2_T	ATAAACCATGTTTAGGGCTC	TGTTACTATGCACTAACAGT	98°C/10 sec	60°C/15 sec	72°C/15sec
QPT2_S	AAAACCATGTTTAGAGCTA	AAGTTCTATGCACTAACGGG	98°C/10 sec	60°C/15 sec	72°C/15sec
QPT1_T	CTGCAATAGTCCACCCTAATGC	ACTTCCTAGCGCGAATCTGG	98°C/10 sec	64°C/15 sec	72°C/15sec
QPT1_S	TGCAATAGTGCACCCTCATGC	GGCCACAAAGCGGGTACCGG	98°C/10 sec	66°C/15 sec	72°C/15sec
hptII	GTGTACGCCCGACAGTCCCGGC	CCCGATTCCGGAAGTGCTTGAC	98°C/10 sec	63°C/10 sec	72°C/15sec

All reactions were initiated with a 30 sec denaturation at 98°C for 30 sec, and terminated with a 7 min extension at 72°C after 33 cycles. Reactions were conducted using Phusion Taq and reaction buffer (New England Biolabs – cat. #M0530L).

```
WTQPT2_T   74 ...TGTCAGCAATAGCCACCAAGAATACAAGAGTGGAGTCATTAGAGGTGAAGCCA... 126
           26 ...   S  A  I  A  T  K  N  T  R  V  E  S  L  E  V  K  P... 42

T8            ...TGTCAGCAATAGCCACCAAGAATACAAAGAGTGGAGTCATTAGAGGTGAAGCCA...
T21           ...TGTCAGCAATAGCCACCAAGAATACAAAGAGTGGAGTCATTAGAGGTGAAGCCA...
K19 allele 1  ...TGTCAGCAATAGCCACCAAGAATACAAAGAGTGGAGTCATTAGAGGTGAAGCCA...
K19 allele 2  ...TGTCAGCAATAGCCACCAAGAATACA-GAGTGGAGTCATTAGAGGTGAAGCCA...


WTQPT2_S   74 ...TGTCAGCAATAGCCACCAAGAATACAAGAGTGGAGTCATTAGAGGTGAAACCA... 126
           26 ...   S  A  I  A  T  K  N  T  R  V  E  S  L  E  V  K  P... 42
T8            ...TGTCAGCAATAGCCACCAAGAATAC--GAGTGGAGTCATTAGAGGTGAAACCA...
T21           ...TGTCAGCAATAGCCACCAAGAATACAAAGAGTGGAGTCATTAGAGGTGAAACCA...
```

Figure 3.1 CRISPR/Cas9-induced knockout mutations in *QPT2_T* and *QPT2_S*. The 20 bp sequence targeted by the nuclease is underlined and the PAM site is shown in red type. The mutations found in the three lines that were characterized in the greenhouse and field are shown. Inserted nucleotides and the positions of deleted nucleotides (dashes) are represented in green type. The numbers in black type correspond to the *QPT2* cDNA sequences beginning at the start ATG codon; orange numbers represent amino acid position. The nucleotides shaded gray highlight the single nucleotide polymorphism where *QPT2_T* and *QPT2_S* differ in this region.

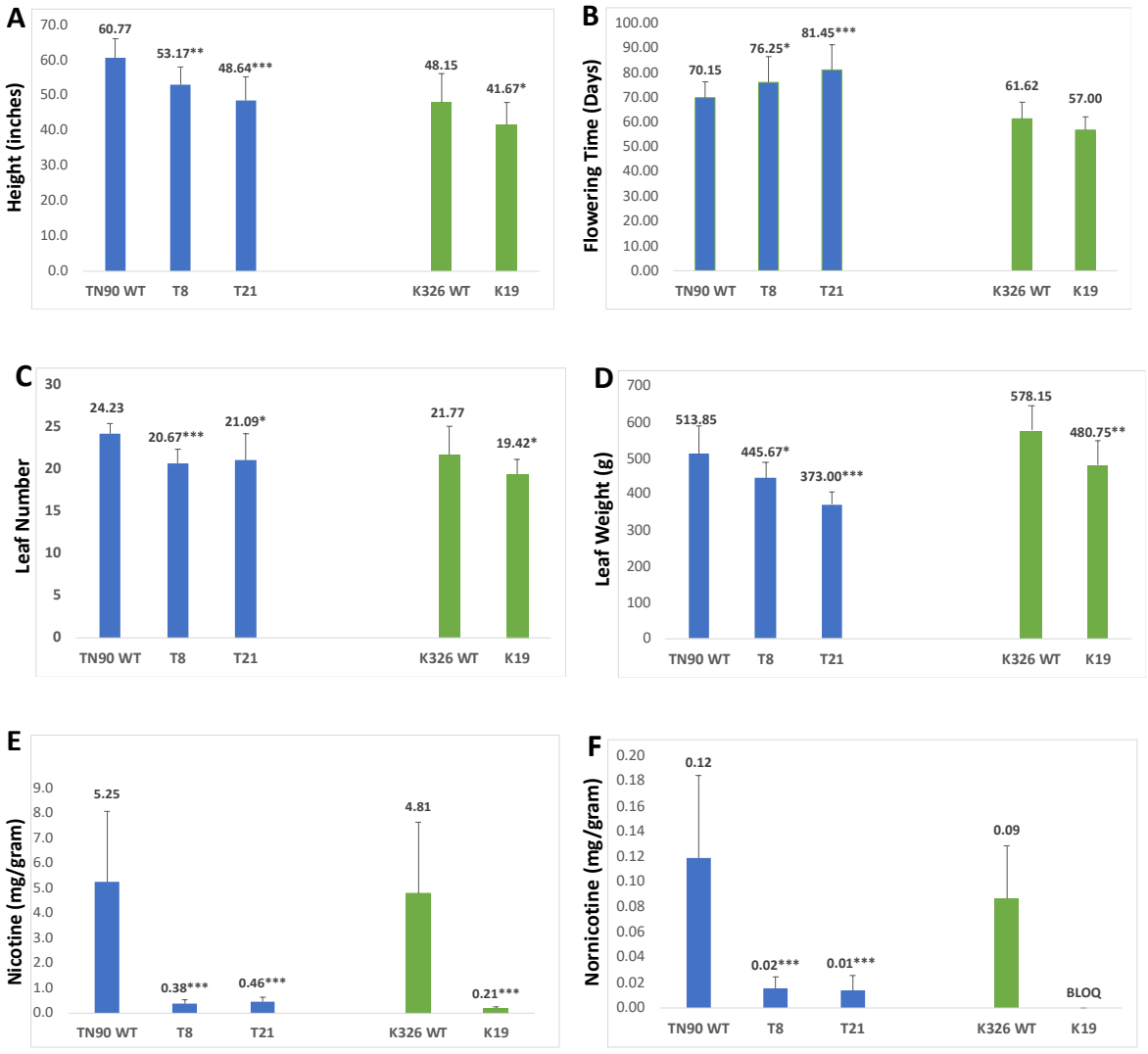
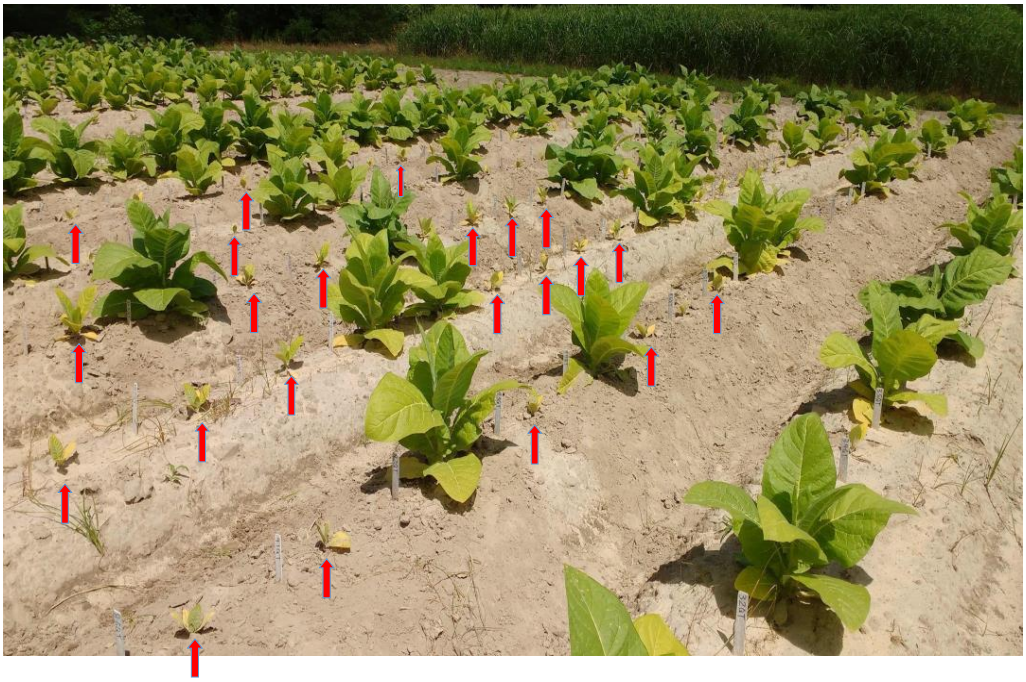


Figure 3.2 Growth characteristics and alkaloid content of greenhouse-grown tobacco plants possessing knockout mutations in *QPT2* genes. Blue bar graphs show data comparisons among mutant lines T8 and T21 and their corresponding TN90 WT control; green bar graphs depict the K19 mutant and its K326 WT control. Measurements include: plant height **(A)**, flowering time **(B)**, leaf number **(C)**, leaf weight **(D)**, nicotine **(E)** and nornicotine **(F)**. For flowering time, days = days post transplant and transfer to the greenhouse. Means and standard error bars are shown above each genotype. Asterisks indicate significant differences between *qpt2* mutant lines and their corresponding WT control at $P < 0.05$ (*), $P < 0.01$ (**) and $P < 0.001$ (***) as determined by t-tests. BLOQ, below level of quantification.

A



B

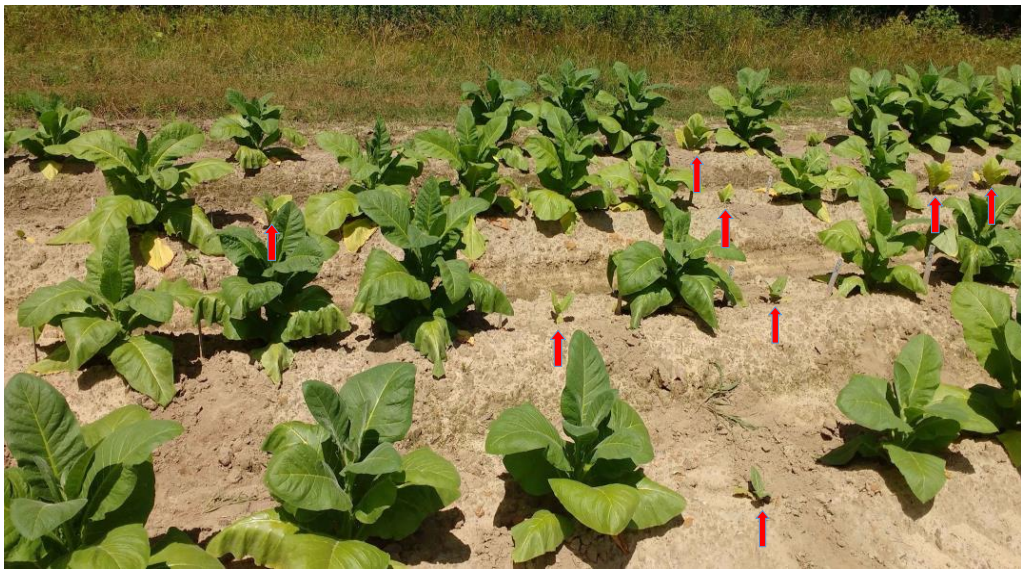


Figure 3.3 Tobacco plants harboring *qpt2* knockout mutations display a severe growth phenotype when grown in a field environment. (A) Control and mutant lines T8 and T21 in the TN90 background; **(B)** Controls and mutant line K19 in the K326 background. Tester and control lines were randomized within rows. Red arrows point to plants homozygous for *qpt2* mutations; full-sized plants correspond to control and other entries. Picture was taken 32 days after transplant.

A

B

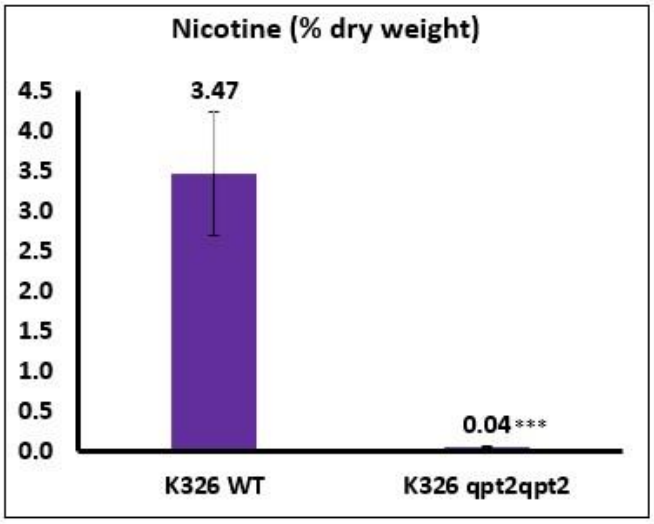


Figure 3.4 K19 (qpt2_t/qpt2_t) border row plants on the day of field harvest (110 days after transplant). (**A**) Average nicotine content from 19 topped K19 border plants (K326 qpt2qpt2) and 19 topped K326 WT plants grown in a separate part of the field is shown on the accompanying graph (**B**). Means ± standard errors of means are shown. The difference in nicotine content was significant at $P < 0.001$ as determined by a t-test.

Chapter 4

Genome Editing Mediated Knockout of *Methylputrescine Oxidase* (*MPO*) and *A622* Gene Function in *Nicotiana tabacum*

Introduction

The pyridine alkaloids of tobacco (*Nicotiana tabacum* L.) are among the most studied group of plant secondary compounds in plants. Nicotine constitutes greater than 90% of the total alkaloid pool in most tobacco genotypes, and is primarily responsible for the pharmacological response experienced by users of tobacco products. In decreasing order of relative abundance, the remaining major alkaloids in tobacco include anatabine, nornicotine, and anabasine. Alkaloid levels in tobacco are influenced by environmental conditions, interactions with plant pests, and plant genetics.

Although nicotine is the primary compound that gives the users of tobacco products the pharmacological effect they seek, there are several circumstances where it would be desirable to develop products using tobacco plants that produce and accumulate very low levels of nicotine. For example, some studies have shown that the use of low-nicotine cigarettes as a component in smoking cessation strategies can help smokers who are trying to quit (Hatsukami et al., 2010; Donny et al., 2014). Other reports have demonstrated that by lowering the nicotine levels below a critical threshold in tobacco products, they can no longer initiate or maintain an addiction response (Benowitz and Henningfield, 1994; Benowitz et al., 2007). Studies such as these may ultimately influence regulatory agencies, such as the U.S. Food and Drug Administration (FDA), who have been given the authority to determine what acceptable levels of various tobacco constituents (including nicotine) will be allowable in cigarettes and other tobacco products. The

World Health Organization (2015) has recommended that nicotine levels of cigarette tobacco filler be reduced to non-addictive levels of 0.4 mg/g, or below.

Tobacco alkaloid levels are also of interest because of their roles as precursors in the production of tobacco specific nitrosamines (TSNAs), a potent group of recognized carcinogens (Hecht, 1998, 2003; Hecht and Hoffman, 1989). The most problematic TSNAs are *N*-nitrosonornicotine (NNN) and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), which are derived through nitrosation reactions with nornicotine and an oxidative derivative of nicotine (such as pseudooxynicotine), respectively, during the curing, storage, and combustion of tobacco. Because tobacco alkaloids serve as essential precursors in TSNA formation, low alkaloid tobacco plants have also been shown to produce reduced amounts of TSNAs within the cured leaf (Xie et al., 2004).

As detailed in the Literature Review (Chapter 1), numerous reports have shown that mutating select steps in the alkaloid biosynthetic pathway can lead to a reduction in the amount of nicotine and other pyridine alkaloids that are produced in the plant. At the onset of this thesis project, two of the steps of the alkaloid pathway whose potential as targets for producing low nicotine tobaccos that had not been adequately explored included those catalyzed by methylputrescine oxidase (MPO) and the NADPH-dependent isoflavone reductase-like enzyme known as A622.

The MPO enzyme is encoded by two closely related isoforms, designated *MPO1.1* and *MPO1.2* by Kajikawa et al. (2017). *MPO1.1* originated from the *N. sylvestris* ancestor of *N. tabacum*, whereas as *MPO1.2* originated from the *N. tomentosiformis*-like progenitor species. *MPO1.1* and *MPO1.2* share approximately 97% nucleotide and amino acid identity. *In vitro* assays of recombinant MPO1.1 enzyme produced in *E. coli* were used to demonstrate enzymatic

function. These assays showed that MPO1.1 utilized *N*-methylputrescine as a substrate more efficiently than any other diamine tested (Heim et al., 2007; Katoh et al., 2007). The tobacco genome also contains another set of closely related genes, designated *MPO2.1* and *MPO2.2*. The predicted MPO2 enzymes share 88% amino acid identity and 96% homology to their MPO1 counterparts (Katoh et al., 2007). Subsequent studies revealed that MPO2 enzymes preferentially accept nonmethylated amines as substrates over their corresponding *N*-methylated versions. Furthermore, transcript analyses showed that *MPO1* genes are coordinately regulated with other alkaloid biosynthetic genes and are highly expressed in the root, in contrast to *MPO2* transcripts that are expressed at low levels constitutively throughout the plant. These observations prompted one group of researchers to propose changing the nomenclature of MPO2 to the more generalized term diamine oxidase (DAO) 1 (Naconsie et al., 2014). The *MPO2.1* (*DAO1.1*) gene originated from *N. sylvestris* and the *MPO2.2* (*DAO1.2*) gene originated from the *N. tomentosiformis*-like ancestor (Kajikawa et al., 2017).

When anti-*MPO* RNAi constructs were introduced into tobacco hairy root cultures, 93 – 99% reductions in nicotine were accompanied by 5- to 10-fold increases in anatabine content, making this normally low abundance species the predominant alkaloid in these materials (Shoji and Hashimoto, 2008). Similarly, BY-2 culture cells which are naturally low in *MPO* gene activity also produce anatabine as the most abundant alkaloid species. In addition to the *MPO* gene family, RNAi suppression of *ODC* and *PMT* genes also results in low nicotine plants with exceptionally high anatabine levels (Chintapakorn and Hamill, 2003; Wang et al., 2009; DeBoer et al., 2011; Dalton et al., 2016). As shown in Fig. 1 of Chapter 1, these three gene families encode enzymes responsible for the synthesis of the pyrrolidine ring of nicotine. Of the four most abundant alkaloids in tobacco, anatabine is the only one that solely utilizes the pyridine ring

pathway for the synthesis of both of its ringed structures. It has been proposed that inhibition of *ODC, PMT* and *MPO* expression results in a reduction in the pool of pyrrolidine ring precursors required for nicotine (and nornicotine) synthesis. The insufficient supply of pyrrolidine rings, in turn, is believed to lead to an overaccumulation of pyridine ring precursors, which by default become incorporated into anatabine (Dewey and Xie, 2013).

A622 was the name given to an enzyme that has been proposed to function in the pyridine branch of alkaloid biosynthesis. The *A622* gene was first discovered in the same study that lead to the identification of *PMT1* (Hibi et al., 1994). Later studies demonstrated that the tobacco genome possesses exactly two *A622* isoforms, one originating from the *N. sylvestris* progenitor, and the other from the *N. tomentosiformis* ancestral species (Shoji et al., 2002; Sierro et al. 2014; Kajikawa et al., 2017). The expression pattern of *A622* genes mimics that of *PMT* and other alkaloid biosynthetic genes (Shoji et al., 2002; Cane et al., 2005; Kidd et al., 2006), an observation that lead to the speculation that it was also a member of the pathway. Defining the specific reaction catalyzed by A622 enzymes, however, has been problematic. *In vitro* enzyme assays using recombinant A622 protein, NADPH and potential substrates failed to reveal the enzyme's specific function (Shoji et al., 2002).

In the wild species *N. glauca* that accumulates anabasine as the most predominant pyridine alkaloid, RNAi mediated suppression of *A622* caused substantial reductions in anabasine, nicotine and anatabine in otherwise normal looking plants (DeBoer et al., 2009). In *N. tabacum,* however, researchers who attempted to use RNAi constructs to suppress *A622* were unable to regenerate whole plants where *A622* had been down regulated. As an alternative, the effects of *A622* suppression were studied by placing the anti-*A622* RNAi constructs under a β-estradiol inducible promoter and introducing it into tobacco hairy roots and BY-2 cell cultures

(Kajikawa et al., 2009). Upon induction, pyridine alkaloid levels were greatly reduced and a large increase in the compound nicotinic acid β-N-glucoside (NaNG) was observed. NaNG has been characterized in plants as a nicotinic acid detoxification product, as opposed to a metabolic intermediate. These results suggested that in the absence of A622, nicotinic acid may accumulate to the level where it becomes toxic to the cell (Kajikawa et al, 2009).

In this project, custom-designed meganuclease and CRISPR/Cas9 genome editing tools were used to introduce knockout mutations into *MPO* and *A622* genes, respectively, in both burley (TN90) and flue-cured (K326) tobacco varieties. In addition to the wild-type version of these cultivars, knockout mutations were introduced into TN90 and K326 lines that possessed EMS-induced mutations in the three major *BBL* genes, *BBLa*, *BBLb* and *BBLc.* The effects of *MPO* and *A622* mutagenesis (both alone and in combination with *BBL* mutations) on plant growth characteristics and alkaloid content were measured in greenhouse grown plants.

Materials And Methods

Meganuclease-Mediated Knockout of *MPO* Genes

A proprietary meganuclease construct (generated by Precision Biosciences, Durham, NC) was strategically designed to recognize the 22mer sequence 5'-ATCTATGGGTTACACAATATGC-3' that is perfectly conserved in *MPO1.1*, *MPO1.2* and *MPO2.2*. Due to a single polymorphism located at position 15 of the 22mer sequence in *MPO2.1*, this *MPO* isoform only matches 21 of the 22 bp of the *MPO*-specific ARCUS recognition site. The meganuclease construct was cloned downstream of an enhanced CaMV 35S promoter, then inserted into the plant expression vector pCAMBIA2300 (https://www.abcam.com/pcambia2300-plant-expression-vector-ab275758.html). The *MPO*-targeting vector was transformed into *Agrobacterium* strain LBA

4404 and introduced into tobacco varieties K326, TN90 and their triple mutant *bbl* counterparts K326 222 and TN90 222 (Lewis et al. 2020) using standard *Agrobacterium*-based transformation protocols (Horsch et al. 1985). DNA sequence analyses of the PCR products using isoform-specific primers (Table 4.1) were conducted to determine whether an induced mutation(s) was introduced at that locus. Given that only a single polymorphism occurs with the 22 bp recognition sequence of *MPO2.1*, genotyping was conducted on this *MPO* isoform as well. In most cases, examination of the resulting sequence chromatograms enabled the categorization of the individual *MPO* genes as falling into one of the following categories: (1) homozygous WT at both alleles; (2) heterozygous for a meganuclease-induced mutation at one allele and WT at the other; (3) homozygous for the same induced mutation at both alleles; or (4) heterozygous for one specific meganuclease-induced mutation at one allele and possessing a different meganuclease-induced mutation at the other allele. Because previous studies suggested that *MPO1.1* and *MPO1.2* are the predominant, if not sole, sources of MPO enzyme activity in tobacco plants that contribute toward alkaloid production (Naconsie et al., 2014), priority was given to T_0 individuals where at least one knockout mutant allele in both *MPO1.1* and *MPO1.2* could be identified. T_0 generation transgenic plants that were identified as possessing induced mutations in *MPO* genes (as determined by DNA sequence analysis) were self-pollinated and genotyped using primers specific to the *nptII* selectable marker gene, then at each *MPO* locus to identify plants that had lost the meganuclease construct via segregation and were fixed for deleterious mutations at each *MPO* locus of interest.

CRISPR/Cas9 Mediated Knockout of *A622* Genes

To permanently disable *A622-S* and *A622-T* gene function, CRISPR/Cas9 constructs were created using the CRISPR/Cas9 vector pRGEB31 (obtained from the non-profit company Addgene), targeting regions conserved in both *A622* isoforms. Three separate pRGEB31-based constructs were generated that targeted the following three *A622*-specific sequences near the 5' end of the gene: target site 1 = 5'-CTGTCGGAGGACAGCAATTT-3'; target site 2 = 5'-GTGGTGATTTCCACTGTCGG-3'; target site 3 = 5'-ATCAAAGCAATTAAAGAAGC-3'. Each CRISPR/Cas9 construct was transformed into TN90 and K326 using *Agrobacterium*-mediated transformation and hygromycin as the selection agent. As described above for *MPO* mutagenesis, DNA sequence analyses of the PCR products using isoform-specific primers that flank the target site of *A662-S* and *A662_T* (Table 4.1) were conducted to determine whether an induced mutation(s) was introduced at that locus. T_0 plants of interest were self-fertilized and T_1 progeny were genotyped with primers specific to the *hptII* selectable marker gene, then at each *A662* locus to identify plants that had lost the pRGEB31 vector insert via segregation and were fixed for deleterious mutations at both *A662-S* and *A622-T*.

Evaluation of Greenhouse Grown Plants

For both the *MPO* and *A662* experiments, a minimum of 10 T_2 generation plants for each genotype of interest were grown in the greenhouse for analysis (with the exception of the *mpo* mutant line K23 which was only represented by 5 individuals due to poor seed set). Plants were grown in 10" pots using a standard soil mix. At the first sign of floral bud development, the date was recorded as the time of flowering and the plants were topped. Suckers were removed by hand for a 10-day period, after which a similar sized upper stalk position leaf was harvested,

dried, and ground for alkaloid analysis as previously described (Lewis et al. 2015). Other measurements taken at harvest included total leaf number, plant height, and total leaf weight. Statistical analysis was conducted using SAS 9.2 software (SAS Institute, Cary, NC).

Results

Genome Editing Mediated Mutagenesis of *MPO* Loci

The fixed mutant lines selected for analysis are listed in Table 4.2, and the specific meganuclease-induced mutations found in each line are shown in Figure 4.1. In the K326 WT background, two lines (K11 and K23) were produced that carried mutant *mpo1.1*, *mpo1.2* and *mpo2.2* alleles. Two independent lines were also obtained in K326 222 (*bbla/bblb/bblc*). In KB19, all four *MPO* genes possessed knockout mutations. The fact that we were able to recover a mutation in *MPO2.1* in this plant was somewhat unexpected given that there is a polymorphism in the 22 bp ARCUS recognition site in this gene (Fig. 4.1). The other mutant line in K326 222, designated KB20, is mutant at both *MPO1* loci, and wild type at the two *MPO2* genes. Our genome editing-mediated targeted mutagenesis effort in the burley market type TN90 yielded two lines, one in TN90 WT (T23) and the other in TN90 222 (TB7). Both lines carry knockout *mpo1.1*, *mpo1.2* and *mpo2.2* alleles.

To determine the effect of the *mpo* mutations on overall plant growth and alkaloid accumulation, the progeny of T_1 generation plants whose allelic complement of mutations had been verified by DNA sequence analysis were grown to maturity in a greenhouse. As shown in Figure 4.2, minimal differences were observed in plant height, flowering time and leaf number among the *mpo* mutant lines. Although there were no significant differences in leaf weight between the two K326 lines containing *mpo* mutations (K11 and K23) versus wild-type K326,

when combined with the triple mutant *bbla/bblb/bblc* trait, decreases of 18% and 30% were observed in lines KB19 and KB20, respectively. In the TN90 background, total leaf weight was reduced by 15% in *mpo* mutant lines both with and without the addition of the *bbl* mutations.

Alkaloid analysis was conducted on leaves in a similar upper position from plants that had been topped a previous ten days, during which time the suckers were removed by hand. As shown in Figure 4.3, *mpo* mutations resulted in nicotine reductions between 93 – 96% in the wild-type K326 and TN90 backgrounds, and dropped to greater than 99% when the *mpo* mutations were combined with the three *bbl* mutations. Comparisons between KB19 and KB20 were of particular interest since the former possessed mutations in all four *MPO* genes, whereas the latter was only mutated at the two *MPO1* loci. None of the four alkaloids measured were lower in KB19 compared with KB20, suggesting that *MPO2.1* and *MPO2.2* do not play a significant role in alkaloid biosynthesis.

The effects of *MPO* mutagenesis on the anatabine content was particularly noteworthy. Similar to RNAi-mediated *MPO* suppression in hairy roots (Shoji and Hashimoto, 2008), knocking out *MPO* gene function in the K326 and TN90 WT backgrounds resulted in a 3- to 7-fold higher accumulation of anatabine compared to their respective controls. However, when *MPO* mutations were introduced into the triple *bbl* mutant K326 222 and TN90 222 backgrounds, the anatabine content dropped to a level that was numerically lower than their wild-type counterparts (though not to an extent deemed statistically significant).

Nornicotine levels were significantly lower in all of the lines possessing *MPO* mutations in comparison to their wild-type controls. Because the nornicotine content was below the limits of quantification for four of the lines, however, we could not assess whether combining *mpo* with *bbl* mutations had an impact on this trait beyond that attainable using the *mpo* mutations alone.

Interestingly, the anabasine content in lines K11 and K23 with only *mpo* mutations was nearly double that observed in K326 wild-type, but this phenomenon was not observed in the TN90 background. Regardless, in each of the K326 222 and TN90 222 backgrounds, the amount of anabasine observed was either equivalent to the WT control (KB19), or below the level of quantification (KB20 and TB7) (Fig. 4.3).

Genome Editing Mediated Mutagenesis of *A622 Loci*

Young T_0 plants transformed with CRISPR/Cas9 constructs targeting three distinct 20 bp sequences at the 5' end of *A622* were analyzed for induced mutations at the cleavage site by PCR and DNA sequence analysis. The number of transgenic plants analyzed for each construct and background, along with the editing efficiency, is shown in Table 4.3. CRISPR/Cas9 vectors directed against targets sites 1 and 2 were exceptionally efficient, with nearly every T_0 plant possessing at least one indel within *A622-S* and/or *A622-T.* In contrast, the construct targeting site 3 yielded no mutations in either *A622* isoform. The high level of mutagenesis efficiency is even more apparent when one looks at each of the four *A622* alleles within a given plant (Table 4.4). T_0 plants transformed with the construct directed against target site 2 resulted in several individuals possessing mutations in all four *A622-S* and *A622-T* alleles. These plants grew slowly in the lab, however, and most of them died from what appeared to be a *Pythium* infection in the soil.

We were ultimately able to generate transgene-free lines with double-homozygous mutant *a622-s* and *a622-t* loci in both K326 and TN90. In K326, lines designated C2, C8 and C21 were derived from a T_0 plant possessing mutations at target site 2, and in TN90, lines

designated F14, F21, and F30 originated from a T_0 plant possessing mutations in target site 1. The specific mutations found in each mutant line are shown in Figure 4.4.

The effects of *A662* mutations on plant growth characteristics are shown in Figure 4.5. Average plant height of the *a622* mutant lines was reduced between 16 – 25% in the TN90 background. Paradoxically, *a622* mutants in the K326 background were 14 – 18% taller than the wild-type control (Fig. 4.5A). Another difference between the effect of *a622* mutations in TN90 versus K326 was observed in the average date to flowering. Flowering time was extended an average of 13 – 32 days across the three mutant lines in TN90, while no differences in flowering were observed in the K326 background (Fig. 4.5B). Two of the three mutant TN90 lines (F14 and F21) averaged ~3 fewer leaves per plant, while no significant differences in leaf number were seen in the K326 materials (Fig. 4.5C). Among the growth parameters measured, the most dramatic differences were observed in the total leaf weight at harvest. Major reductions in leaf biomass were associated with *A622* inactivation in both TN90 and K326 (Fig. 4.5D). A 39 – 50% reduction in total leaf weight was observed in TN90, and a 20 – 26% reduction was observed in K326.

The effects of the *A622* mutations on the four major alkaloids is shown in Fig 4.6. Nicotine levels were reduced by a factor of ~400-fold in both the K326 and TN90 backgrounds (in order to view the values on the same graph, a log scale was used on the y-axis representing nicotine content in Fig. 4.6A). The levels of nornicotine, anatabine, and anabasine were all below the levels of quantification in all *a622* mutant lines (Fig. 4.6B-D).

Discussion

Using genome editing-based knockout of *MPO* and *A622* genes, we demonstrated that dramatic reductions in the nicotine content of the leaf can be achieved in whole tobacco plants grown in a greenhouse environment. One characteristic of the low nicotine tobaccos developed by *MPO* disruption is that these plants display an accompanying increase in anatabine to levels well above that which is found in normal tobacco plants. Because the pharmacological effects of anatabine and/or its derivatives have been minimally investigated in comparison to nicotine, the high anatabine trait could prove detrimental to regulatory approval by the FDA, or consumer acceptance. We conclude that combining *mpo* mutations with *bbl* mutations confers two marked advantages: (1) the nicotine levels in *mpo* + *bbl* mutant plants is significantly lower than that attainable by the *mpo* and *bbl* mutations individually; and (2) the potentially problematic elevated anatabine trait associated with *MPO* inhibition becomes ameliorated when combined with the three *bbl* mutations.

Investigators who have studied the *MPO* gene family in tobacco have raised questions regarding which members actively contribute toward alkaloid production. Specifically, due to the low level of *MPO2* transcript accumulation in the alkaloid-producing cells of the root, together with the preference for recombinant MPO2 enzymes to accept non-*N*-methylated amine substrates over their corresponding *N*-methylated versions, it has been proposed that *MPO2* genes/enzymes may contribute little, if any, toward alkaloid biosynthesis (Naconsie et al., 2014). Our observations that none of the four alkaloids assayed in line KB19 (*mpo1/1mpo1.1, mpo1.2*/*mpo1.2 mpo2.1/mpo2.1, mpo2.2/mpo2.2*) were lower than that found in KB20 (*mpo1/1mpo1.1, mpo1.2*/*mpo1.2 MPO2.1/MPO2.1, MPO2.2/MPO2.2*), are supportive of the

hypothesis that *MPO2* genes and their corresponding enzymes are not involved in pyridine alkaloid biosynthesis.

As emphasized in Chapter 1, the only way to determine whether a given alkaloid reduction strategy shows legitimate promise as a means to develop commercially acceptable tobacco cultivars that can reliably meet the 0.4 mg/g threshold proposed by the FDA is through field analysis. Very recently, a study was published involving the field testing of K326 plants that contained an RNAi construct directed against the *MPO* family, including lines that also possessed the triple mutant *bbla/bblb/bblc* trait (Kernodle et al. 2022). Although the lines possessing the anti-RNAi *MPO* construct + mutant *bbl* loci showed a moderate yield penalty, they averaged less than 0.4 mg/g nicotine and displayed a favorable grade index (Kernodle et al., 2022). These results suggest that the genome edited *mpo* mutant + *bbl* lines developed in our study may represent a viable ***nontransgenic*** means for producing acceptable ultra-low nicotine tobacco cultivars. The series of *mpo* mutant lines that were tested in the greenhouse (Table 4.2) are being grown in the field for the first time in 2022.

The results of the greenhouse evaluation of our *A662* mutant materials suggest that mutations in this step of the pathway can severely reduce alkaloid accumulation. However, they also imposed a greater growth penalty than was observed when mutating *MPO* (or combining *mpo* + *bbl* mutations). During the course of this study, a report was published where *A622* was similarly knockout out using CRISPR/Cas9 technology (Burner et al., 2022). A field trial conducted using the mutant *A622* lines showed that under field conditions, overall plant growth was vastly reduced, causing the authors to conclude that knocking out *A622* gene function is incompatible with the production of economically viable tobacco plants. Their findings are very reminiscent of the results that we observed when knocking out *QPT2* genes (Chapter 3).

Overall Conclusions

Chapters 2 – 4 described four different strategies designed to produce ultra-low nicotine tobaccos in a manner that retains acceptable yield and quality standards. Three of the strategies proved to be of either questionable value (knockout of minor *BBL* genes), or were clearly associated with severe growth defects (*QPT2* and *A622* inactivation). The most promising strategy involved combining mutations in *MPO* genes with mutations in the three major *BBL* genes. Field evaluations will ultimately determine whether this mutant combination can reliably produce a cured leaf product with less than 0.4 mg/g without overly compromising quality and yield.

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Figures and Tables

Table 4.1 PCR Primers used in this study.

MPO Genes	Primer Sequence
MPO1_S_F2	5’ TCACTATGACCAGTGCATGC 3’
MPO1_S_R2	5’ TCCCAAACACTACCATTGCAG 3’
MPO1_T_F2	5’ CCCTTTTGATGCTTGGTTGT 3’
MPO1_T_R2	5’ TCGACCATTTGACATTTTGC 3’
MPO2_T_F1	5’ TCCAATATCTGCTAGCTCAAAACA 3’
MPO2_T_R1	5’ GTTAAAGTGTGTGTGTTTGGC 3’
MPO2_S_F2	5’ TCGCATCCCCAACCTAGTAAC 3’
MPO2_S_R2	5’ ACAGTTGTCGGAAACGAACC 3’
A622_S	
A622_S_F	5’-ATTACATACATCGACAATAGAAGAA-3’
A622_S_R	5’-AATTTATCTCGTTTGTGTGGTAAT-3’
A622_T	
A622_T_F	5’-TTATATACATCGACAATATAACTTG-3’
A622_T_R	5’-AATTTGTCTCTTTCGTATGGTATA-3’
NptII	
NptII_F	5'-GAACAAGATGGATTGCACGC-3'
NptII_R	5'-AGAAGGCGATAGAAGGCGAT-3'
HptII	
HptII-F	5'-GTGTACGCCCGACAGTCCCGGC-3'
HptII-R	5'-CCCGATTCCGGAAGTGCTTGAC-3'

Table 4.2 Meganuclease induced mutations in tobacco *MPO* genes. Some lines are segregating for two mutations at select *MPO* loci. The e allele mutations at these loci are separated by a forward slash (/). Del=deletion; Ins=insertion. Lines labeled 222 posses EMS-induced knockout mutations in the genes *BBLa*, *BBLb*, and *BBLc* alternative as described in Lewis et al., 2020.

Line	**MPO1.1**	**MPO2.1**	**MPO1.2**	**MPO2.2**
K11 (K326 WT background)	7 bp del	WT	1 bp ins / 8 bp del	222 bp del
K23 (K326 WT background)	1 bp ins	WT	49 bp del + 3 bp ins	22 bp del + 5 bp ins
KB19 (K326 222 background)	4 bp del / 1 bp ins	1 bp del	7 bp del	25 bp ins + 4 bp del / 48 bp del + 1 bp ins
KB20 (K326 222 background)	25 bp del	WT	11 bp del / 87 bp del	WT
T23 (TN90 WT background)	1 bp ins	WT	190 bp del + 4 bp ins	1 bp ins / 27 bp del + 16 bp ins
TB7 (TN90 222 background)	1 bp del / 14 bp del	WT	19 bp del + 3 bp ins	1 bp ins

Table 4.3. CRISPR/Cas9-mediated mutagenesis of tobacco *A622* genes. Efficiency is defined as the number of T_0 plants that were shown to possess at least one induced mutation in either *A622-S* or *A622-T* over the total number of T_0 plants examined.

Construct	TN90		K326	
	transgenic plants	Efficiency (S&T)	transgenic plants	Efficiency (S&T)
A622 pRGEB31-Target1	8	8/8 (100%)	6	6/6 (100%)
A622 pRGEB31-Target2	12	11/12 (92%)	10	10/10 (100%)
A622 pRGEB31-Target3	11	0	16	0

Table 4.4. Genotypes of tobacco plants transformed with constructs targeting *A622* genes. A lowercase s or t indicates a mutated *A622-S* and *A622-T* allele, respectively. Numbers shown represent the total number of T_0 plants from a given transformation experiment possessing the indicated *A622* phenotype. A "chimeric" plant is one that possesses an induced mutation, but at a sub-stoichiometric level suggesting that the mutation is not found in all cells of the plant and therefore may not be heritable.

	Construct	sstt	Sstt	ssTt	SSTt	SsTT	SsTt	SSTT	Chimeric
TN90	A622 pRGEB31-Target1	0	0	1	2	0	3	0	2
	A622 pRGEB31-Target2	5	1	1	0	0	1	1	3
	A622 pRGEB31-Target3	0	0	0	0	0	0	16	0
K326	A622 pRGEB31-Target1	0	0	2	1	0	1	0	2
	A622 pRGEB31-Target2	2	0	0	1	0	1	0	6
	A622 pRGEB31-Target3	0	0	0	0	0	0	16	0

***MPO1.1* mutations**

```
WT Seq          …CGAAATTTTTGAGAAGAGCTGCATTTCTGAAGCACAATCTATGGGTTACACAATATGCACCTGGAGAAGATTTTCCAGGAGGAGAGTTCC…
K11             …CGAAATTTTTGAGAAGAGCTGCATTTCTGAAGCACAAT-------TTACACAATATGCACCTGGAGAAGATTTTCCAGGAGGAGAGTTCC…
K23             …CGAAATTTTTGAGAAGAGCTGCATTTCTGAAGCACAATCTATGGGTTTACACAATATGCACCTGGAGAAGATTTTCCAGGAGGAGAGTTCC…
KB19 allele 1   …CGAAATTTTTGAGAAGAGCTGCATTTCTGAAGCACAATCTATGGGTTTACACAATATGCACCTGGAGAAGATTTTCCAGGAGGAGAGTTCC…
KB19 allele 2   …CGAAATTTTTGAGAAGAGCTGCATTTCTGAAGCACAATCTATGGGTTACA----ATGCACCTGGAGAAGATTTTCCAGGAGGAGAGTTCC…
KB20            …CGAAATTTTTGAGAAGAGCTGCATTTCTGA-------------------------TGCACCTGGAGAAGATTTTCCAGGAGGAGAGTTCC…
T23             …CGAAATTTTTGAGAAGAGCTGCATTTCTGAAGCACAATCTATGGGTTTACACAATATGCACCTGGAGAAGATTTTCCAGGAGGAGAGTTCC…
TB7   allele 1  …CGAAATTTTTGAGAAGAGCTGCATTTCTGAAGCACAATCTATG--------------CACCTGGAGAAGATTTTCCAGGAGGAGAGTTCC…
TB7   allele 2  …CGAAATTTTTGAGAAGAGCTGCATTTCTGAAGCACAATCTATGGGT-ACACAATATGCACCTGGAGAAGATTTTCCAGGAGGAGAGTTCC…
```

***MPO1.2* mutations**

```
WT Seq          …CGAAATTTTTGAGAAGAGCTGCATTTCTGAAGCACAATCTATGGGTTACACAATATGCACCTGGAGAAGAATTTCCAGGAGGAGAGTTCC…
K11   allele 1  …CGAAATTTTTGAGAAGAGCTGCATTTCTGAAGCACAATCTATGGGTTTACACAATATGCACCTGGAGAAGAATTTCCAGGAGGAGAGTTCC…
K11   allele 2  …CGAAATTTTTGAGAAGAGCTGCATTTCTGAAGCACAATCTATGGGTTA--------GCACCTGGAGAAGAATTTCCAGGAGGAGAGTTCC…
K23             …CGAAATTTTTGAGAAGAGCTGCATTTCTGAAGCACAATCTATGGGTTACTTC……49 bp deletion……
KB19            …CGAAATTTTTGAGAAGAGCTGCATTTCTGAAGCACAAT-------TTACACAATATGCACCTGGAGAAGAATTTCCAGGAGGAGAGTTCC…
KB20  allele 1  …CGAAATTTTTGAGAAGAGCTGCATTTCTGAAGCACAATCTATGGGT……87 bp deletion……
KB20  allele 2  …CGAAATTTTTGAGAAGAGCTGCATTTCTGAAGCACAATCTATGGGTTAC-----------CTGGAGAAGAATTTCCAGGAGGAGAGTTCC…
T23             ……190 bp deletion……ATTTCTGGAGAAGAATTTCCAGGAGGAGAGTTCC…
TB7             …CGAAATTTTTGAGAAGAGCTGCATTTCTGAAGCACAATCTATCAT----------------TGGAGAAGAATTTCCAGGAGGAGAGTTCC…
```

***MPO2.1* mutation**

```
WT Seq          …CTAAGTTTTTGAGAAGAGCTGCATTTTTGAAGCACAATCTATGGGTTACAAAATATGCACCCGGAGAAGATTTTCCCGGGGGAGAGTTCC…
KB19            …CTAAGTTTTTGAGAAGAGCTGCATTTTTGAAGCACAATCTATGGGT-ACAAAATATGCACCCGGAGAAGATTTTCCCGGGGGAGAGTTCC…
```

***MPO2.2* mutations**

```
WT Seq          …CTAAGTTTTTGAGAAGAGCTGCATTTTTGAAGCACAATCTATGGGTTACACAATATGCACCCGGAGAAGATTTTCCAGGGGGAGAGTTCC…
K11             ……222 bp deletion……
K23             …CTAAGTTTTTGAGAAGAGCTGCATTTTTGAAGCACAATCTATGGGTTAAGCAT----------TTTTCCAGGGGGAGAGTTCC…
KB19 allele 1   …CTAAGTTTTTGAGAAGAGCTGCATTTTTGAAGCACAATCTA----TTACACAATATGCACCCGGAGAAGATTTTCCAGGGGGAGAGTTCC…
                                                  ⇓ 25 bp insertion (near perfect duplication)
                                          (ATGCATTTTTGAAGCACAATCTGGA)

KB19 allele 2   …CTAAGTTTTTGAGAAGAGCTGCATTTTTGAAGCACAATCTATGGGTTACAG……48 bp deletion……
T23  allele 1   …CTAAGTTTTTGAGAAGAGCTGCATTTTTGAAGCACAATCTATGGGTTTACACAATATGCACCCGGAGAAGATTTTCCAGGGGGAGAGTTCC…
T23  allele 2   …CTAAGTTTTTGAGAAGAGCTGCATTTTTGAAGCACAATCTATGGGTTAC----------------------------AGGGGGAGAGTTCC…
                                                        ⇓ 16 bp insertion
                                                  (GGAGAAGATTAGGGTT)

TB7             …CTAAGTTTTTGAGAAGAGCTGCATTTTTGAAGCACAATCTATGGGTTTACACAATATGCACCCGGAGAAGATTTTCCAGGGGGAGAGTTCC…
```

Figure 4.1. Meganuclease-induced mutations found in the *MPO* genes of the lines developed in this study. The 22 bp region shaded in yellow represents the custom designed meganuclease recognition site. Short deletions are represented by dashes; long deletions are shown as dotted lines. Insertion events are shown in bold, italicized type. The single nucleotide polymorphism in the target region of *MPO2.1* is highlighted in turquoise.

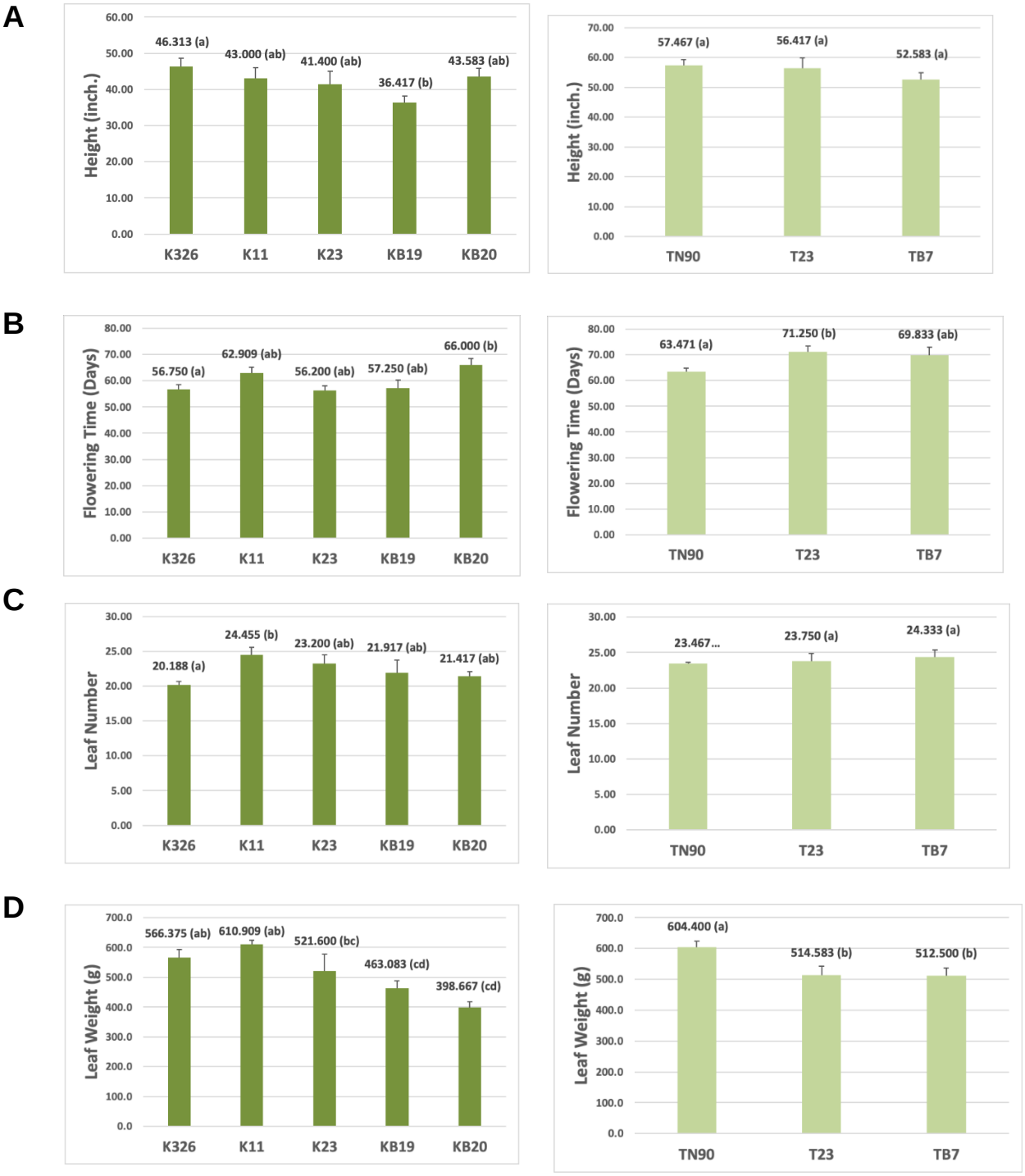

Figure 4.2. Height (A), Flowering Time (B), Leaf Number (C) and Total Leaf Weight (D) of lines possessing *MPO* mutations. Flowering time is presented as days after transplant from the lab to the greenhouse. Means ± standard errors of means with the same letter are not significantly different at a 5% significance level using Tukey-Kramer HSD grouping. The number of individuals assayed per genotype is defined in Figure 4.3.

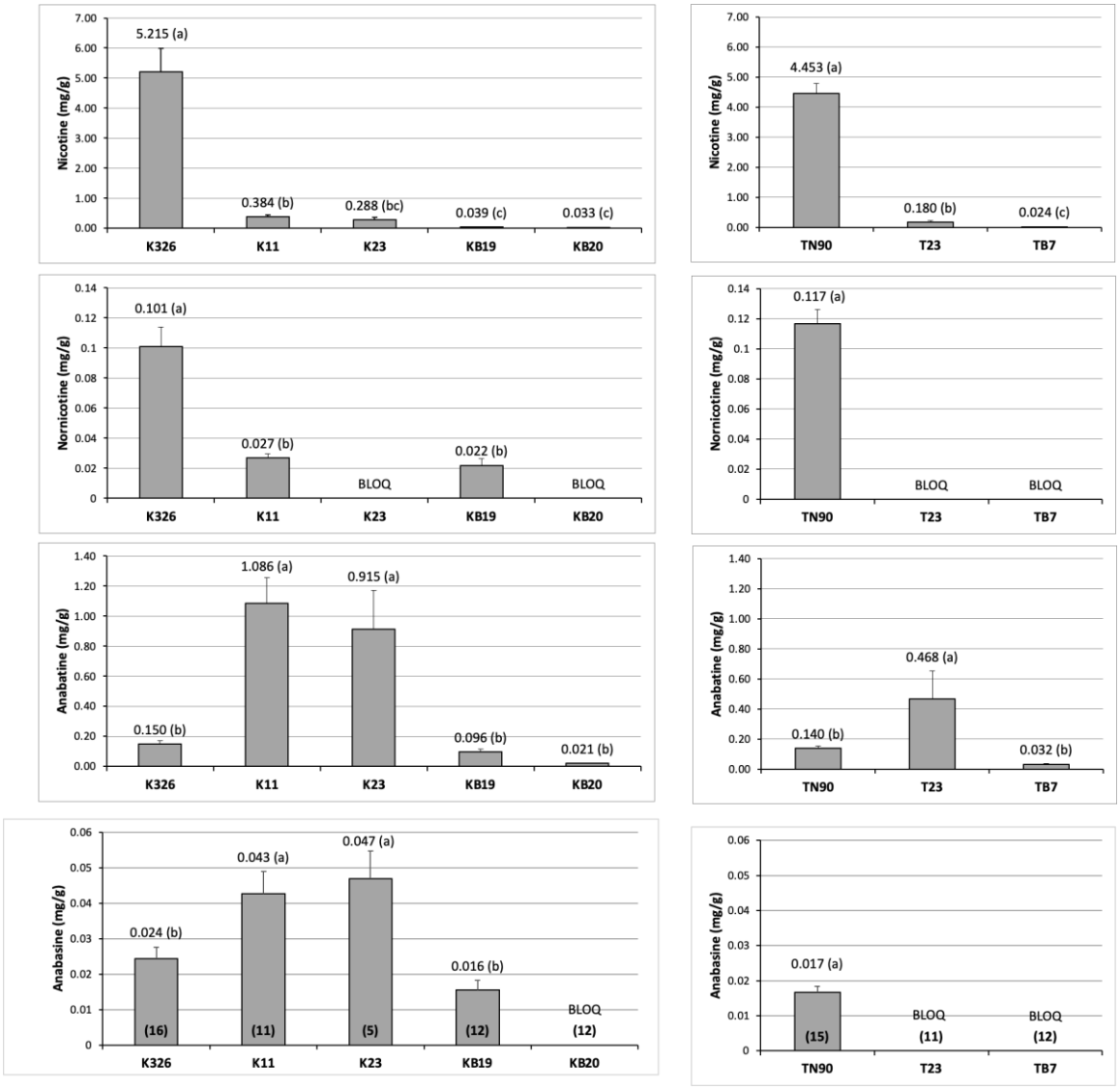

Figure 4.3. Alkaloid means of plants possessing meganuclease-induced mutations in *MPO* genes. Left column shows *mpo* mutant analysis in the flue-cured lines K326 (K11 and K23) and K326 222 (KB19 and KB20. Right column displays *mpo* mutant analysis in the burley lines TN90 (T23) and TN90 222 (TB7). The specific *mpo* mutations present in the lines represented on the x-axis are defined in Table 4.2. Standard errors are shown. Means ± standard errors of means with the same letter are not significantly different at a 5% significance level using Tukey-Kramer HSD grouping. BLOQ = below level of quantification. Number of individuals per group is indicated within the bars at the bottom of the anabasine determinations.

A. K326

A622_S

AGCAAGTTGATGTGGTGATTTCCACTGTCGGAGGACAGCAATTTACTGATCAAGTGAACATCATCAA (WT SEQ)
AGCAAGTTGATGTGGTGATTTCCA-----CGAGGACAGCAATTTACTGATCAAGTGAACATCATCAA (5 bp deletion)

A622_T

AGCAAGTTGATGTGGTGATTTCCACTG-TCGGAGGACAGCAATTTGCTGATCAAGTGAACATCATCAA (WT SEQ)
AGCAAGTTGATGTGGTGATTTCCACTG**A**TCGGAGGACAGCAATTTGCTGATCAAGTGAACATCATCAA (1 bp insertion)

B. TN90

A622_S

CAAGGCAATCAAGCAAGTTGATGTGGTGATTTCCACTGT-CGGAGGACAGCAATTTACTGATCA (WT SEQ)
CAAGGCAATCAAGCAAGTTGATGTGGTGATTTCCACTGT**T**CGGAGGACAGCAATTTACTGATCA (1 bp insertion)

A622_T

CAAGGCAATCAAGCAAGTTGATGTGGTGATTTCCACTGT-CGGAGGACAGCAATTTGCTGATCA (WT SEQ)
CAAGGCAATCAAGCAAGTTGATGTGGTGATTTCCACTGT**T**CGGAGGACAGCAATTTGCTGATCA (1 bp insertion)

Figure 4.4. CRISPR/Cas9-induced mutations found in *A622* genes of the lines developed in this study. **(A)**, mutations found in K326 lines C2, C8 and C21; **(B)**, mutations found in TN90 lines F14, F21, and F30. The 20 bp region shaded in yellow represents the enzyme recognition site. The PAM site is underlined. Deletions are represented by dashes; insertions are shown in bold type.

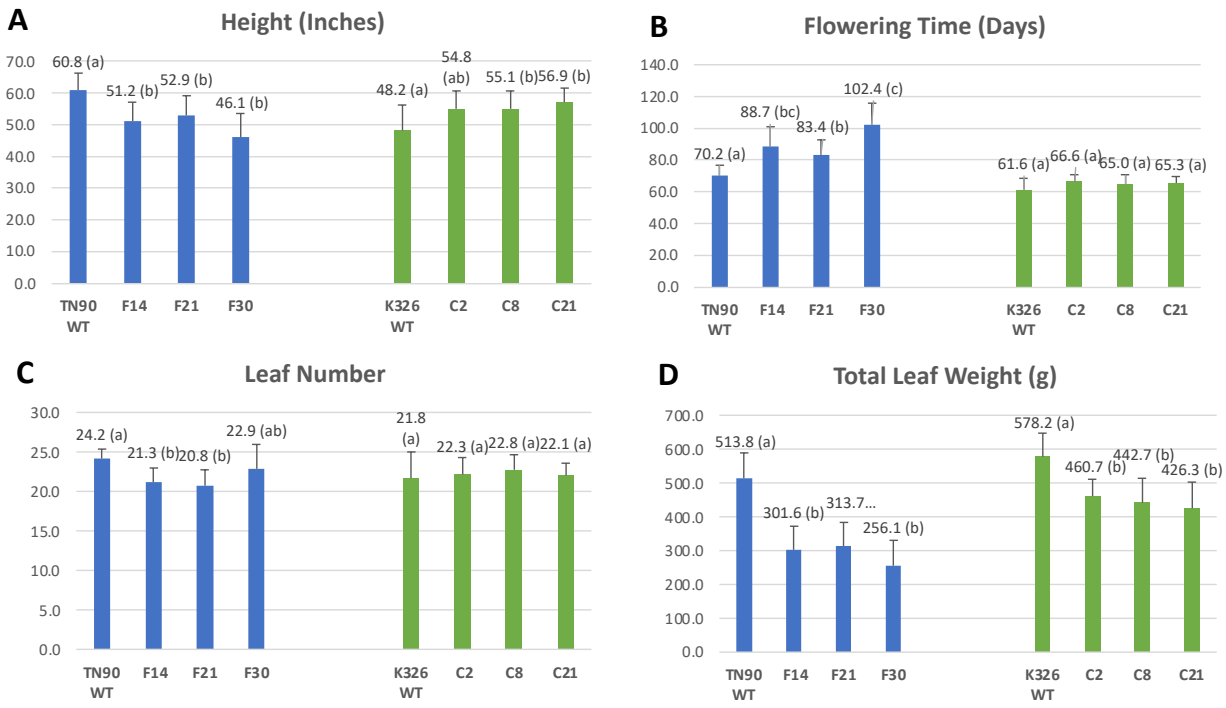

Figure 4.5. Height (A), Flowering Time (B), Leaf Number (C) and Total Leaf Weight (D) of lines possessing *A622* mutations. Flowering time is presented as days after transplant from the lab to the greenhouse. Bar graphs of plants in the TN90 background are colored blue; those in K326 are shown in green. Means ± standard errors of means with the same letter are not significantly different at a 5% significance level using Tukey-Kramer HSD grouping. The number of plants per genotype ranged from 9 – 12.

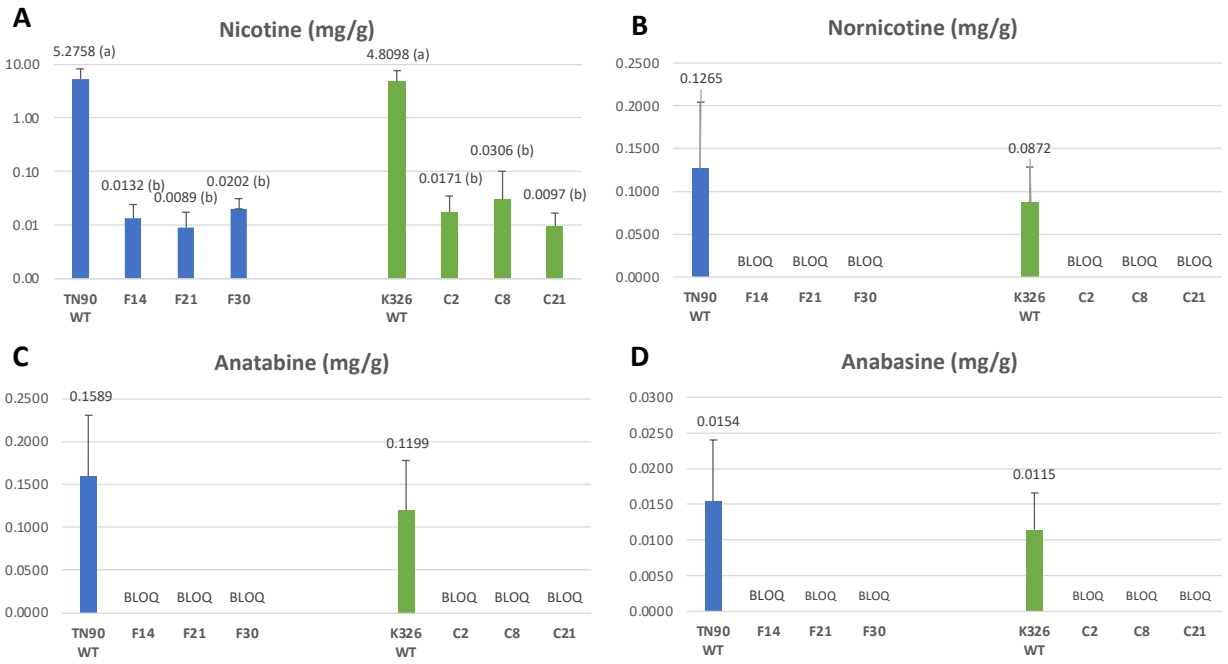

Figure 4.6. Alkaloid means of plants possessing CRISPR/Cas9-induced mutations in *A622* genes. (A), Nicotine; **(B)**, Nornicotine; **(C)**, Anatabine. Bar graphs of plants in the TN90 background are colored blue; those in K326 are shown in green. The specific *a622* mutations present in the lines represented on the x-axis are defined in Fig. 4.4. Due to the great differences in values between the wild-type and mutant line, the y-axis for nicotine content **(A)** is presented as a log scale. Standard errors are shown. Means ± standard errors of means with the same letter are not significantly different at a 5% significance level using Tukey-Kramer HSD grouping. BLOQ = below level of quantification. Number of individuals per genotype ranged from 10 – 12.

APPENDIX

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Specialty section:

frontiers in Plant Science

ORIGINALRESEARC H
published:03April202 0
doi:10.3389/fpls.2020.0036 8

This article was submitted to
Plant Biotechnology, a section of the journal
Frontiers in Plant Science

Received: 23 January 2020
Accepted: 13 March 2020
Published: 03 April 2020

Citation:
Lewis RS, Drake-Stowe KE,
Heim C, Steede T, Smith W and
Dewey RE (2020) Genetic and Agronomic Analysis of Tobacco
Genotypes Exhibiting Reduced
Nicotine Accumulation Due to Induced Mutations in Berberine
Bridge Like (BBL) Genes.
Front. Plant Sci. 11:368. doi: 10.3389/fpls.2020.00368

Genetic and Agronomic Analysis of Tobacco Genotypes Exhibiting Reduced Nicotine Accumulation Due to Induced Mutations in *Berberine Bridge Like* (*BBL*) Genes

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Genetic methodologies for reducing nicotine accumulation in the tobacco plant (*Nicotiana tabacum* L.) are of interest because of potential future regulations that could mandate lowering of this alkaloid in conventional cigarettes. Inactivation of tobacco genes such as the *Berberine Bridge Like* (*BBL*) gene family believed to encode for enzymes involved in one of the latter steps of nicotine biosynthesis could be a viable strategy for producing new tobacco cultivars with ultra-low leaf nicotine accumulation. We introduced deleterious mutations generated via ethyl methanesulfonate treatment of seed or gene editing into six known members of the *BBL* gene family and assembled them in different combinations to assess their relative contribution to nicotine accumulation. Significant reductions (up to 17-fold) in percent leaf nicotine were observed in genotypes homozygous for combined mutations in *BBL-a*, *BBLb*, and *BBL-c*.

The addition of mutations in *BBL-d1*, *BBL-d2*, and *BBL-e* had no additional significant effect on lowering of nicotine levels in the genetic background studied. Reduced nicotine levels were associated with reductions in cured leaf yields (up to 29%) and cured leaf quality (up to 15%), evidence of physiological complexities within the tobacco plant related to the nicotine biosynthetic pathway. Further nicotine reductions were observed for a *BBL* mutant line cultivated under a modified production regime in which apical inflorescences were not removed, but at the expense of further yield reductions. Plants in which *BBL* mutations were combined with naturally occurring recessive alleles at the *Nic1* and *Nic2* loci exhibited further reductions in percent nicotine, but no plant produced immeasurable levels of this alkaloid. Findings may suggest the existence of a minor, alternative pathway for nicotine biosynthesis in *N. tabacum*. The described genetic materials may be of value for the manufacture of cigarettes with reduced nicotine levels and for future studies to better understand the molecular biology of alkaloid accumulation in tobacco.

Keywords: tobacco, *Nicotiana tabacum*, nicotine, alkaloids, gene editing, regulation

INTRODUCTION

Nicotine is a highly studied plant natural product produced in significant quantities by the species *Nicotiana tabacum* L., commonly known as tobacco, and numerous other members of the *Nicotiana* genus. This pyridine alkaloid is synthesized in tobacco roots and subsequently translocated to aerial plant parts in processes stimulated by plant wounding or loss of the apical inflorescence. Nicotine accumulation likely plays a role in natural plant defense against herbivores. Nicotine also plays an important role in human society as it is the primary addictive substance in manufactured tobacco products such as combustible cigarettes which have well-studied toxicant profiles. The United States Food and Drug Administration (FDA) lists 93 tobacco and tobacco smoke chemical constituents designated as "harmful and potentially harmful" due to their association with carcinogenesis, addiction, or respiratory, cardiovascular, reproductive, or developmental toxicity (United States Food and Drug Administration, 2012). Nicotine *per se* is not recognized as a carcinogen, but the World Health Organization (2015) and the United States Food and Drug Administration (2018) have recommended a mandated lowering of nicotine levels in combustible cigarettes to nonaddictive levels in order to reduce overall addiction to such products and to lower corresponding toxicant exposure. The specific concentrations at which nicotine becomes non-addictive in combustible cigarettes may be difficult to determine and may vary amongst individuals, but Benowitz and Henningfield (1994) have predicted tobacco filler nicotine contents of between 0.02 and 0.03% to be below a "sub-threshold level of addiction." The World Health Organization (2015) has recommended lowering of nicotine content of the tobacco filler to below 0.04%. Percent nicotine on a dry weight basis in conventional tobacco cultivars typically ranges from between 1.0 and 5.0%, with observed variability being due to market type (burley, flue-cured, dark, cigar, or Oriental), plant genetics, growing environment, and stalk position. Manufacturers blend sourced cured leaf to produce cigarette tobacco filler with between 1.0 and 2.0% nicotine on a dry weight basis.

Various methods of chemical extraction have reportedly been used to achieve 80–98% reductions in nicotine content of tobacco filler (Seligman, 1983; Philip Morris United States, 1994; Coffa et al., 2016). Increased costs associated with chemical extraction, and the potential for co-extraction of compounds that positively affect organoleptic properties, make these approaches unattractive, however. Use of modified plant genetics is a preferred route to achieve reduced cigarette nicotine levels, if low-nicotine cultivars can be developed without substantially reducing cured leaf yields or otherwise undesirably altering chemical composition.

Genetic approaches to develop new tobacco cultivars with reduced potential for nicotine accumulation include the use of (1) genetic variability that naturally exists within *N. tabacum* or closely related species, (2) genetic variability induced by gene editing or mutagen treatment, or (3) novel variation generated via genetic engineering (reviewed by Lewis, 2018).
Wide variation exists for alkaloid accumulation amongst diverse tobacco materials in the United States *Nicotiana* Germplasm Collection, ranging from 0.02 to 6.55% on a dry weight basis (Sisson and Saunders, 1982). The ultra-low nicotine levels of 0.04% recommended by the World Health Organization are not routinely observed amongst the lowest-alkaloid material, however. Recessive alleles at the *Nic1* and *Nic2* (also known as *A* and *B*) loci have been found to contribute to major reductions in nicotine and associated alkaloids (nornicotine, anabasine, and anatabine) from between 1.5 and 4.5% to approximately 0.20–0.45% (Legg and Collins, 1971; Lewis, 2018). This allelic variability is well known to be associated with reduced cured leaf yields and quality, however (Chaplin and Weeks, 1976).

Knowledge of specific genes identified to be involved in nicotine biosynthesis (Dewey and Xie, 2013) allows for the use of technologies such as RNA interference to lower their expression and achieve corresponding reductions in nicotine accumulation (Lewis, 2018).
Commercialization of "GMO" tobacco cultivars is subject to variable levels of complicating regulatory oversight around the world, however. As an alternative to genetic engineering, gene silencing achieved via induced mutation or gene editing might be used to realize reductions in nicotine. Because of the polyploid nature of *N. tabacum*, mutations in multiple gene copies are often necessary to achieve a desired phenotype. The increased complexity of mutation breeding in a polyploid species can be offset by the fact that outcomes of such breeding approaches are not considered regulated articles in many parts of the world.

The *Berberine Bridge Like* (*BBL*) gene family was previously identified to encode for enzymes involved in one of the final steps of the *N. tabacum* nicotine biosynthetic pathway, although their precise role is currently not understood (Kajikawa et al., 2011). This family is comprised of six closely related members, three of which are expressed to significant degrees (*BBL-a*, *BBLb*, and *BBL-c*) and three of which are lowly expressed (*BBL-d1*, *BBL-d2*, and *BBL-e*) (Kajikawa et al., 2017). The objectives of the current research were to establish nearly isogenic versions of elite flue-cured and burley tobacco cultivars that differed for the presence/absence of deleterious mutations in each of these six known *BBL* genes, and to evaluate these materials for altered alkaloid profiles and potential corresponding changes in yield and cured leaf quality. It was also of interest to determine if further reductions in nicotine could be achieved by (1) growing these modified tobaccos under a production regime where apical inflorescences are not removed, and (2) combining induced *BBL* mutations with the naturally occurring recessive alleles at the *Nic1* and *Nic2* loci.

MATERIALS AND METHODS

Development and Testing of Nearly Isogenic Lines Carrying Mutations in BBL-a, BBL-b, and BBL-c

Treatment of tobacco seeds with ethyl methanesulfonate was previously used to introduce genome-wide mutations into burley tobacco breeding line DH98-325-6 (Lewis et al., 2010) and single nucleotide substitution mutations leading to truncated protein products were identified in

the three most highly expressed *BBL* genes (*BBL-a*, *BBL-b*, and *BBLc*) (Lewis et al., 2015) (**Table 1**). We subsequently used the backcross breeding method to transfer the three deleterious *BBL* mutations to tobacco cultivars K326, TN 90, and TN90 SRC. K326 is a popular flue-cured tobacco cultivar marketed as a fertile inbred line. TN 90 is a historically important burley tobacco cultivar also marketed as a fertile inbred line. TN 90 SRC (TN 90 **S**table **R**educed **C**onverter) is a nearly isogenic version of TN 90 that was produced by transferring deleterious mutations in three nicotine demethylase genes (*CYP82E4*, *CYP82E5*, and *CYP82E10*) (Lewis et al., 2010) to TN 90 using seven generations of backcrossing. These three gene mutations dramatically reduce the demethylation of nicotine to form nornicotine, a secondary alkaloid considered undesirable because of its association with the accumulation of the carcinogen *N*-nitrosonornicotine in cured tobacco leaves (Lewis et al., 2008).

K326, TN 90, and TN 90 SRC were each initially hybridized with an individual plant in which the *BBL-a*, *BBL-b*, and *BBL-c* truncation mutations were initially assembled in triple homozygous conditions (Lewis et al., 2015). Each F_1 was then backcrossed to their respective recurrent parents, and selection for all three mutations was carried out using Kompetitive Allele Specific PCR (KASP) markers. The backcross breeding method was carried out to the BC_7F_1 generation for each of the three lines, at which time triple heterozygous mutant genotypes were self-pollinated to produce the BC_7F_2 generation. BC_7F_2 individuals were genotyped to identify all eight possible homozygous genetic combinations (**Supplementary Table 1**), which were then self-pollinated to produce BC_7F_3 seed of the nearly isogenic lines (NILs) for each genetic background. Numerical nomenclature is hereafter used to refer to each of these genotypes, where a "0" indicates the homozygous "wild-type" condition at a particular locus, "2" designates the homozygous mutant condition at a particular locus, and "1" indicates a heterozygote (**Supplementary Table 1**). For example, the designation "K326 (210)" indicates a line homozygous for the mutant genotype at the *BBL-a* locus,

TABLE 1 | Deleterious mutations identified and studied for six *BBL* genes in tobacco.

Gene	% Amino acid Similarity to BBL-a	Mutation	Position from ATG	Amino acid Change
BBL-a	–	G/A	681	W227Stop
BBL-b	92.8	G/A	438	W146Stop
BBL-c	82.6	C/T	448	Q150Stop
BBL-d1	64.0	Indel (−106, +11 bp)	163	Frameshift mutation
BBL-d2	65.4	Indel (−2, +19 bp)	271	Frameshift mutation
BBL-e	85.4	Indel (−19, +11 bp)	263	Frameshift mutation

heterozygous for the mutant genotype at the *BBL-b* locus, and homozygous for the wild type allele at the *BBLc* locus.

The eight K326-derived NILs were evaluated for yield, physical cured leaf quality, and cured leaf chemistry in comparison with K326, NC95, and corresponding NC95 isolines MAFC5

(*Nic1*/*Nic1 nic2*/*nic2*) (Chaplin, 1986), LMAFC34 (*nic1*/*nic1 Nic2*/*Nic2*) (Chaplin and Burk, 1984), and LAFC53
(*nic1*/*nic1 nic2*/*nic2*) (Chaplin, 1975). An F_1 hybrid between K326 and K326 (222), designated as K326 (111) was also included. Experiments were carried out at three North Carolina field locations (Upper Coastal Plain Research Station, Rocky Mount; Cunningham Research Station, Kinston; and the
Oxford Tobacco Research Station, Oxford) during 2016 and 2017, for a total of six field environments. Experimental units at each location consisted of single 20-plant rows managed according to standard flue-cured production practices for North Carolina. Intra-row spacing was 56 cm at all three locations, while inter-row spacing was 122 cm at the Oxford and Rocky Mount locations and 112 cm at the
Kinston locations.

To investigate the level of leaf green color immediately prior to harvest of the top leaves (an indicator of leaf ripening), the chlorophyll contents of the upper most leaves of K326, K326 (222), NC95, MAFC5, LMAFC34, and LAFC53 were estimated using a handheld Konica Minolta SPAD-502 meter (Konica Minolta, Tokyo, Japan) according to manufacturer's instructions. One measurement was taken for the top leaf of each of 10 plants per plot and averages were calculated for each plot.

Leaves were harvested in four separate harvests (primings) and flue-cured. Each priming was weighed to generate yield data, and official USDA quality grades were assigned by a former USDA grader. Cured leaf quality was also assessed using the 2017 North Carolina Flue-Cured Tobacco Grade Index (North Carolina Cooperative Extension Service, 2017). Value per hundred weight (\$ cwt^{-1}) and value (\$) ha^{-1}were calculated based on the 2017 flue-cured tobacco price index (North Carolina Cooperative Extension Service, 2017). Plot values for grade index and \$ cwt^{-1} were calculated using a weighted average over all four primings. Fifty-gram de-stemmed cured leaf samples were prepared for each plot by compositing cured leaf from each priming on a weighted-mean basis. Ovendried samples were ground to pass through a 1-mm sieve and analyzed for alkaloid profiles (expressed as a percentage of dry weight) as previously outlined by Lewis et al. (2015). Percent reducing sugars were analyzed according to the method of Davis (1976).

The eight TN 90-derived and eight TN 90 SRC-derived *BBL* mutant NILs lines were evaluated at two locations representative of burley tobacco producing environments in North Carolina during the 2016 and 2017 growing seasons: the Mountain Research Station at Waynesville, and the Upper Mountain Research Station at Laurel Springs. Materials were evaluated in comparison with TN 90, TN 90 SRC, Burley 21, and the corresponding Burley 21 isolines HI Burley 21 (*Nic1*/*Nic1 nic2*/*nic2*), LI Burley 21 (*nic1*/*nic1 Nic2*/*Nic2*), and LA Burley 21 (*nic1*/*nic1 nic2*/*nic2*) (Legg et al., 1970). Experimental units were single 20 plant rows managed according to suggested production practices for burley tobacco production in North Carolina. Row and plant spacing were 122 cm and 46 cm, respectively. To investigate the level of leaf green color immediately prior to harvest, the chlorophyll contents of the upper most leaves of TN 90, TN 90 SRC, TN 90 (222), TN 90 SRC (222), Burley 21, HI
Burley 21, LI Burley 21, and LA Burley 21 were estimated using a SPAD-502 meter. The top of leaf of each of 10 plants per plot was measured, and averages were calculated for each plot.

Plots were stalk cut at maturity, speared onto sticks, and aircured in structures protected from rainfall. After curing, leaves were stripped into four stalk positions, weighed, and assigned a USDA grade by a former USDA tobacco grader. A grade index was also assigned using the

index of Bowman et al. (1989). Plot values for grade index were calculated using a weighted average over all four stalk positions. Fifty-gram cured leaf samples were prepared for each plot, processed, and analyzed for alkaloid profiles as previously described.

Development and Testing of Nearly Isogenic Lines Carrying Mutations in BBL-d1, BBL-d2, and BBL-e

To investigate the potential role of three additional, but lowly expressed, members of the *BBL* gene family in nicotine biosynthesis (Kajikawa et al., 2017), gene editing was used to introduce deleterious mutations into *BBL-d1*, *BBL-d2*, and *BBL-e* (**Table 1**). Two proprietary ARCUS sequence-specific nuclease (Precision Biosciences, Durham, NC, United States) were strategically designed to introduce mutations into the three minor *BBL* genes, one specific for *BBL-e* and the other specific for *BBL-d1* and *-d2*. TN90 SRC (222) was a source of tobacco leaf explants used in *Agrobacterium*-based transformation to introduce the transgene construct carrying the ARCUS nuclease gene. Transgenic plants identified as possessing mutations in the three minor genes were self-pollinated and, after selection against plants carrying transgene insertions, all possible homozygous mutant combinations were identified in the TN90 SRC (222) genetic background. Plants were self-pollinated to produce stable lines that were designated using the previously described nomenclature (**Supplementary Table 1**). These materials were included in the 2017 burley tobacco field experiments (two environments) and evaluated as previously described.

Testing of Low Alkaloid Flue-Cured Tobacco Genotypes Under Topped and Non-topped Production Regimes

K326 and NC95 were evaluated, along with their respective lownicotine NILs K326 (222) and LAFC53 (*nic1*/*nic1 nic2*/*nic2*), in a 2018 field experiment to investigate the extent to which nicotine levels might be further reduced by growing tobacco lines with a reduced genetic potential for alkaloid accumulation under a nonconventional production regime where plants are not topped (i.e., the apical inflorescence is not removed). This experiment was carried out in a field environment near Clayton, NC, using recommended tobacco production methods for the region (with the exception of non-topping). The experimental design was a split plot design with four replications. The main plot factor was production regime (topped versus non-topped), while the subplot factor was genotype. Experimental units were five plant rows with intra-row plant spacing and row spacing of 56 and 112 cm, respectively. All plants within the topped portion of the experiment were topped on the same day after greater than 95% of the plants flowered. Plants were generally topped 1–2 leaves below the lowest flowering branch, where the smallest remaining leaves were at least 20 cm in length and 10 cm in width. Immediately post-topping, each plant was treated with a downstalk application of Prime + EC (Syngenta Crop Protection, Greensboro, NC, United States) according to manufacturer's instructions in order to suppress lateral meristem development (suckering). Chemical sucker control was supplemented with hand removal, when necessary.

Plots were harvested in four primings, according to the rate of ripening, from the bottom to the top of the stalk. Four to six leaves were harvested in each priming. Per plot fresh leaf weights were recorded in the field and converted to kg ha^{-1} fresh weight yields. Five leaves from each priming for each plot were collected, oven dried, and analyzed for individual stalk position

alkaloid profiles as previously described. Alkaloid percentages for a composite sample for each plot were calculated based on the relative weights of each priming.

Combining BBL Mutations With Recessive Alleles at the Nic1 and Nic2 Loci

It was of interest to determine whether nicotine accumulation of K326 (222) could be further lowered by incorporating the recessive alleles at the *Nic1* or *Nic2* loci into this genetic background. K326 (222) was initially hybridized with LAFC53 (*nic1*/*nic1 nic2*/*nic2*). BC_2F_1 progeny were subsequently developed after backcrosses to K326 (222) accompanied with selection for the homozygous mutant condition at the *BBLa*, *BBL-b*, and *BBL-c* loci using the previously described KASP markers and selection for the recessive *nic1* and *nic2* alleles using SNP markers described by Adams et al. (2016). *bbl-a*/*bbla bbl-b*/*bbl-b bbl-c*/*bbl-c Nic1*/*nic1 Nic2*/*nic2* BC_2F_1 individuals were self-pollinated and triple homozygous *bbl* mutant BC_2F_2 plants were identified via genotyping that were either *Nic1*/*Nic1 nic2*/*nic2*, *nic1*/*nic1 Nic2*/*Nic2*, or *nic1*/*nic1 nic2*/*nic2*. Such BC_2F_2 plants were self-pollinated to produce BC_2F_3 families that were evaluated in comparison with K326, K326 (222), and LAFC53 for nicotine accumulation in a single 2019 field environment near Clayton, NC. Plants were managed according to standard flue-cured production practices for North Carolina. The experimental design was a completely randomized design with each genotype being represented by between 12 and 37 plants. The top two leaves of each plant were harvested 21 days after topping, air cured, and analyzed for alkaloid profiles as previously described.

Data Analysis

Analyses of variance appropriate for analyzing randomized complete block designs or split plot designs were carried out for all measured traits using PROC MIXED of SAS 9.3 (SAS Institute, Cary, NC, United States) according to Littell et al. (1996). Environments were considered as random effects, while genotype and production regime (topped versus nontopped) were considered as fixed effects. Means separations were performed via appropriate F-tests or by using Fisher's least significant difference test ($\alpha = 0.05$).

RESULTS

Evaluation of K326 Flue-Cured Tobacco Isolines Containing Mutations in BBL-a, BBL-b, and BBL-c

Evaluation of eight K326 homozygous *BBL* mutant lines and corresponding controls in six field environments indicated that that the addition of no single *BBL* gene mutation had a significant effect to lower nicotine accumulation (**Figure 1**). Only genotypes with mutations in both *BBL-a* and *BBL-b* exhibited significant ($P < 0.05$) reductions in nicotine as compared to K326. The K326 (220) and K326 (222) mutant lines accumulated 0.45 and 0.38% nicotine, respectively, as compared to 2.69% for K326. Progressive decreases in nicotine were observed as the number of recessive alleles at the *nic1* and *nic2* loci increased in the genetic background of NC95. Some small significant ($P < 0.05$) reductions were observed for percent levels in the triple homozygous

mutant line K326 (222) were not lower than anatabine in single the NC95. *nic1*/*nic1 nic2*/*nic2* isoline of flue-cured tobacco cultivar NC mutant lines, but not for anabasine (**Figure 1**). Extreme reductions in percent anatabine were measured for K326 (220) and K326 (222). More modest, but still significant, reductions were found for percent anabasine for these two genotypes. Similar to nicotine, progressive decreases in anatabine and anabasine accumulation were found with the addition of recessive alleles at the *nic1* and *nic2* loci in an NC95 genetic background. Significant ($P < 0.05$) increases in percent nornicotine were observed for the double mutant genotypes K326 (220) and K326 (222) (**Figure 1**). No significant changes in percent reducing sugars were found amongst the K326 isolines or amongst the NC95 isolines.

Prior to harvest of the last priming, the difference in leaf green color (as measured by a SPAD meter) between K326 and K326 (222) was found to be non-significant ($P > 0.05$) (**Figure 2**). In comparison, the NC95 isolines MAFC5 (*Nic1*/*Nic1 nic2*/*nic2*) and LAFC53 (*nic1*/*nic1 nic2*/*nic2*) were found to be significantly more green than NC95.Significant observed reductions in percent nicotine for K326 (220) and K326 (222) were associated with significantly lower ($P < 0.05$) redeaf yields (17.0 and 29.5% respectively) (**Figure 1**). Significant reductions in cured leaf quality also accompanied lower nicotine levels in K326 (222) as reflected by significantly lower grade index and cwt value. These characters were reduced by 11.3 and 14.5% relative to K326, respectively. No significant differences in yield were detected amongst the NC95 isolines, but cured leaf quality of MAFC5 and LAFC53 were significantly reduced relative to NC95. Cured leaf quality of the low nicotine mutant genotype K326 (222) was significantly better than that for LAFC53.

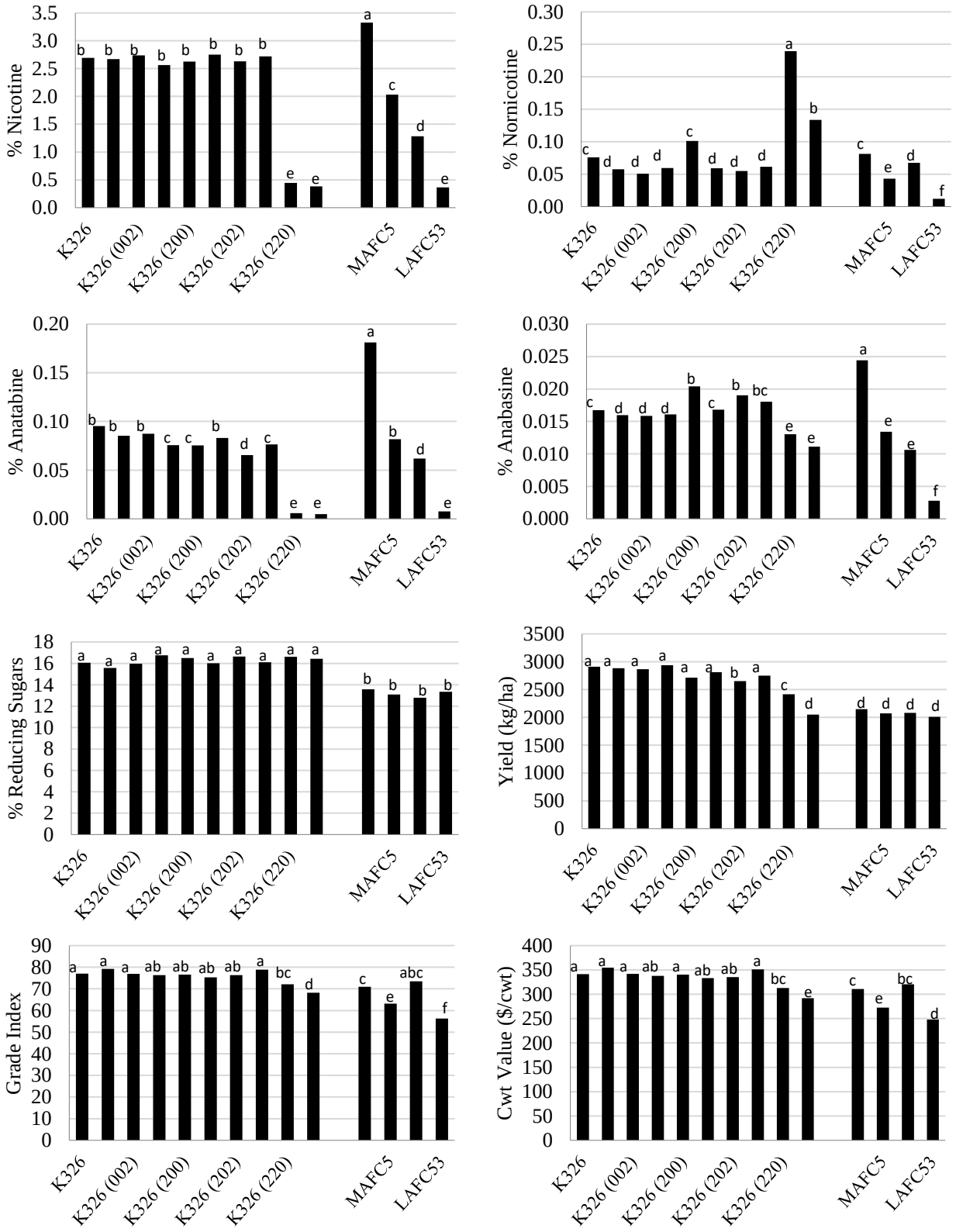

Figure 1. Entry means for flue-cured genetic materials evaluated for alkaloid levels, yield, and quality determinations. Means are averaged over six 2016 and 2017 North Carolina field environments. Means with different letters are significantly different from each other at the $P < 0.05$ level of significance.

Evaluation of TN 90 Burley Tobacco Isolines Containing Mutations in BBL-a, BBL-b, and BBL-c

The *BBL-a*, *BBL-b*, and *BBL-c* mutations were also evaluated in all possible homozygous combinations in the genetic background of burley tobacco cultivar TN 90 in a total of four field environments. Relative to TN 90, the only TN 90 single mutant line exhibiting a slight significant ($P < 0.05$) reduction in percent nicotine was TN 90 (002) (**Figure 3**). Similar to the K326 genetic background, dramatic reductions in nicotine were only realized in double mutant genotypes TN 90 (220) and TN 90 (222). Significant ($P < 0.05$) differences were detected between the *nic1*/*nic2* isolines of burley tobacco cultivar Burley 21, with LI Burley 21 and LA Burley 21 exhibiting the second-lowest and lowest nicotine levels of the group, respectively (**Figure 3**). Percent nicotine for TN 90 (222) was not lower than that for LA Burley 21.

Relative to TN 90, small significant ($P < 0.05$) reductions in anatabine were detected for single mutant line TN 90 (002) and double mutant line TN 90 (202) (**Figure 2**). Dramatic reductions in anatabine were observed only for the double mutant genotypes TN 90 (220) and triple mutant TN 90 (222). Progressive decreases in percent anatabine were measured as the number of recessive alleles at the *nic1* and *nic2* loci increased in the Burley 21 genetic background. Relative to TN 90, a small significant reduction in anabasine content was observed for single *BBL* mutant line TN 90 (002), and a significant modest increase was measured for single *BBL* mutant line TN 90 (200). A significant modest decrease in percent anabasine was measured for the triple homozygous mutant TN 90 (222). Nornicotine accumulation was found to be significantly greater, relative to TN 90, in mutant lines TN 90 (200) and TN 90 (220).

Prior to stalk harvesting of burley tobacco plots, the degree of leaf greenness (as measured by a SPAD meter) between TN 90 and TN 90 (222) was found to be non-significant ($P > 0.05$) (**Figure 2**). In comparison, only the Burley 21 isoline HI Burley 21 (*Nic1*/*Nic1 nic2*/*nic2*) was found to be significantly less green than Burley 21. LA Burley 21 (*nic1*/*nic1 nic2*/*nic2*) exhibited the greatest average SPAD meter rating, but this was not significantly greater than that for Burley 21.

Nicotine reductions in double and triple homozygous mutant lines TN 90 (220) and TN 90 (222) were associated with significant ($P < 0.05$) reductions in cured leaf yields of 13.2 and 24.5%, respectively (**Figure 3**). Reductions in nicotine for these mutant genotypes was also found to be associated with significant ($P < 0.05$) reductions in cured leaf quality (as reflected by grade index) of 6.2 and 15.5%, respectively. Cured leaf quality of the low percent nicotine genotype TN 90 (222) was not significantly different than that for LA Burley 21, the *nic1*/*nic1 nic2*/*nic2* isoline of Burley 21.

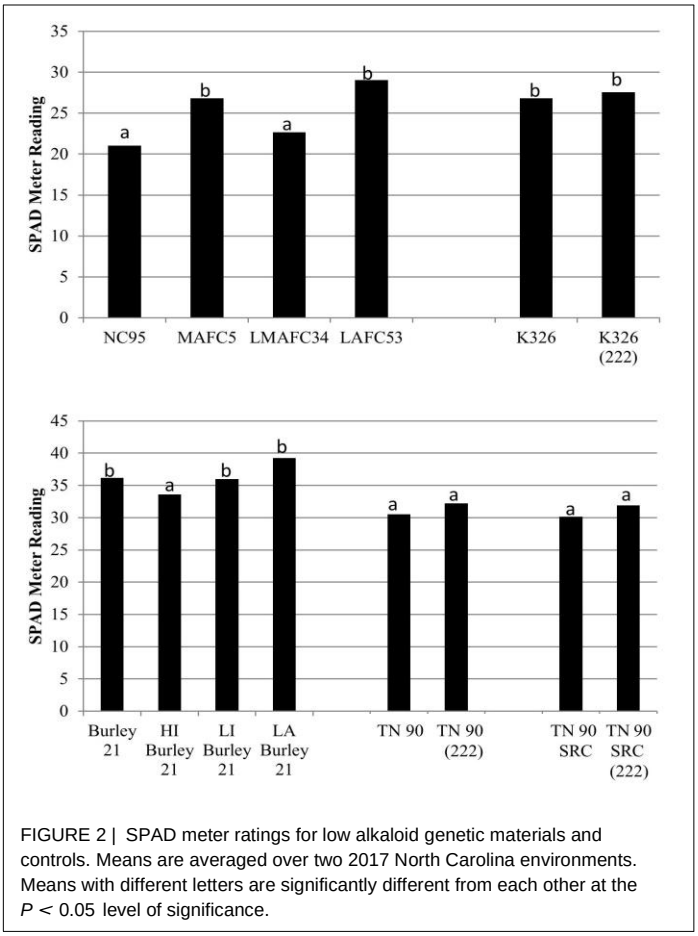

FIGURE 2 | SPAD meter ratings for low alkaloid genetic materials and controls. Means are averaged over two 2017 North Carolina environments. Means with different letters are significantly different from each other at the $P < 0.05$ level of significance.

Evaluation of TN 90 SRC Burley Tobacco Isolines Containing Mutations in BBL-a, BBL-b, and BBL-c

The *BBL-a*, *BBL-b*, and *BBL-c* mutations were also evaluated in all possible homozygous combinations in a TN 90 SRC genetic background. As mentioned previously, the TN 90 SRC genotype differs from TN 90 in that TN 90 SRC carries deleterious mutations in each of three nicotine demethylase genes (Lewis et al., 2010), a genetic combination that dramatically reduces the demethylation of nicotine to form nornicotine. Relative to TN 90 SRC, percent nicotine was only significantly ($P < 0.05$) reduced in TN 90 SRC (220) and TN 90 SRC (222) (**Figure 4**).

Similar to the other genetic backgrounds, percent anatabine was dramatically and significantly ($P < 0.05$) reduced in the double and triple homozygous mutant genotypes TN 90 SRC (220) and TN 90 SRC (222), relative to TN 90 SRC (**Figure 4**). Slight significant reductions in percent anatabine were measured for TN 90 SRC (200) and TN 90 SRC (202). Relative to TN 90 SRC, the only mutant isoline exhibiting significantly ($P < 0.05$) lower anabasine accumulation was the triple homozygous mutant line, TN 90 SRC (222). No significant differences were observed between the TN 90 SRC mutant isolines for the accumulation of nornicotine (**Figure 4**).

The green color of TN90 SRC (222) was not significantly greater than that for TN 90 SRC immediately prior to stalk harvest (**Figure 2**). Nicotine reductions in double and triple homozogyous mutant lines TN 90 SRC (220) and TN 90 SRC (222) were accompanied by significant ($P < 0.05$) reductions in cured leaf yields of 11.7 and 22.5%, respectively (**Figure4**). Cured leaf quality as measured by grade index was also significantly ($P < 0.05$) reduced by 8.7% in the triple homozygous mutant genotype as compared to TN 90 SRC.

Evaluation of TN 90 SRC Burley Tobacco Isolines Possessing Mutations in up to Six BBL Genes

Relative to TN 90 SRC (222), no additional significant changes in the accumulation of nicotine, nornnicotine, anatabine, or anabasine were found in genotypes in which additional *BBL* mutations (*bbl-d1*, *bbl-d2*, or *bbl-e*) were added to the genetic background of TN 90 SRC (222) (**Figure 5**).

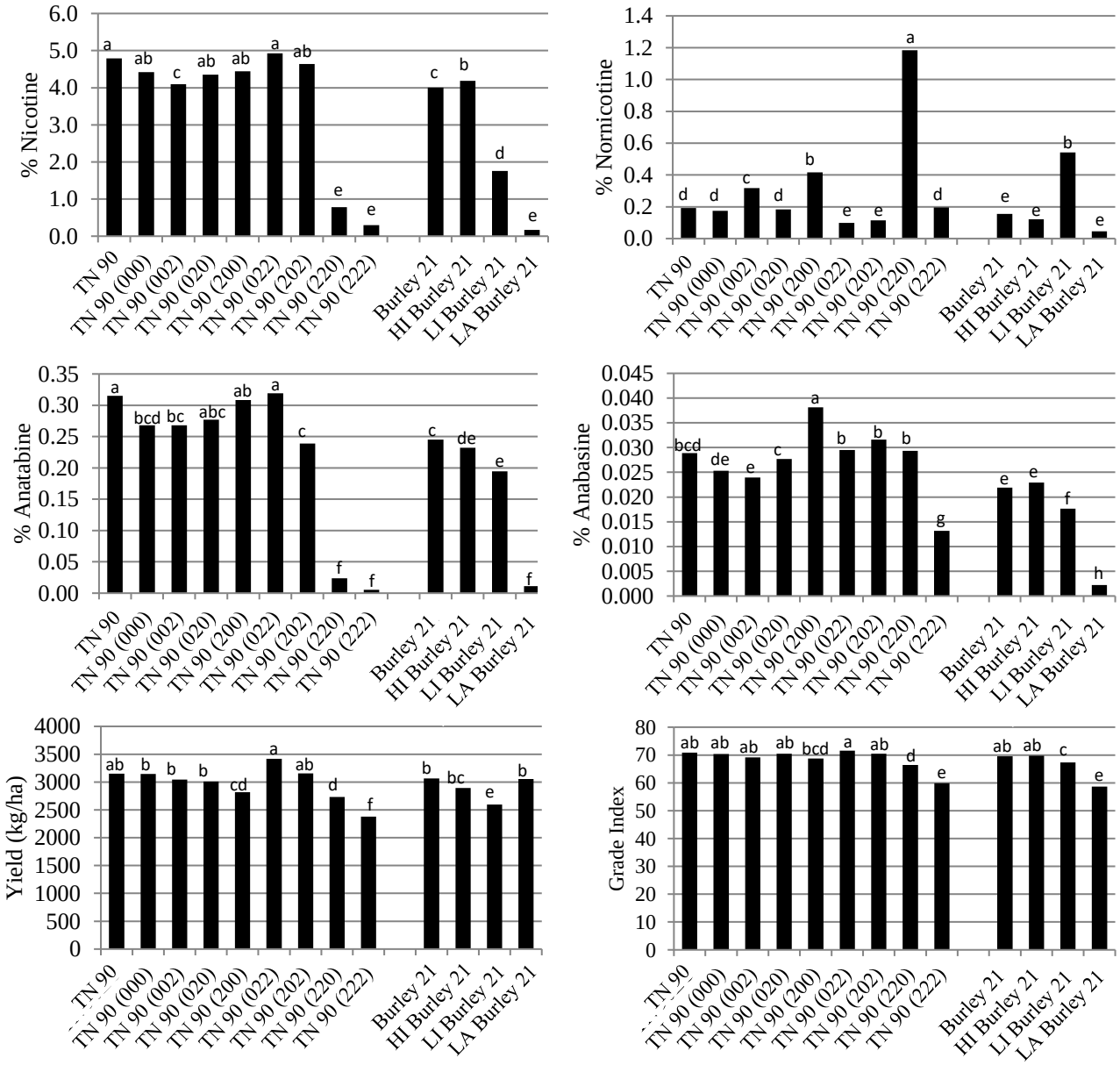

Figure 3. Entry means for TN90 burley tobacco genetic materials evaluated for alkaloid levels, yield, and quality determinations. Means are averaged over four 2016 and 2017 North Carolina field environments. Means with different letters are significantly different from each other at

Yield was numerically lower in most materials containing at least four mutations, although these reductions were not statistically significant. Cured leaf quality as measured by grade index was significantly lower for two genotypes carrying at least four *BBL* mutations as compared to TN 90 SRC (222) (**Figure 5**).

Testing of Low Alkaloid Flue-Cured Tobacco Genotypes Under Topped and Non-topped Production Regimes

K326 and NC95 were evaluated in comparison with their lowalkaloid NILs K326 (222) and LAFC53 (*nic1*/*nic1 nic2*/*nic2*) under a conventional production regime (where all plants were topped at flowering) and under a non-conventional production regime in which flowers were not removed. Averaged over all tested genotypes, the untopped regime resulted in significantly lower ($P = 0.0038$) lower nicotine levels (averaged over all stalk positions) as compared to the production regime in which all plants were topped (**Figure 6**). Untopped K326 and NC95 exhibited significantly lower ($P < 0.0001$) percent nicotine (averaged over all stalk positions) relative to topped K326 and NC95, respectively (**Figure 6**). Although untopped K326 (222) and LAFC53 exhibited numerically lower percent nicotine for composite samples relative to topped K326 (222) and LAFC53, these differences were not statistically significant ($P > 0.05$). The lowest numerical average percent nicotine was measured for untopped LAFC53 (0.067%).

Averaged over all genotypes, green leaf yields of untopped tobacco were 37.4% lower than green leaf yields of topped tobacco (significantly different at $P = 0.0015$). Each of the four tested genotypes was significantly lower yielding ($P < 0.0002$) under the untopped versus the topped production regime (**Figure 6**).

Average percent nicotine for each individual stalk position for each of the four tested genotypes is presented in **Figure 7**. For the topped tobacco entries, the general trend was one of increasing nicotine accumulation as the stalk position increased from lowest to highest. The lowest percent nicotine for topped tobacco (0.077%) was observed for the lowest stalk position for genotype K326 (222). For untopped tobacco, the trend was one of decreasing nicotine content as the stalk position increased for K326 and NC95 (**Figure 7**). These trends were not obvious and the range in nicotine levels between stalk positions was substantially less for the low alkaloid genotypes K326 (222) and LAFC53. The lowest average measured percent nicotine in the experiment was for the uppermost stalk position for untopped LAFC53 (0.046%).

Combining BBL Mutations With Recessive Alleles at the Nic1 and Nic2 Loci

Genotypes of the *bbl-a*/*bbl-a bbl-b*/*bbl-b bbl-c*/*bbl-c* genetic combination generally exhibited lower, but not significantly lower, alkaloid levels (with the exception of nornicotine) when combined with the recessive alleles at only the *Nic2* locus in a K326 genetic background (**Figure 8**). Significant reductions ($P < 0.05$) reductions in all alkaloids ($P < 0.05$) were observed for *bbl-a*/*bbl-a bbl-b*/*bbl-b bbl-c*/*bbl-c* individuals also carrying the recessive alleles at only the *Nic1* locus. The lowest alkaloid levels were measured for *bbl-a*/*bbl-a bbl-b*/*bbl-b bbl-c*/*bbl-c* plants that

were also homozygous for the recessive alleles at both the *Nic1* and *Nic2* loci (**Figure 8**). The average nicotine content of plants with this genotype was very low (0.014%).

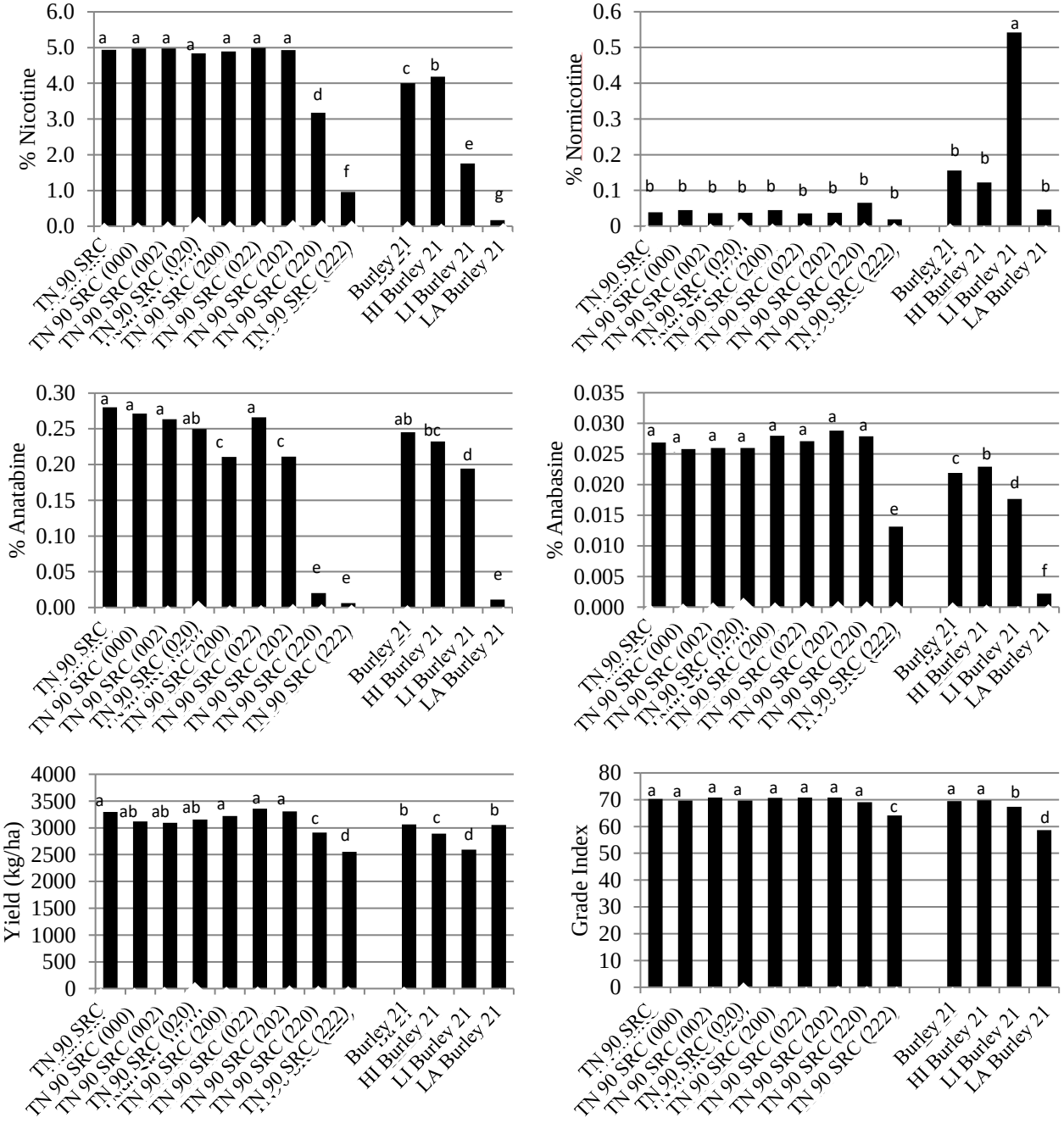

Figure 4. Entry means for TN90 SRC burley tobacco genetic materials evaluated for alkaloid levels, yield, and quality determinations. Means are averaged over four 2016 and 2017 North Carolina field environments. Means with different letters are significantly different from each other at the $P < 0.05$ level of significance.

DISCUSSION

The potential for mandated lowering of nicotine levels in conventional cigarettes by regulatory agencies has caused a growing interest in genetic methodologies that might be used to reduce levels of this alkaloid in the tobacco plant. Ultra-low levels of nicotine (0.04% or below) for cigarette tobacco filler are currently being recommended, but there are no commercially available or publicly disclosed tobacco cultivars of any market type that routinely produce cured leaf with nicotine content this low under conventional production regimes (Lewis, 2018).

Extensive knowledge currently exists regarding enzymes directly involved in nicotine biosynthesis (Dewey and Xie, 2013). It might therefore seem straightforward to interfere with expression of associated genes to reduce nicotine to zero or close to zero. Very recently, Schachtsiek and Stehle (2019) reported on "nicotine-free tobacco" developed using CRISPR-Cas9-based gene editing of members of the *BBL* gene family. However, this work was carried out using poorly described leaf samples from non-topped plants of an unknown tobacco cultivar that were grown in pots in a greenhouse. In addition, this prior research did not determine the relative importance of the six known *BBL* genes on alkaloid accumulation and rigorous evaluations of corresponding effects on tobacco yield and quality were not carried out. Our results demonstrate how conclusions drawn from research related to tobacco alkaloid accumulation can differ according to whether the plants were evaluated in greenhouse versus field environments. Indeed,

as shown here, greenhouse experiments can lead to misleading information for the tobacco regulatory and manufacturing communities.

Thus far, there are no published reports where silencing of a single target gene family has resulted in a nicotinefree tobacco genotype when grown under field conditions and conventional production regimes. In research described here, we assembled all possible homozygous

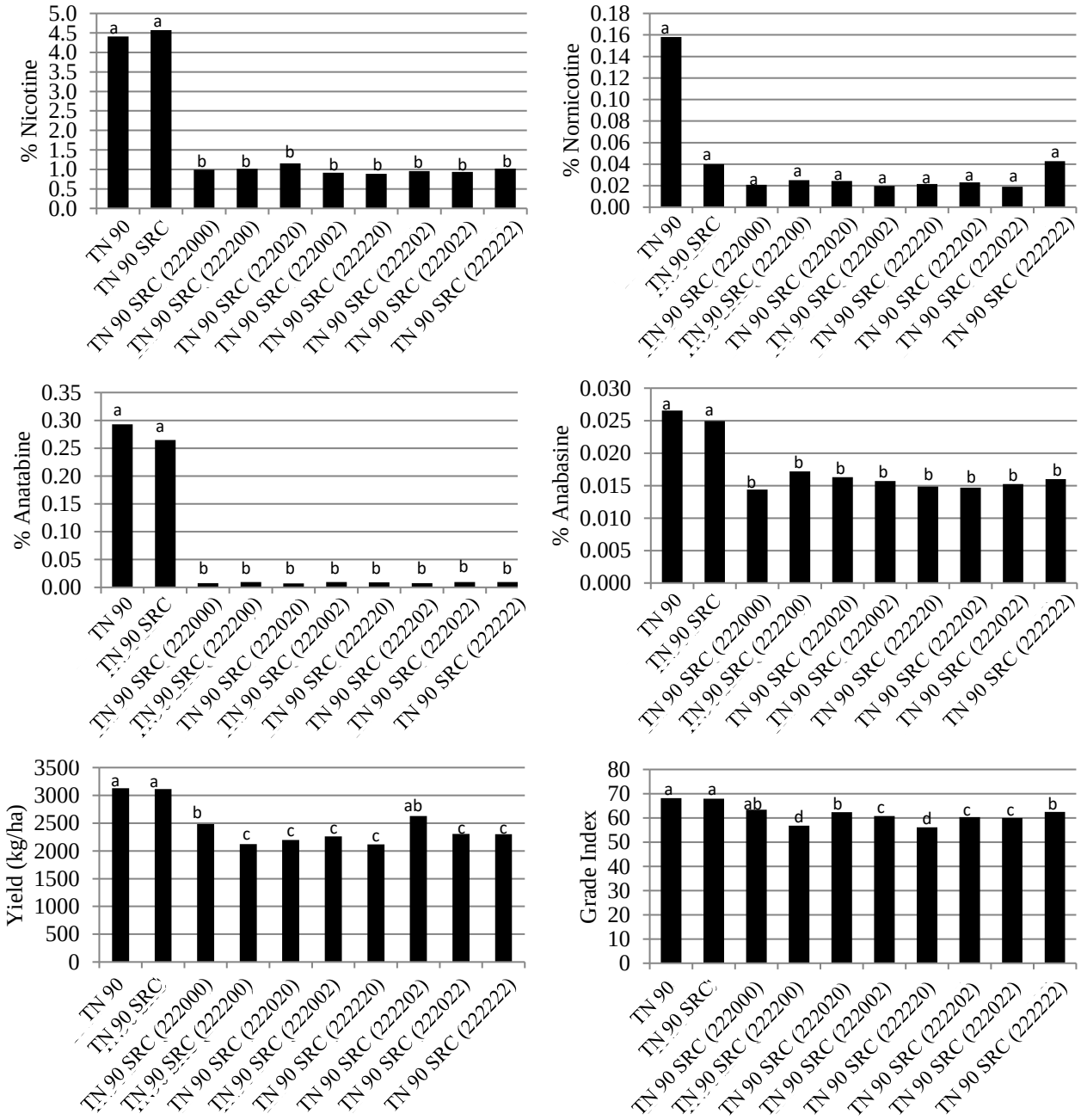

Figure 5. Entry means for TN 90 SRC burley tobacco genetic materials evaluated for alkaloid levels, yield, and quality determinations. Means are averaged over two 2017 North Carolina field environments. Means with different letters are significantly different from each other at the $P < 0.05$

combinations of truncation mutations in the most highly expressed members of the *BBL* gene family involved in one of the later steps of nicotine biosynthesis. The lowest-nicotine *BBL* mutant

combinations resulted in reductions of between 5.8 and 17-fold. Averaged over all stalk positions, the lowest-nicotine *BBL* mutant combination exhibited 0.297% nicotine in the cured leaf. Dramatic reductions in nicotine accumulation were only observed in materials homozygous for mutations in the two most highly expressed *BBL* genes, *BBL-a* and *BBL-b*. Mutations in *BBL-c* resulted in only slight additional reductions in nicotine when combined with mutations in *BBL-a* and *BBL-b*. Mutations in *BBL-d1*, *BBL-d2*, and *BBL-e* appeared to have no significant effect on nicotine levels, either alone, or in combination with other mutations in the TN 90 SRC genetic background that was studied. In no genetic background did the lowest nicotine *BBL* mutant combination exhibit percent nicotine that was numerically lower than that for the conventionally developed low-alkaloid *nic1*/*nic1 nic2*/*nic2* breeding lines LA Burley 21 and LAFC53. Even the lowest nicotine stalk position of the best mutant combination was not beneath the recommended target nicotine level of 0.04%.

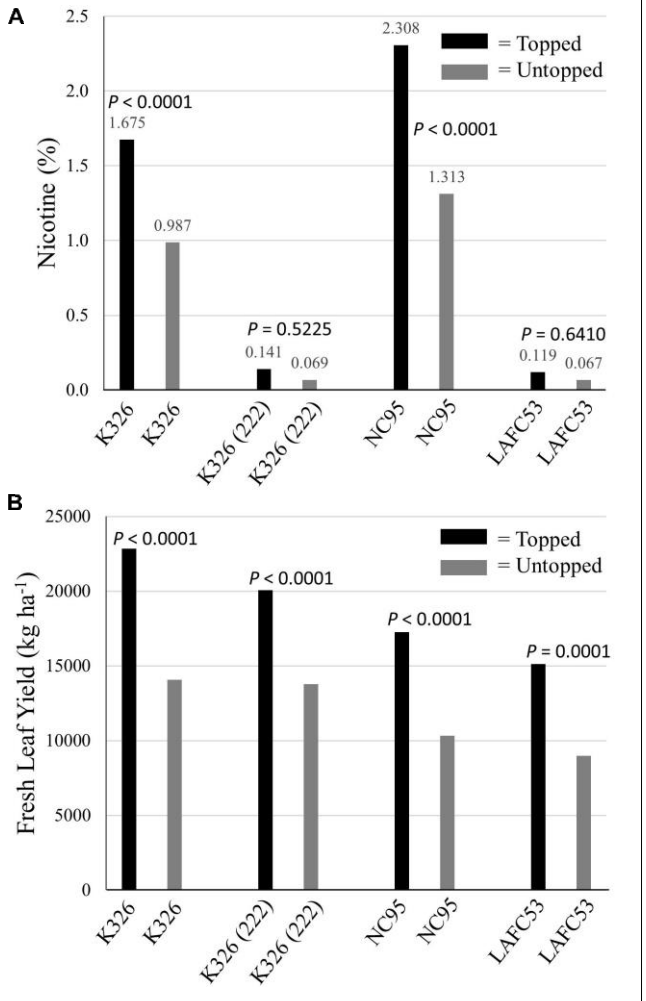

FIGURE 6 | Percent nicotine(A) and fresh leaf yields(B) for four tobacco genotypes evaluated under topped and untopped production regimes. *P*-values for testing the level significance between two means within a genotype are indicated above the bars.

A typical objective of metabolic engineering is to achieve a desired chemical change without otherwise affecting other chemical attributes, growing efficiency, or derived product quality. Prior research has revealed great difficulty in using modified plant genetics to reduce nicotine levels without altering metabolic profiles or negatively affecting tobacco cured leaf yields or quality (Chaplin and Weeks, 1976; Kudithipudi et al., 2017). Similar to the *nic1*/*nic2* system of nicotine reduction, the *BBL* mutant system was also found to reduce cured leaf yields by up to 29%, with associated reductions in cured quality of up to 15%. The reasons for observed associations between reduced nicotine and these measures of commercial significance are unknown, but such data is evidence of complexities associated with plant biochemical and physiological processes that should not be underestimated. It is possible that modified harvesting and curing regimes may lessen the negative impact of this allelic variability on cured leaf quality in low nicotine materials. It may also be important to study the potential for increased insect herbivory on tobacco plants with reduced nicotine accumulation, as increased insect feeding has been observed on *Nicotiana* plants with silenced nicotine biosynthetic genes (Steppuhn et al., 2004).

Nolke et al. (2018) reported increases in polyamine content to be correlated with delays in maturation and senescence in a study of *nic1*/*nic2* isolines of burley tobacco, with problems being most apparent in the lowest alkaloid material. Delayed field ripening of tobacco can translate into reduced cured leaf quality. It is worthwhile to point out that the increased green leaf color frequently associated with the *nic1*/*nic2* type of genetic variation was not as obvious in the *BBL* mutant material. This may have contributed to less severe reductions in cured leaf quality for the *BBL* mutant material as compared to that observed for some of the *nic1*/*nic2* genetic stocks.

No *BBL* mutant combination exhibited percent nicotine lower than that exhibited by the *nic1*/*nic1 nic2*/*nic2* NILs evaluated in the current research. We were, however, able to combine mutations in *BBL-a*, *BBL-b*, and *BBL-c* with the recessive alleles at the *Nic1* and *Nic2* loci. Such genotypes were found, on average, to exhibit nicotine levels in the top leaves harvested 21 days after topping that were lower than those observed for triple *BBL*mutant materials by themselves. Because these genotypes were not inbred lines, we did not carry out evaluation of this material for yield and quality.

BBL enzymes clearly play a role in nicotine biosynthesis, but their precise function is yet to be completely understood. Tobacco genotypes with deleterious mutations in the three most highly expressed *BBL* genes also exhibit severe reductions in anatabine and modest reductions in anabasine, thus indicating a role for BBL-a, BBL-b, and BBL-c enzymes in the biosynthesis of these alkaloids. Because the biosynthesis of nicotine, anatabine, and anabasine all involve condensation reactions with a nicotinic acid derivative, it is reasonable to presume that *BBL-a*, *BBL-b*, and *BBL-c* encode for enzymes involved with the activation of nicotinic acid or play a role in the completion of condensation reactions involving a pyridine ring. It was previously reported that a novel alkaloid designated as dihydrometanicotine (DMN) accumulated in the roots of tobacco plants with interrupted BBL enzyme activity (Kajikawa et al., 2011; Lewis et al., 2015). As pointed out by Kajikawa et al. (2011), the chemical structure of DMN suggests that the condensation of the *N*-methylpyrrolinium cation and pyridine rings may precede the biochemical step catalyzed by *BBL* enzymes. Although it is possible that DMN may be a substrate for BBL enzymes, its failure to be oxidized to nicotine or other known alkaloids in *in vitro* assays suggests the possibility that DMN is instead a stable derivative of the authentic, presumably unstable BBL substrate (Kajikawa et al., 2011).

There are currently no reported experiments where genetic manipulation of the tobacco plant has resulted in reductions of nicotine to non-measurable levels in field-grown plants. In research reported here, we abolished enzyme functionality for all known members of the *BBL* family for which corresponding genes can be identified in public genomic sequence databases. Likewise, use of RNA interference to dramatically reduce expression of alternative gene families involved in nicotine biosynthesis has not resulted in reductions of tobacco nicotine content to non-

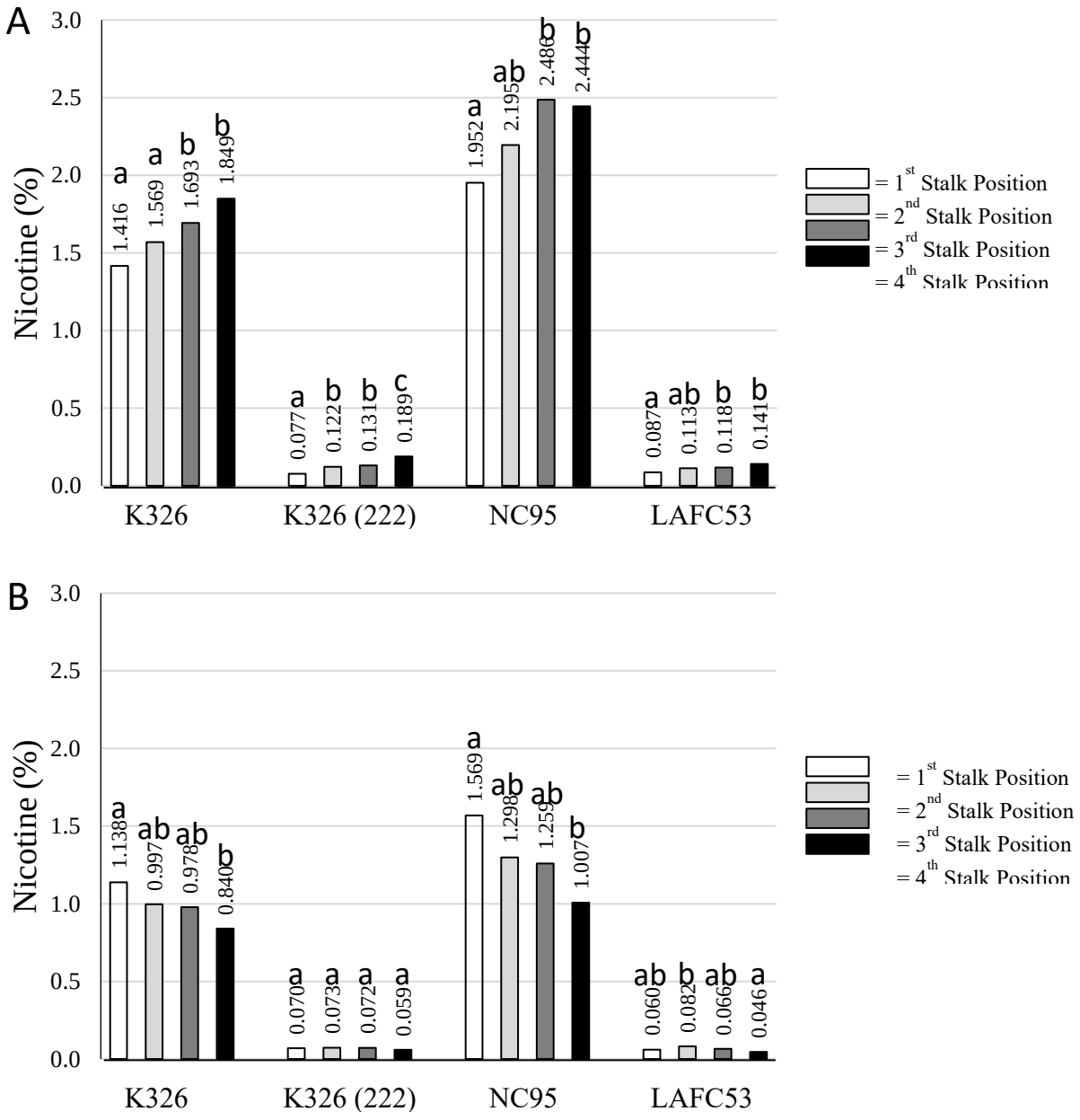

Figure 7. Percent nicotine for four different stalk positions for each of four different tobacco genotypes evaluated under topped (A) and untopped (B) production regimes. Means with different letters within a genotype are significantly different from each other at the $P < 0.05$ level of significance.

detectable levels (Lewis, 2018). Tobacco plants homozygous for deleterious mutations in all known nicotine demethylase genes normally expressed in the leaves or roots also similarly still accumulate appreciable amounts of nornicotine (Lewis et al., 2010). This leads to interesting speculation about the complete genetic and biochemical basis of nicotine and nornicotine production and accumulation in tobacco. The specific enzymatic or chemical steps involved in the condensation reaction between a nicotinic acid-derived intermediate and the *N*-methylpyrrolinium cation in the formation of nicotine are still not understood (Kajikawa et al., 2011). The accumulation of appreciable amounts of nicotine in the absence of any believed functional BBL enzyme activity suggests that some nicotine and its demethylated counterpart, nornicotine, may be biosynthesized via an alternative route. Heim et al. (2007) suggested the possibility of an alternative route for nornicotine biosynthesis that more directly involves putrescine and that is independent of nicotine. In the present research, we observed significantly elevated levels of nornicotine in tobacco lines possessing specific *BBL* mutant combinations, supporting the possible role of a currently uncharacterized route for biosynthesis of this alkaloid. The possible role of promiscuous activity by enzymes currently not identified to be primarily involved with nicotine biosynthesis cannot be ruled out for affecting the accumulation of the

remaining nicotine in tobacco genotypes silenced for a single gene family. The observation of further reductions in nicotine when mutations in *BBL-a*, *BBL-b*, and *BBL-c* were combined with the recessive alleles at the *Nic1* and
Nic2 loci may be informative, however. This finding suggests that gene(s) controlling the

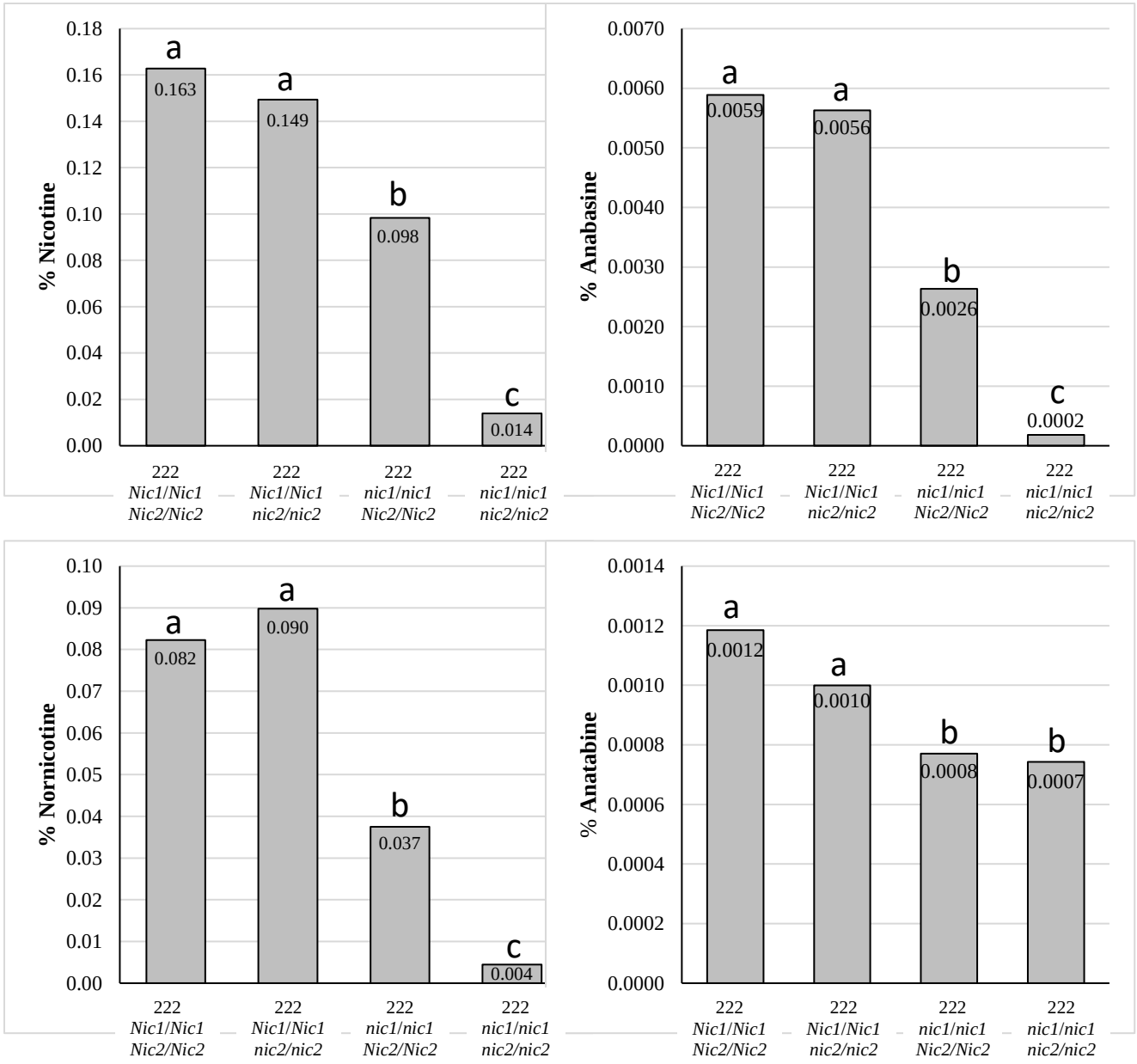

Figure 8. Mean alkaloid contents on a percent dry weight basis for top two leaves of BC_2F_3 individual plants fixed for mutations in *BBL-a*, *BBL-b*, and *BBL-c*, but segregating for recessive alleles at the *Nic1* and *Nic2* loci. Samples were collected 21 days after topping. Number of plants in each genotypic class is indicated within parenthesis. Genotypic means are indicated on individual bars, and means with the same letters are not significantly different from each other at the $P > 0.05$ level of significance as determined by *t*-tests.

accumulation of the majority of the remaining nicotine in genotypes homozygous for the *BBL*

mutations are likely positively regulated by transcription factors reported to reside at the *Nic1* and *Nic2* loci (Shoji et al., 2010; Humphry et al., 2018; Pramod et al., 2019).

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**.

AUTHOR CONTRIBUTIONS

KD-S, WS, and CH performed the genotyping. TS performed the chemical analyses. RL and RD designed the research.
RL analyzed the data and wrote the manuscript. All authors reviewed the manuscript.

FUNDING

This research was partially supported by the 22nd Century Group, Inc.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at:
https://www.frontiersin.org/articles/10.3389/fpls.2020.00368/ full#supplementary-material

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Conflict of Interest: NCSU has licensed specific genetic materials described in this manuscript to 22nd Century Inc.

ACKNOWLEDGMENTS

This research was partially supported by 22nd Century Group, Inc.