Switchgrass (*Panicum virgatum* L.) pollen-mediated gene flow and transgene bioconfinement strategies

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Dedication

This dissertation is dedicated to my lovely wife, Meghan, and our sons, Christian, William, and Reagan, as well as my parents, Bill and Barbara Millwood, my aunt, Martha Jones, my brothers, Ricky Millwood, Rusty Millwood, and Ryan Millwood, parents-in-law John and Christine Mooney and to all family and friends who have given support, advice, love and prayers.

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Abstract

Switchgrass (*Panicum virgatum* L.) is a native North American grass under development as a bioenergy feedstock. Genetic engineering has been employed to introduce transgenic traits for economical biofuel production. However, potential transgene movement from switchgrass raises regulatory and environmental concerns. Experimental studies are needed to determine the potential for transgene flow from switchgrass. The aims of this research are to estimate pollen dispersal and pollination distances for transgenic switchgrass, and evaluate genetically engineered male sterility as a transgene bioconfinement strategy. A three-year field experiment was performed to estimate switchgrass pollen dispersal and pollination distances using a modified Nelder wheel design. Switchgrass (cv. 'Alamo') plants, with whole plant and pollen orange fluorescent protein expression (OFP), were planted as a pollen source. Pollen traps and pairs of nontransgenic Alamo 2 pollen-receptor plant clones were positioned every 10 m extending to 20 m, 30 m, 30 m, and 100 m respective to the north, south, west, and east (direction of the prevailing wind). The finding of the study suggested that switchgrass pollen dispersal is inversely related to distance, and pollination can readily occur up to 100 m (maximum distance measured). To examine transgene bioconfinement, a proof-of-concept study was designed and tested in the model plant tobacco (*Nicotiana tabacum* L.). Targeted expression of the *Eco*RI endonuclease was driven by the tomato pollen-specific LAT52 promoter leading to selective male sterility the elimination of viable transgenic pollen. Nine *Eco*RI transgenic events were produced with normal morphology, and *Eco*RI expression was specific to pollen and negligible in other tissues. Bioconfinement efficacy was examined in glasshouse experiments by hand-crossing transgenic plants to nontransgenic male-sterile and emasculated tobacco. The results demonstrated at or near 100% transgene bioconfinement for one or more generations.

Transgenic lines were further examined in field trials where *Eco*RI tobacco plants were placed in plots with a male-sterile plants. Once again, transgene bioconfinement was observed at or near 100% in four *Eco*RI lines. These results suggest *Eco*RI-driven male sterility is a safe and effective approach for transgene bioconfinement, and this strategy should be translatable to switchgrass as well as other plant species.

Table of contents

Chapter 1: Introduction 1

1.1 Background 2

1.2 Transgene flow from genetically engineered crops 3

1.3 Switchgrass – a potential next-gen genetically engineered crop 8

1.4 Objectives 10

References 12

Appendix 21

Tables 21

Chapter 2: Pollen-mediated gene flow from transgenic to non-transgenic switchgrass (*Panicum virgatum* L.) in the field 24

2.1 Abstract 25

2.2 Introduction 26

2.3 Results 30

2.3.1 Transgenic lines and strategy 30

2.3.2 Plant growth and productivity 31

2.3.3 F_1 progeny analysis 31

2.3.4 Pollen dispersal 32

2.4 Discussion 33

2.5 Conclusions 38

2.6 Materials and methods 39

2.6.1 Transgenic line selection and analysis 39

2.6.2 Field experiment 40
2.6.3 Plant growth and productivity 41
2.6.4 Pollen collection and screening 42
2.6.5 Seed collection, germination and screening 42
2.7 Acknowledgments 43
References 45
Appendix 58
Tables and figures 58
Supplementary tables and figures 61
Chapter 3: Gene use restriction technologies for transgenic plant bioconfinement 69
3.1 Abstract 70
3.2 Introduction 70
3.3 Gene use restriction technologies defined 73
3.4 Components of GURTs 74
3.4.1 The target gene 74
3.4.2 Promoter of the target gene 75
3.4.3 Trait switch gene 75
3.4.4 Genetic switch 76
3.4.5 Functionality of GURTs: putting all the components together 77
3.5 Benefits of GURTs 78
3.5.1 Intellectual property protection 78
3.5.2 Bioconfinement 79
3.5.3 Comparison of IPP- and bioconfinement-oriented GURTs 80

3.6 Designing GURTs for bioconfinement 81
3.6.1 Host plants and system design 81
3.6.2 Choosing appropriate components 83
3.6.3 Disrupter gene 84
3.6.4 Excisor gene 84
3.6.5 Promoter 85
3.6.6 Tuning GURTs 86
3.7 Perspectives and future outlooks 87
3.8 Acknowledgements 89
References 90
Appendix 107
Tables and figures 107
Chapter 4: Engineered selective plant male sterility through pollen-specific expression of the *Eco*RI restriction endonuclease 111
4.1 Abstract 112
4.2 Introduction 113
4.3 Results 116
4.3.1 Transgenic plants: survival and *Eco*RI inheritance and expression 120
4.3.2 Plant morphology 118
4.3.3 Bioconfinement 118
4.4 Discussion 120
4.4.1 Pollen-expressed *Eco*RI as a bioconfinement tool 120
4.4.2 Comparison of LAT52-*Eco*RI-mediated male sterility with other methods 121

4.5 Conclusions 125
4.6 Experimental procedures 126
4.6.1 Vector construction and plant transformation 126
4.6.2 Polymerase chain reaction (PCR) and Southern blot analyses 127
4.6.3 Quantitative reverse-transcriptase PCR (qRT-PCR) 128
4.6.4 Plant productivity 129
4.6.5 Glasshouse bioconfinement experiments 130
4.6.6 Field bioconfinement experiment 132
4.7 Acknowledgement 133
References 134
Appendix 147
Tables and figures 147
Supplementary materials and methods 153
Supplementary tables and figures 155
Chapter 5: Conclusions 161
Vita 166

List of tables

Table 1-1. Genetically engineered switchgrass: nonregulated and regulated United States Department of Agriculture, Animal and Plant Health Inspection Service, Biotechnology Regulatory Services permitted releases. 21

Table 2-1. Growth characteristics of pollen-source and pollen-receptor switchgrass plants grown under field conditions. 58

Table 2-S1. Soil analysis from field site collected from the pollen-source plot and seven pollen-recipient plots. 61

Table 2-S2. PCR progeny screen of F_1 seedlings collected from pollen-recipient plots in the field 62

Table 2-S3. Mean meteorological data collected for the growing seasons of 2012 and 2013 at Oliver Springs, Tennessee. 63

Table 3-1. Comparison of GURTs aiming to intellectual property protection and bioconfinement 147

Table 4-1. Growth characteristics of transgenic *Eco*RI tobacco plants grown under glasshouse conditions (means ± SE). 147

Table 4-2. Screening of progeny seedlings germinated from seeds produced by hybridization experiments performed in the glasshouse and field. 148

Table 4-S1. Segregation analysis of T_2 progeny based on the hygromycin-resistant assay..... 155

Table 4-S2. Hygromycin selection results of F_1 and BC_1F_1 progeny seedlings collected from maternal emasculated 'Xanthi' plants hand-crossed with experimental pollen donors..... 156

List of figures

Figure 2-1. Relationship between transgenic F1 switchgrass seedlings collected from individual pollen-recipient plots and distance from the transgenic switchgrass pollen-source plot (Poisson regression, F1,15 = 12.98, $P < 0.003$)..... 59

Figure 2-2. Average number of orange fluorescent protein (OFP)-tagged switchgrass pollen grains detected as a function of distance (Poisson regression, F1,8 = 288.38, $P < 0.0001$) under field conditions..... 60

Figure 2-S1. Southern blot analysis of T_0 transgenic switchgrass events containing stable integration of the T-DNA..... 64

Figure 2-S2. Sample of orange fluorescent protein (OFP)-tagged switchgrass pollen from source plants. A), white light, B), TxRed filter, C), merged image 65

Figure 2-S3. A sample gel from PCR analysis for the *pporRFP* (OFP) gene that was performed on F_1 switchgrass progeny (lanes 1–12) collected from field study pollen-recipient plots 66

Figure 2-S4. Experimental layout of field study located in Oliver Springs, Tennessee, U.S.A. Transgenic switchgrass (cultivar 'Alamo', clone ST1) served as a pollen-source (10 rows × 10 plants) while non-transgenic switchgrass (cultivar 'Alamo', clone Alamo 2) plants were placed in pollen-recipient plots planted at 10 m intervals..... 67

Figure 2-S5. Average number of orange fluorescent protein (OFP)-tagged switchgrass pollen grains detected as a function of distance in the north, south, and east directions (East, $R^2 = 0.953$; North, $R^2 = 0.990$; West, $R^2 = 0.930$; and South $R^2 = 0.993$) 68

Figure 3-1. A schematic diagram of signal cascade of GURTs..... 108

Figure 3-2. A schematic diagram describing fundamental designs of GURTs..... 109

Figure 3-3. Schematic diagrams showing V- and T-GURTs for bioconfinement 110

Figure 4-1. Transformation vector T-DNA used in plant transformation experiments and genomic PCR results 149

Figure 4-2. Quantitative RT-PCR of the *Eco*RI transcript in transgenic plants in various tissues 150

Figure 4-3. Phenotype of a representative T_1 transgenic event (E4) expressing *Eco*RI in pollen and a nontransgenic tobacco ('Xanthi') 151

Figure 4-4. Average number of seeds produced per pod from transgenic lines expressing *Eco*RI in pollen under glasshouse and field conditions 152

Figure 4-S1. Southern blot analysis of T_2 transgenic *Eco*RI tobacco plants 157

Figure 4-S2. Quantitative RT-PCR analysis of the *Eco*RI transcript of mature pollen in transgenic plants 158

Figure 4-S3. Pollen grains produced per flower from *Eco*RI tobacco plants grown under greenhouse conditions 159

Figure 4-S4. Progeny screening method using MS medium supplemented with 50 $mg.L^{-1}$ hygromycin 160

Chapter 1: Introduction

1.1 Background

Genetically engineered (GE) crops have provided economic, agronomic, and environmental benefits to consumers as well as large- and small-scale farmers. (NASEM, 2016). Yet, debate continues over potential adverse impacts related to transgene movement (transgene flow) from GE crops. Transgene flow can occur through the dispersal of transgenic pollen to nearby sexually compatible wild or weedy (WW) relatives as well as seed or vegetative propagule dispersal during harvest and transport or through natural means (e.g. wind, water, or animals). Each carries with it regulatory and environmental concerns should transgenes move to unmanaged populations. One concern is a transgene could provide a selective advantage to a recipient population, leading to new weeds or increased weediness that could negatively impact local plant populations or lead to problematic weed control issues. (Londo et al., 2010; Kwit et al., 2011). Some have suggested that transgene flow is certain to occur, and transgenes are unlikely to be removed once they have escaped their intended locations (Marvier and Van Acker 2005). These concerns have led to intensive research efforts focused on the risks associated with transgene flow and transgene persistence in the environment.

The movement of transgenes has been documented for several GE plant species (regulated and nonregulated) that contain transgenes for only a few traits (reviewed by Kwit et al., 2011; Ellstrand et al., 2013; Price and Cotter, 2014; Gressel, 2015; Tsatsakis et al., 2017; Ellstrand, 2018). The majority of GE crops contain either a single or stacked traits for viral-, insect-, or herbicide-resistant varieties, and transgene movement to unmanaged populations could lead to undesirable outcomes. For example, if virus- or insect-resistant transgenes move beyond cultivation, then the transgene-recipient population would gain protection from either disease or

herbivory. Transgenic hybrids/volunteers could outcompete other plants within a population when disease or herbivory pressure is high. Although, it should be noted there is no documentation of such occurrence in the literature (NASEM, 2016). Additionally, should herbicide-resistant transgene movement occur, transgenic hybrids could be protected against selective sweeps (e.g. herbicide sprays) leading to an increase in herbicide-resistant hybrids/volunteers within a population (Kwit et al., 2011). This scenario has occurred previously and led to weed management problems (Zapiola et al., 2008; Greene et al., 2015; Zapiola and Mallory-Smith, 2017). In addition, herbicide resistant transgene flow may not be detrimental in all environments, because no fitness advantage is gained in natural ecosystems where herbicide application does not occur (Gressel, 2015).

Indeed, many cases of and transgenic plants have been identified outside of their intended location (Price and Cotter, 2014), and certain transgenes have been persisted in the environment more than others (Ellstrand, 2018). In following examples, transgene flow has led to transient transgenic volunteers that germinated from seedbanks established from previous field trials, and in a very few cases hybrids/volunteers have established as persistent free-living transgenic populations (Ellstrand, 2018). Yet, the movement of transgenes beyond cultivation has been of little consequence, and no evidence exists to link GE crops to adverse environmental impacts (NASEM, 2016).

1.2 Transgene flow from genetically engineered crops

To date, a small number of transient volunteer plants that contained either single or stacked traits for insect or herbicide resistance in maize (*Zea mays* L) have been documented in South Korea

(Lee et al., 2009; Park et al., 2010; Han et al., 2014; Ellstrand, 2018), and herbicide-resistant wheat (*Triticum aestivum* L.) volunteers were found in the United States (US) states of Montana, Oregon, and Washington (Ellstrand, 2018 and references therein). These GE volunteer populations are likely the result of seed spillage during harvest or transport. In addition, regulated GE maize volunteers, likely germinated from prior field trial seedbanks, have been identified in the US states of Iowa and Nebraska. These volunteers contained genes that code for protein, enzymes, and molecules used to produce pharmaceutical or industrial compounds (NASEM, 2016). These GE volunteers were destroyed following their discovery, and there are currently no reports of persistent GE maize or wheat populations beyond cultivation.

Transgene flow has also been report for GE virus-resistant papaya in the US state of Hawai′i. Seeds from feral papaya populations located on the islands of Oahu and Hawai′i were collected and 22% of seeds were positive for transgenes (Manshardt et al., 2016). Cross-pollination with GE plants growing in nearby plantations was the likely route of transgene movement. There was no indication of negative agronomic or ecological impacts associated with transgene flow from GE papaya.

GE herbicide-resistant and insect-resistant cotton (*Gossypium hirustum* L.) hybrids were identified hundreds of kilometers from known cultivated sites when wild cotton populations were sampled in northern Mexico from 2002 through 2008 (Wegier et al., 2011). GE seed dispersal was determined to be the likely path of transgene flow leading volunteer establishment and subsequent cross-pollination with local WW cotton populations. To this point, there are no reports of negative impacts associated with these populations.

Herbicide-resistant (glyphosate) alfalfa seeds were found in 27% of feral population sites sampled in the US states of California, Idaho, and Washington (Greene et al., 2015). The primary route of transgene flow likely occurred via seed spillage (Greene et al., 2015) during the harvest of seed production fields. Pollen-mediated transgene flow may have also played a significant role due to the presence of nearby feral alfalfa populations growing within the pollination range of GE alfalfa fields (Greene et al., 2015). These glyphosate-resistant hybrid/volunteer populations have become problematic to control in ruderal environments, because glyphosate is typically used for weed control. An eradication plan that excludes glyphosate application is needed to eliminate roadsides populations (NASEM, 2016) and to minimize further transgene dispersal into the environment. Furthermore, new management strategies are needed to prevent future GE seed spillage during harvest and transport.

Several reports of transgene flow have been documented with GE canola (*Brassica napus* L.). Pollen-mediated gene flow can readily occur, because canola varieties, such as *B. napus*, can readily hybridize with many WW relatives including *B. rapa* L., *Raphanus raphanistrum* L., *Sinapis arvenis* L, and *Erucastrum gallicum* (Willd.) O.E. Schulz (Warwick et al., 2003). F_1 hybrids between GE herbicide-resistant canola and *B. rapa* L. were identified in Québec, Canada (Warwick et al., 2003). Multi-generation backcrossed hybrids were later found within populations indicating transgenic individuals were present in the population for some time (Warwick et al., 2008). In addition, transgenic seed spillage has been a major route of transgene flow for canola. Free-living transgenic herbicide-resistant populations have been document along transport lines in Manitoba, Canada (Knispel and McLachlan, 2010), the US state of North Dakota (Schafer et al., 2011), and Japan (Aono et al., 2006). These studies suggest that GE

hybrids/volunteers canola populations can be established via pollen- or seed-mediated transgene flow. Although is no apparent risk associated with GE herbicide-resistant canola (Warwick et al., 2008; NASEM, 2016), these populations, like herbicide-resistant alfalfa, could lead to weed control issues if herbicide-resistance renders commonly used herbicides useless.

There are also several reports of transgene flow involving creeping bentgrass. Regulated field trials of GE herbicide-resistant creeping bentgrass (glyphosate resistant) were planted on 162 ha in the US state of Oregon in 2002. The following year strong winds moved GE pollen and seeds out of cultivated areas, and once discovered, the field trials were terminated after harvest (Zapiola and Mallory-Smith, 2017). In the following years, seeds and leaf tissues were collected *in situ* from creeping bentgrass and WW relatives found growing in the region. Surveys were conducted nearly every year from 2003 through 2016, and transgenic intergenic and interspecies hybrids were found each year testing occurred (Reichman et al., 2006; Zapiola et al., 2008; Zapiola and Mallory-Smith, 2017). In recent years, glyphosate-resistant creeping bentgrass has become a major weed in irrigation canals, because glyphosate is the only herbicide approved for canal weed control and other control measure have been ineffective (NASEM, 2016). An eradication plan should be developed that excludes glyphosate application otherwise there is a strong likelihood that GE creeping bentgrass will remain in the environment and problematic to control (NASEM, 2016).

It is important to understand the commonalities and circumstances that led to previous incidents of transgene flow in order to develop sound risk assessment and management plans for current and future GE crops. The plant species involved in these examples have one or more of the

following common characteristics. They are known to have previously established feral populations near cultivated sites, have small seeds that are easily dispersed (e.g. shatter during harvest), have nearby sexually compatible WW, long-distance pollen dispersal, perennial growth, and transgenic traits are neutral or beneficial in the receiving environment (Ellstrand, 2018). As these examples illustrate, GE-glyphosate resistance can lead to problematic weed management when volunteers/hybrids are established in a ruderal ecosystem where glyphosate application is commonly used for weed control.

Although the list of current transgenes released in the environment have thus far been of minimal consequence, the next generation of GE traits may lead to entirely different outcomes. Next-gen GE crops will be endowed with traits, such as increased growth rate, drought tolerance, and disease resistance. For example, intensive research efforts have begun to genetically engineer these transgenic traits into grass species for improved bioenergy production. Bioenergy crops, such switchgrass (*Panicum virgatum* L.), have similar reproductive biology to that of creeping bentgrass. Using this example as a guide, it is apparent that gene flow from other GE grass species is likely to occur. Should pollen- or seed-mediated transgene flow occur with next generation transgenic traits, there is concern transgenic hybrid/volunteers could colonialize new areas beyond their natural habitat. This scenario carries with it potential ecosystem consequences should these plant become invasive and negatively impact native plant populations (Kausch et al., 2010, Kwit et al., 2012). For next-gen GE crops, relevant risk assessment should focus on the biology of the crop (reviewed in Andersson & de Vicente 2010), the location of sexually compatible WW relative present in the region of cultivation (Ellstrand 2003), exposure pathways

(e.g. pollen and seed dispersal; Liu and Stewart, 2019) as well as the effects of the associated transgenic trait in the proposed release environment.

1.3 Switchgrass – a potential next-gen genetically engineered crop

In all bioenergy visions for the United States (US) dedicated biomass crops such as switchgrass play a prominent role (US Department of Energy, 2016). Switchgrass has become an attractive biofuel feedstock candidate because of its high biomass yield and its ability to grow with little input on low fertility soils (Sanderson et al., 1996). However, the plant cell walls of switchgrass have proven to be recalcitrant to biofuel (e.g. ethanol) conversion. Genetic engineering has been employed to address this problem, and breakthroughs have produced GE switchgrass that reportedly produce significantly more biofuel (Xu et al., 2011; Fu et al., 2011a; Fu et al., 2011b; Saathoff et al., 2011; Shen et al., 2012; Baxter et al., 2014; Poovaiah et al., 2014; Baxter et al., 2015; Wuddineh et al., 2016; Baxter et al., 2016). It is clear that transgenic approaches will be a key component in switchgrass biofuel feedstock development. In fact, from 2008-2019 a total of 37 "Biotechnology Release Permits" have been issued for GE switchgrass (Table 1-1) by the US Department of Agriculture's Animal and Plant Health Inspection Service (APHIS), Biotechnology Regulatory Services (BRS). In addition, two GE switchgrass (Table 1-1) cultivars have been classified as nonregulated using through the BRS "Am I Regulated" process. The majority of transgenic traits currently under development in switchgrass are related to altered cell wall biosynthesis to improve the conversion efficiency from biomass to biofuel (Table 1-1). Other traits include delayed flowering, increased biomass, and abiotic stress (e.g. drought) tolerance (Table 1-1).

To meet bioenergy goals widespread plantings of switchgrass are projected in the central and eastern portions of the US (US Department of Energy, 2016). The inclusion of GE cultivars raises regulatory and environmental concerns (Stewart, 2007; Kausch et al., 2010; Kwit et al., 2012), because switchgrass is perennial grass that is native to North America, east of the Rocky Mountains (Zhang et al., 2011). In addition, switchgrass is a wind-pollinated species and mostly self-incompatible which forces outcrossing (Martinez-Reyna and Vogel, 2002). The presence of GE switchgrass in its native region would likely lead to transgene flow to native or wild populations (Stewart, 2007, Kausch et al., 2010, Kwit and Stewart, 2012). Recent modelling efforts suggest most switchgrass pollen will travel up to 200 m, but pollen may move up to 6.5 km in strong wind conditions (Ecker et al., 2013). It is likely that the release of GE switchgrass would lead to transgene flow similarly to that of creeping bentgrass where hybridization was documented at 21 km from creeping bentgrass cultivation (Watrud et al., 2004). Furthermore, seed-mediated transgene flow is likely to occur in a similar manner to that of transgenic canola, alfalfa, and creeping bentgrass. These lessons are important to remember as risk management strategies are considered. When above-ground switchgrass biomass is harvested, each switchgrass plant will contain thousands of seeds that would practically be impossible to contain en route to biorefineries. It is conceivable that GE volunteer populations could be established outside cultivation; which would extend its pollination range and allow recurrent cross-pollination with WW populations or other nearby agronomic non-GE switchgrass fields. North America is the geographic center of diversity for switchgrass, and if past lessons are worth heeding; this fact would lead to added concerns from ecologists and regulators regarding potential transgene flow. Therefore, it is clear that gene flow is a real concern for GE switchgrass. To address future management or environmental issues (Kwit and Stewart, 2012;

Ecker et al., 2013; Millwood et al., 2017; Chang et al., 2018), and it would be wise to include bioconfinement technology (*sensu* Millwood et al., 2016) and/or mitigation technologies during the GE crop development phase (Moon et al., 2010; Sang et al., 2013; Ding et al., 2014; Gressel 2015).

1.4 Objectives

Given the increased interest in switchgrass as a bioenergy feedstock and efforts to produce GE cultivars, studies to gain detailed knowledge of switchgrass gene flow should be undertaken. Up to this point, little has been documented regarding switchgrass pollen biology as well as pollen dispersal and pollination distances. These data would prove valuable for the environmental risk assessment of switchgrass and help in to develop risk management plans. In addition, the testing and deployment of bioconfinement technologies may be necessary to fully realize the potential of switchgrass as a GE bioenergy crop. The overall goals of these experiments described in this dissertation are two-fold: 1) Gather experimental data to gain knowledge of transgene flow in switchgrass with the aim to address potential risks associated with large agronomic GE switchgrass plantings, and 2) Evaluate a novel bioconfinement strategy for the elimination or reduction of pollen-mediated gene flow by linking male sterility to any engineered transgenic trait. Specifically, the following three objectives will be explored to gain knowledge on the drivers of switchgrass pollen-mediated gene flow and to explore effective transgene bioconfinement strategies that have potential for deployment in switchgrass.

1. Pollen-mediated gene flow from transgenic to non-transgenic switchgrass (*Panicum virgatum* L.) in the field

2. Gene use restriction technologies for transgenic plant bioconfinement
3. Engineered selective plant male sterility through pollen-specific expression of the *Eco*RI restriction endonuclease

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Appendix

Tables

Table 1-1. Genetically engineered switchgrass: nonregulated and regulated United States Department of Agriculture, Animal and Plant Health Inspection Service, Biotechnology Regulatory Services permitted releases.

Company or Institution	Release Type	Effective Date	Expiration Date	Release Location	Phenotypes
Joint Bioenergy Institute	BRS Permit	4/1/2019	4/1/2020	CA	Altered lignin biosynthesis, decreased ferulic acid, lignin levels decreased
Joint Bioenergy Institute	BRS Permit	3/7/2019	3/7/2020	CA	Altered lignin biosynthesis, decreased ferulic acid, lignin levels decreased
Joint Bioenergy Institute	BRS Permit	4/1/2017	4/1/2019	CA	Altered lignin biosynthesis, decreased ferulic acid, lignin levels decreased
University of Tennessee	BRS Permit	4/1/2017	4/1/2018	TN	Growth rate increased, lignin levels decreased
Afingen, Inc.	BRS Permit	6/30/2016	6/29/2017	CA	Altered lignin biosynthesis, increased stalk strength
University of Tennessee	BRS Permit	6/21/2016	6/20/2019	TN	Growth rate increased
University of Tennessee	BRS Permit	6/21/2016	6/20/2019	TN	Growth rate increased
University of Tennessee	BRS Permit	5/30/2016	5/30/2019	TN	Growth rate increased, lignin levels decreased
University of Tennessee	BRS Permit	5/25/2016	5/24/2019	TN	Increased growth rate, reduced or no flowering
ERDC-CRREL	BRS Permit	5/1/2016	5/1/2019	NY	Hygromycin resistant, rdx degradation, resist tnt phytotoxicity
University of Tennessee	BRS Permit	6/12/2015	6/12/2018	TN	Growth rate increased, decreased lignin level
University of Tennessee	BRS Permit	4/13/2015	4/13/2018	TN	Fluorescent protein visual marker, lignin levels decreased
University of Tennessee	BRS Permit	10/4/2014	10/4/2017	TN	Lignin levels decreased
Agrivida, Inc.	BRS Permit	4/22/2014	4/22/2017	IN	Phenotype classified as confidential business information
University of Tennessee	BRS Permit	4/1/2014	4/1/2017	TN	Growth rate increased, lignin levels decreased

Table 1-1 (continued)

Company or Institution	Release Type	Effective Date	Expiration Date	Release Location	Phenotypes
University of Tennessee	BRS Permit	6/21/2013	6/21/2016	TN	Growth rate increased
University of Tennessee	BRS Permit	6/21/2013	6/21/2016	TN	Growth rate increased
University of Tennessee	BRS Permit	5/25/2013	5/25/2016	TN	Fluorescent protein visual marker, increased growth rate, reduced or no flowering
Ceres, Inc.	Nonregulated	1/02/2013	N/A	N/A	Water use efficiency enhanced
Plant Gene Expression Center	BRS Permit	6/1/2012	6/1/2015	CA	Improved biofuel, increased biomass, reduced lignin content
Ceres, Inc.	BRS Permit	5/15/2012	5/15/2015	TX	Biomass conversion efficiency
Ceres, Inc.	BRS Permit	5/6/2012	5/6/2015	TX	Drought tolerance increased, nitrogen utilization efficiency increase
Ceres, Inc.	BRS Permit	5/2/2012	5/2/2015	AZ, TX	Drought tolerance increased
Ceres, Inc.	BRS Permit	5/1/2012	5/1/2015	TX	Phenotype classified as confidential business information
Ceres, Inc	Nonregulated	4/24/2012	N/A	N/A	Biomass increased, Biomass conversion efficiency
University of Tennessee	BRS Permit	4/13/2012	4/13/2015	TN	Fluorescent protein visual marker, lignin levels decreased
University of Tennessee	BRS Permit	10/4/2011	10/4/2014	TN	Lignin levels decreased
Ceres, Inc.	BRS Permit	6/25/2011	6/25/2014	TX	Drought tolerance increased
Ceres, Inc.	BRS Permit	6/17/2011	6/17/2014	TX	Biomass conversion efficiency
Institute For Advanced Learning And Research	BRS Permit	6/8/2011	6/8/2014	VA	Altered lignin biosynthesis, fertility altered, growth rate increased, increased stalk strength, senescence altered, yield increased, phosphinothricin tolerant
University of Tennessee	BRS Permit	5/25/2011	5/25/2013	TN	Visual marker
Ceres, Inc.	BRS Permit	7/12/2010	7/12/2013	GA	Nitrogen utilization efficiency increase
Ceres, Inc.	BRS Permit	6/30/2010	6/30/2013	AZ, TX	drought tolerance increased, nitrogen utilization efficiency increase, sterile
Ceres, Inc.	BRS Permit	6/21/2010	6/21/2013	TN	Nitrogen utilization efficiency increase

Table 1-1 (continued)

Company or Institution	Release Type	Effective Date	Expiration Date	Release Location	Phenotypes
Plant Gene Expression Center	BRS Permit	4/1/2010	4/1/2013	CA	Increased biomass, reduced lignin content
Ceres, Inc.	BRS Permit	5/6/2009	5/6/2012	AZ, TX	drought tolerance increased, fertile, nitrogen utilization efficiency increase, sterile
University of Tennessee	BRS Permit	4/17/2009	4/17/2012	TN	Fluorescent protein visual marker, lignin levels decreased
Ceres, Inc.	BRS Permit	6/24/2008	6/24/2011	TX	Drought tolerance increased
Ceres, Inc.	BRS Permit	6/24/2008	6/24/2011	TX	Sterile

Sources: https://www.aphis.usda.gov/aphis/ourfocus/biotechnology/permits-notifications-petitions/SA_Permits/Status-Update, https://www.aphis.usda.gov/aphis/ourfocus/biotechnology/am-i-regulated/Regulated_Article_Letters_of_Inquiry

Chapter 2: Pollen-mediated gene flow from transgenic to non-transgenic switchgrass (*Panicum virgatum* L.) in the field

A version of this chapter has been published in the journal BMC Biotechnology with the following authors: Reginald J. Millwood, Madhugiri Nageswara-Rao, Rongjian Ye, Ellie Terry-Emert, Chelsea R. Johnson, Micaha Hanson, Jason N. Burris, Charles Kwit and C. Neal Stewart Jr.. Reginald J. Millwood collected data, performed data analyses, and drafted and revised the manuscript. Madhugiri Nageswara-Rao collected pollen data and participated in the manuscript draft. Rongjian Ye, Ellie Terry-Emert, Chelsea R. Johnson assisted with the field experiment and progeny data collection. Micaha Hanson assisted with the field experiment and pollen data collection. Jason N. Burris performed the Southern blot analysis. Charles Kwit and C. Neal Stewart Jr. supervised the study and participated in manuscript drafts and revisions. Journal article citation: Millwood, R., Nageswara-Rao, M., Ye, R., Terry-Emert, E., Johnson, C.R., Hanson, M., Burris, J.N., Kwit, C. and Stewart, C.N., Jr. (2017) Pollen-mediated gene flow from transgenic to non-transgenic switchgrass (*Panicum virgatum* L.) in the field. BMC Biotechnol. 17, 40. doi: 10.1186/s12896-017-0363-4

2.1 Abstract

Switchgrass is C_4 perennial grass species that is being developed as a cellulosic bioenergy feedstock. It is wind-pollinated and considered to be an obligate outcrosser. Genetic engineering has been used to alter cell walls for more facile bioprocessing and biofuel yield. Gene flow from transgenic cultivars would likely be of regulatory concern. In this study we investigated pollen-mediated gene flow from transgenic to nontransgenic switchgrass in a 3-year field experiment performed in Oliver Springs, Tennessee, U.S.A. using a modified Nelder wheel design. The planted area (0.6 ha) contained sexually compatible pollen source and pollen receptor switchgrass plants. One hundred clonal switchgrass ‘Alamo’ plants transgenic for an orange-

fluorescent protein (OFP) and hygromycin resistance were used as the pollen source; whole plants, including pollen, were orange-fluorescent. To assess pollen movement, pollen traps were placed at 10 m intervals from the pollen-source plot in the four cardinal directions extending to 20 m, 30 m, 30 m, and 100 m to the north, south, west, and east, respectively. To assess pollination rates, nontransgenic Alamo 2 switchgrass clones were planted in pairs adjacent to pollen traps. In the eastward direction there was a 98% decrease in OFP pollen grains from 10 to 100 m from the pollen-source plot (Poisson regression, $F_{1,8} = 288.38$, $P < 0.0001$). At the end of the second and third year, 1,820 F_1 seeds were collected from pollen recipient-plots of which 962 (52.9%) germinated and analyzed for their transgenic status. Transgenic progeny production detected in each pollen-recipient plot decreased with increased distance from the edge of the transgenic plot (Poisson regression, $F_{1,15} = 12.98$, $P < 0.003$). The frequency of transgenic progeny detected in the eastward plots (the direction of the prevailing wind) ranged from 79.2% at 10 m to 9.3% at 100 m. In these experiments we found transgenic pollen movement and hybridization rates to be inversely associated with distance. However, these data suggest pollen-mediated gene flow is likely to occur up to, at least, 100 m. This study gives baseline data useful to determine isolation distances and other management practices should transgenic switchgrass be grown commercially in relevant environments.

2.2 Introduction

Switchgrass (*Panicum virgatum* L.) is a perennial C4 bunchgrass that is native to North America and is widely distributed from Canada to Mexico where it occasionally reaches “common” status in certain prairies (Weaver et al., 1934; Howe et al., 2002; Baer et al., 2005; Haught and Myster, 2008), marshes (Ford and Grace, 1998), conservation reserve program settings (Mulkey et al.,

2006; Adler et al., 2009), and along roadsides and waste places (Radford et al., 1968; Ahrens et al., 2011). Elsewhere, it is typically not a large component of natural areas (Grelen and Duvall, 1966; Byrd,1980) and is often found in much lower densities than those grown in agronomic settings. Traditionally switchgrass has been grown as a forage crop (Heaton et al., 2004; Parrish and Fike, 2005), and because it is highly adaptable, it has served a variety of purposes including conservation, wildlife habitat, prairie restoration, erosion control, and in ornamental gardens (Heaton et al, 2004; Kwit and Stewart, 2012; Nageswara-Rao et al., 2012). Owing to its high biomass yield and suitability for marginal land, switchgrass has been the target of extensive development as a dedicated lignocellulosic biofuel feedstock (Sanderson et al, 1996). With the current drive to meet U.S. government-mandated benchmarks for energy production [16 billion gallons (1 gallon = 3.79 L) cellulosic biofuel by 2022; (US 110th Congress, 2007)], large-scale agronomic plantings of dedicated biofuel crops, including switchgrass, are projected to increase significantly through 2022 (Kwit and Stewart, 2012; Nageswara-Rao et al., 2014; Trainor et al., 2016). Although switchgrass research and development of conventional cultivars has significantly expanded over the last 25 years (Casler et al., 2015), extensive research efforts have been undertaken to produce transgenic switchgrass cultivars with increased biomass, enhanced saccharification efficiency, and modified lignin biosynthesis for improved biofuel properties (Xu et al., 2011; Fu et al., 2011a; Fu et al., 2011b; Saathoff et al., 2011; Shen et al., 2012; Baxter et al., 2014; Baxter et al., 2015; Poovaiah et al., 2014; Wuddineh et al., 2016; Baxter et al., 2016). Indeed, genetic engineering appears to be the only route to overcome biomass recalcitrance and sustainably improve biomass and biofuel production in tandem (Stewart, 2007; Jakob et al., 2009; Kausch et al., 2010).

Although genetic engineering provides an opportunity to increase biomass yields and efficiencies in switchgrass production, transgenes from large-scale switchgrass plantings could escape to (1) nontransgenic switchgrass that might be used for livestock feed or other future uses, and (2) wild relatives in the landscape matrix (Kwit and Stewart, 2012; Stewart, 2007; Kausch et al., 2010). Switchgrass is also being engineered for abiotic and biotic resistance traits (e.g. drought and salt tolerance, as well as pathogen resistance) (Ridley and Mallory-Smith, 2015), which could lead to increased distribution as well as shifted ecological niches if introgressed in conspecific or wild relative lineages. Switchgrass already exhibits a number of characteristics associated with invasiveness (Buddenhagen et al., 2009; Chimera et al. 2010). These include C_4 photosynthesis, a long canopy duration, few known pests and diseases, rapid growth in the spring (growing season), below-ground partitioning of nutrients in the fall (dormant season), and high water-use efficiency (Raghu, 2006). The potential repercussions from habitat shifts include conservation concerns, with two different extremes being (1) the subsequent rapid spread of a transgene throughout the wild population and enhancing the invasiveness of the recipient population, or (2) extinction of the wild population (Kwit and Stewart, 2012; Raghu, 2006; Barney and DiTomaso, 2008; Davis et al., 2010; Barney, 2014; Ecker et al., 2015). The likely route of transgene escape from switchgrass is through pollen-mediated gene flow (Stewart, 2007). It is well documented that wind-mediated pollen- and gene flow readily occurs in many grasses (reviewed by Kausch et al., 2010), and transgene escape and establishment has been documented with herbicide resistant creeping bentgrass (*Agrostis stolonifera* L.) (Belanger et al., 2003; Watrud et al., 2004; Reichman et al., 2006). Switchgrass shares similar characteristics (perennial, wind-pollinated, obligate outcrossing species) with creeping bentgrass, and considering field sites in the U.S. would be located near wild conspecifics and congeners, it is conceivable that pollen-mediated

gene flow would occur (Kwit and Stewart, 2012). Yet to date relatively few studies have been published concerning switchgrass pollen biology and dispersal (Ge et al., 2011; Ecker et al., 2013). The dearth of gene flow data, especially in the U.S. is largely to regulatory conditions: all regulated field trials to date require panicles to be removed at the R0 stage, obviating the ability to assess transgenic pollen movement and hybridization from transgenic pollen donors on non-transgenic pollen recipients.

It is imperative that we increase our existing knowledge on the drivers of transgene spread to satisfy imminent regulatory and conservation concerns regarding future transgenic bioenergy crops; indeed, stewardship of resources is a major reason. For switchgrass, we know little about pollen spread. To address the knowledge gap, we obtained release permits (11-007-106r and 13-046104r-a1) through the United States Department of Agriculture's Biotechnology Regulatory Services for the release of transgenic switchgrass expressing an orange fluorescent protein gene and a hygromycin resistance marker gene in order to perform a small multi-year pollen dispersal and cross-pollination field study. The study site was located in a secluded area surrounded by forest on all sides. Although the study was small in size, this experiment provided opportunity to gather much needed empirical data on transgenic switchgrass pollen dispersion and pollination distances under field conditions over a 3-year period in which permit conditions allowed plants to flower and set seed. These findings will aid in management practices for future cultivation of transgenic switchgrass, will be useful to both biosafety regulators and conservation biologists, and may also serve as a model for the incorporation of transgenic cultivars of other bioenergy feedstocks into the landscape.

2.3 Results

2.3.1 Transgenic lines and strategy

We used 'Alamo' switchgrass, which is a viable lowland cultivar grown in the southeastern U.S. While switchgrass is known to have various ploidy levels, lowland varieties are diploidized ancient allotetraploids. Our strategy for estimating gene flow in switchgrass was based on using a relatively large number of pollen-source to create a windborne 'pollen cloud' for pollinate pollen recipient plants at various distances and directions. Given that switchgrass is putatively self-incompatible, our goal was for the vast majority of pollen produced by source plants to harbor the transgene. For that reason, we chose transgenic switchgrass lines containing multiple T-DNA insertions to ensure that the vast majority of pollen grains were transgenic. In addition, we also chose lines for which the orange fluorescent protein (OFP) marker gene was clearly detectable in pollen and vegetative tissues (Burris et al., 2009). Multiple transgenic events were screened for T-DNA copy number and OFP fluorescence (Burris et al., 2009) through Southern blot analysis and epifluorescent microscopy (Figure 2-S1 and Figure 2-S2). Based on the above selection criteria, transgenic lines '1– 4' and '2–9' were chosen as pollen donors in the field experiment. Line 1–4' possessed two transgene copies and line '2–9' had six copies (Figure 2-S1), and exhibited OFP fluorescence when screened (Figure 2-S2). Moreover, we observed that practically every pollen grain was clearly OFP-positive, which ensured that the vast majority of pollen grains were transgenic. Assuming disomic inheritance and no association of different copies of the transgenes, 25% and 1.6% of non-transgenic pollen grains are expected in lines "1–4" and "2–9", respectively (based on two and six transgene copies). Considering about 1/3 and 2/3 of the former and the latter plants in pollen donor population, the overall frequency of nontransgenic pollen should be about 9.4%. Since transgenic plants are expected to fertilize

homozygous recessive (for the transgenic locus) pollen recipient plants, the same phenotypic frequencies are expected in hybrid seedlings. Of course, these are expectations on average, and it is possible that the apparent homogeneity of OFP pollen fluorescence observed in Figure 2-S2 is from sampling error, which is to say, we could have missed the non-transgenic pollen in the survey. Alternatively, the happenstance of transgene integration could have led to the observed pollen fluorescence distribution, which was apparently higher than the ~90% expected transgenic from the discussion above. We consider both scenarios in the Discussion section.

2.3.2 Plant growth and productivity

To fully assess the growth conditions, soil parameters such as water pH, buffer value and other micro nutrients were recorded, and the soil fertility was considered to be low-to-marginal (Table 2-S1). The pollen-source plot mean tiller number (44 per plant) and height (107.5 cm) were comparable to plants in pollen-recipient plots (Table 2-1). The pollen-recipient plots had a wide range in means for both tiller number (7–80 tillers per plant) and plant height (74.0–208.6 cm). Above-ground biomass was measured for the 2014 season only (Table 2-1), and the mean weight of pollen-source plants (2.6 g per plant) was low compared to the pollen-recipient plots means (1.0–70.7 g per plant).

2.3.3 F_1 progeny analysis

A total of 1,820 F_1 seeds were collected from pollen-recipient plants and after cold-treatment, 962 germinated (52.9%). The total number of F_1 seedlings analyzed for both the 2013 and 2014 seasons was 962, and a total of 204 F_1 seedlings were deemed to be OFP positive then analyzed via OFP-gene specific PCR with congruent positive results. There were many seeds that did not

germinate, which were not included in the final analysis as transgenic positive or negative. Nonetheless, most of these non-germinating seeds appeared to be small and pale with no detectable OFP-fluorescence in the seed; we deemed them to be non-viable. The number of F_1 seedlings analyzed per pollen-receptor plant in the east direction was 717; 66 from the west, 91 from the south, and 88 from the north. Figure 2-S3, a sample gel from the PCR analysis, and Table 2-S2, the summary Table 2-S2, show that a portion of the progeny from pollen-recipient plants are not transgenic.

The east direction represented the long radius of the pollen-receptor plots as well as the direction of the prevailing wind. When normalized for pollen-receptor tiller number (Table 2-1), data indicated the number of F_1 progeny collected from pollen-receptor plots to be inversely related to distance from the pollen-source plot (Figure 2-1; Poisson regression, $F_{1,15} = 12.98$, $P < 0.003$). From the east direction, the majority of transgenic progeny were collected between 10 and 50 m plots (60.6%) compared to the remaining 40–100 m plots (39.4%). In addition, a greater than seven-fold decrease in transgenic progeny percentage was observed from the 10 m (79.2%) to 100 m (9.3%) plots. The percentages of transgenic F_1 seedling observed in the west (10–30 m; 56.5–66.7%), south (10–30 m; 70.2–6.8%), and north (10–20 m; 32.0–34.2%) directions were similar to those observed in the east direction at similar distances (Table 2-S2).

2.3.4 Pollen dispersal

For all years, the Alamo 2 clonal type plants located in the pollen recipient plot began flowering in mid-July, while the ST1 clonal type plants in pollen-donor plots began booting the last week of July. Anthesis for both plant types overlapped starting in early August and continued until the

last week of September of each growth season. During the pollen sampling period, the average wind speed was 5.2 km/h, the average dew point was 15.7 °C, the average temperature was 26.3 °C and the average humidity was 52.7% (Table 2-S3) at the Oliver Springs study site (Figure 2-S4). OFP-tagged pollen counts collected from the eastward radius (direction of the prevailing wind) decreased as distance increased from the pollen-source plot (Figure 2-2; Poisson regression, $F_{1,8} = 288.38$, $P < 0.0001$). These results indicate that the source pollen is capable of traveling at least 100 m, albeit at low frequency (ca. 0.01% of all switchgrass pollen from the source plants). More importantly, OFP-tagged pollen counts also declined with increased distance to the west ($R^2 = 0.930$), north ($R^2 = 0.990$), and south ($R^2 = 0.993$) directions from the source plants (Figure 2-S5). When OFP-tagged pollen counts (within 30 m) were compared across all the directions, the means of OFP-tagged pollen observed were highest in the eastward direction (104.3) as compared to the west (32.5), south (32.2) and north (29.4). The mean OFP-tagged pollen counts from 10 to 30 m away (104.3) from the source plants in the eastward direction were five-fold higher than mean pollen counts from 40 to 100 m (19.4).

2.4 Discussion

The results presented here suggest that for switchgrass the majority of cross-pollination and transgenic pollen dispersal occur at short distances up to 50 m from the pollen source; however, a significant amount of pollen was detected at 50–100 m (Figure 2-1 and Figure 2-2). In addition, in the outermost pollen-receptor plot, 9.3% of the F_1 seedlings recovered were transgenic. Our study is the first to record empirical data on switchgrass pollen dispersal and pollination distances in the field.

Transgenic cross-pollination percentages presented here in switchgrass as a function of distance from a transgenic pollen source are consistent with findings from other experimental studies concerned with transgene flow from a transgenic pollen source (Ritala et al., 2002; Ma et al., 2004; Messeguer et al., 2004; Wang et al., 2004; Darmency et al., 2009; Kim et al., 2009a; Kim et al., 2009b; Van De Wiel et al., 2009; Rieben et al., 2011; Scorza et al., 2013; Zhao et al., 2013).

While the breeding systems and spatial scales involved in such studies vary widely, the primary conclusion is that the majority of transgenic cross-fertilization with nontransgenic counterparts occurs at short distances. In addition, studies addressing wind-pollinated grasses and having the largest spatial scales indicated transgenic cross-pollination percentages of 0.08% at 25 m from a transgenic source, and 0.006% at 250 m in maize (Van De Wiel et al., 2009), and levels declining to < 1% at 150 m in tall fescue (Wang et al., 2004). In our study, transgenic cross-pollination frequency declined with distance (beyond 50 m) from the transgenic source patch (Figure 2-1), with significant reduction beginning at a distance 50 m from the transgenic source, falling to 9.3% at 100 m.

Our strategy was to purposefully use two clones that could not intraclonally self-pollinate, our experimental design was set up to gather worst-case scenario data: maximizing potential gene flow. A single clonal type of switchgrass in the pollen source plot and a different single clone as pollen receptor plants theoretically allow a singular mating choice given that switchgrass is putatively and obligate outcrosser. This situation is vastly different than that of a typical commercial field inhabited by a synthetic population of heterogeneous genotypes that would be

expected to intermate with each other. Thus, our observed outcrossing rates by distance are likely many-fold higher than that expected in a commercial setting, even if all commercial plants are members of one cultivar. Nonetheless, declining transgenic pollen loads as a function of distance, and the possibility of selfing or apomixis, the former of which has been noted, rarely, in switchgrass (Martinez-Reyna and Vogel, 2002; Liu and Wu, 2012), may have led to nontransgenic seed production which could have influenced the frequency of observed transgenic progeny. Additionally, if the clone used for our receptor plants has any degree of self-compatibility, it is possible that, along with competitive interplays between the two pollen types and less-than-ideal overlap of floral phenologies, could have also influenced these results, which are similar to those exhibited by the wind-pollinated forage grass *Festuca pratensis* Huds. in a similarly designed, albeit nontransgenic, experiment (Rognli et al., 2000). In that study, gene flow between the source and receptor plants of *F. pratensis* was 50% at a distance up to 15 m and showed a rapid decrease to 10% at 75 m, and 5% at 155 m (Rognli et al., 2000).

Previously, we have not observed self-compatibility in our experimental clones in the greenhouse or field prior to these experiments. Nonetheless, we have also not isolated it from other genotypes the way they were isolated in these experiments. Thus, it is possible that selfing in these clones were practically below detection before. There have been prior reports of selfing in switchgrass (Liu and Wu, 2012; Casler et al., 2011) dispelling the view that it is a completely obligate outcrosser. Those findings suggest there can be an occasional breakdown in the self-incompatibility system, which is the most likely explanation of the existence of some nontransgenic progeny. We estimated that at least ca. 90.6% of the pollen cloud was transgenic, with pollen microscopy evidence suggesting it may be even higher. Therefore, the 204 transgenic

progeny (22%) from pollinations from pollen sink plants may be an underestimate of gene flow from the pollen-source population. Multiplying 204 by 0.094 (the expected non-transgenic pollen frequency from the transgenic plants) yields an estimated 19 additional progenies likely sired by transgenic plants. Therefore, of the 78% non-transgenic progeny from 'pollen-sink' plants, once we subtract the 19 progeny sired by transgenics, we are still left with 739 progenies (77%) coming from apparent self-pollination events. Thus, serendipitously, our results may have inadvertently led to increased evidence of selfing in switchgrass.

Our ability to empirically quantify pollen dispersal distances in switchgrass was arguably only feasible via the use of fluorescent protein tagged pollen source plants. In our case, the majority of pollen produced by our source plants expressed OFP, which distinguished switchgrass pollen from that of other heterospecifics at our field site and made fluorescent screening of pollen traps an efficient endeavor. Other methods utilized for the study of pollen movement have limitations, including destructive analysis or radioactive labels (Watrud et al., 2004; Rognli et al., 2000; Reinke and Bloom, 1979; Nilsson et al., 1992; Song et al., 2009). Many researchers have utilized GFP-tagged pollen in transgenic plants to study gene flow (Messeguer et al. 2004; Hudson et al., 2001; Moon et al., 2006). In the present study, OFP was selected as the marker of choice since its expression can be visualized without sample destruction as has been shown with a different OFP variant in tobacco species (Rice et al., 2013). The OFP-tagged pollen method is more rapid and convenient than real-time PCR and other such DNA-based screening methods in which DNA extraction is a prerequisite. The OFP tracking technique has great potential as a tool for gene flow and risk assessment studies as well as for post-release monitoring of transgenic plants (Stewart, 2005). The use of OFP and other fluorescent protein markers may also provide a viable

option in estimating pollen flow of specific pollen donors in systems with concurrent heterospecific pollen flow and where palynological expertise to identify pollen grains to species is lacking.

Switchgrass pollen is diminutive with a mean diameter range of 36–48 μm [36.02 μm (Bragg, 1969), 48.4 μm (Ge et al., 2011), and 43.7 μm (Ecker et al., 2013)]. Switchgrass pollen has short-lived viability after shedding, which also varies under environmental conditions. Greenhouse-grown 'Alamo' switchgrass produced pollen that was viable no longer than 20 min under sunny conditions and 150 min under cloudy conditions with a half-life of 4.3 min and 22.9 min; respectively (Ge et al., 2011). In another study, pollen from greenhouse grown switchgrass plants was exposed to ambient outdoor conditions and viability was completely lost after 60 min for most samples (half-life = 17.3 min) and 100 min for a single sample (Ecker et al., 2013). Short viability periods require pollen to be dispersed quickly for pollination to occur over long distances, yet, depending on wind speeds and other environmental factors, a 60–100 min viability period may allow for pollen dispersal over a large area. Indeed, when switchgrass pollen dispersion was modeled based on 100 min viability, pollen was capable of moving up to 3.5 km in light wind and 6.5 km in stronger wind conditions; the majority of pollen is expected to travel < 200 m (Ecker et al., 2013). In our study we observed a rapid decline in transgenic pollen deposition with increasing distance from the source plot; the majority of pollen deposition occurred within the first 50 m (Figure 2-2, Figure 2-S5). The distance in which the majority of pollen was deposited was nearer to the pollen source than predicted by Ecker et al. 2013; however, the pattern of decline was similar. Our results may differ from the published model

because of the secluded nature of our experimental site in which the forested border may have buffeted wind.

Nonetheless, these results are in agreement with pollen dispersal patterns observed in perennial ryegrass [*Lolium perenne*, (Giddings et al., 1997)] where the bulk of pollen deposition occurred within 10 m from the source. Also, meadow fescue [*Festuca pratensis* (Rognli et al., 2000)] pollen frequency declined rapidly within 75 m of the pollen source.

2.5 Conclusions

In a unique small field study in transgenic switchgrass, the first of its kind in which regulated switchgrass was allowed to flower in the field, we collected data on pollen movement and pollen-mediated transgenic hybridization. Over a 3-year period, the majority of pollen movement and cross-pollination occurred in close proximity to the pollen source, yet there was a significant amount of gene flow detected in our outermost sentinel plots (50–100 m) along with some apparent selfing. As U.S. regulatory agencies have set no allowable limits on the amount of adventitious presence for genetically engineered plants, the potentially acceptable limits of transgene flow are unknown in the U.S. for switchgrass and other crop species. If we used the E.U. upper limit of 0.09% [Regulation (EC) No 1830/2003; (Moon et al., 2011)], it would appear that in our worst-case scenario study, the gene flow observed here would have exceeded E.U. standards. Therefore, we could reasonably expect that at least a 100 m isolation distance would be required between transgenic edges and potential wild or cultivated nontransgenic plants that might receive transgenic pollen. For regulatory and risk assessment purposes, a spatially and genetically heterogeneous design could be useful to predict gene flow from commercial

engineered switchgrass. Given the reproduction biology of this perennial species coupled with potentially fitness-enhancing traits [e.g. drought or salt tolerance, rapid growth rate, and disease resistance (Ridley and Mallory-Smith, 2015)] imbued via genetic engineering, transgene flow might require mitigation by an engineered bioconfinement system [e.g. selective male-sterility (Millwood et al., 2016) or transgene excision (Moon et al., 2011)].

2.6 Methods

2.6.1 Transgenic line selection and analysis

Switchgrass ('Alamo' ST1 clones) plants were genetically engineered through *Agrobacterium*-mediated transformation, as described in Burris et al. (Burris et al., 2009), with a vector containing the *pporRFP* (OFP) gene under the control of the maize ubiquitin (*ZmUbi1*) promoter and the hygromycin phosphotransferase (*hph*) selectable marker gene under the control of the rice actin (*OsAct1*) promoter (Burris et al., 2009). Transgenic T_1 hemizygous plants were grown in a greenhouse (16/8 h day/night and 28/22 ° day/night). Transgenic lines were selectively chosen by evaluating T-DNA stable integration and transgene copy number using Southern blot analysis (Figure 2-S1) methodology as described in Moon et al. (2011), with a few changes. Briefly, genomic DNA was extracted of T_0 transgenic switchgrass events 1–4 and 2–9, along with other p*porRFP* transgenic switchgrass events. Ten micrograms of DNA from each sample was digested with *Bam*HI, which cuts once in the T-DNA, to yield estimates of T-DNA insert number for each line. The blot was probed with ^{32}P-labeled pporRFP gene fragment. To select the best OFP fluorescent events, visual analysis of OFP fluorescent levels was performed by epifluorescent microscopy for roots and sheaths as described by Burris et al. (2009) and pollen (Figure 2-S2) epifluorescence microscopy (Rice et al., 2005; Moon et al., 2011).

2.6.2 Field experiment

To examine transgenic pollen movement and pollination between transgenic and nontransgenic switchgrass plants, a 3-year field experiment was performed under USDA APHIS BRS release permits (11-007-106r and 13046-104r). The field site in Oliver Springs, Tennessee U.S.A. was chosen because of its heavily forested borders that provided a natural barrier from wild or otherwise cultivated switchgrass plants. All experimental plants were transplanted by hand on September 20, 2011, and the total planted area was approximately 0.7 ha. A modified Nelder wheel design (Nelder, 1962) consisted of a transgenic pollen-source plot and several pollen-recipient plots placed at 10 m intervals in the four cardinal directions from the pollen-source (Figure 2-S4). The pollen source and recipient plants had similar growth patterns and flowering times, which assured the highest possibility for transgene flow. The pollen-source plot contained 10 rows planted on 0.3 m centers, and each row contained 10 plants of the same type. Four rows were planted with clonally propagated plants from transgenic line '1–4' (40 plants total) and the remaining six were planted clones from line '2–9' (60 plants total). Post-transplant plant mortality led to the establishment of twenty-six '1–4' plants and fifty '2–9' plants, which remained throughout all experiments. Alamo 2 clones (cultivar 'Alamo') were chosen as the pollen-recipients to ensure cross-pollination would not occur between pollen-receptor plots. The long axis of the experiment was set up parallel with prevailing wind direction (eastward). Two receptor plants, 0.6 m apart from each other, were transplanted at distances in 10 m intervals. The northward plots were at 10 m and 20 m, southward and westward at 10 m to 30 m, and eastward from the source plants at10 m to 100 m (Figure 2-S4).Pollen-receptor plots suffered mortality as well. All plots had at least one plant and plots containing two receptor plants were: west 10 m, south 10 m, east 50 m, east 60 m, east 80 m, and east 90 m.

It is important to note that pollen-donor and pollen-recipient plants are both distinct clonal types originally derived from the cultivar 'Alamo.' These clonal types are sexually compatible with each other. However, clones of the same type are putatively not self-compatible. Specifically, pollen-donor plants are putatively not sexually compatible with other pollen-donor plants, and pollen-receptor plants are putatively not sexually compatible with pollen-receptor plants.

2.6.3 Plant growth and productivity

At the end of each growing season plant height, biomass production, and tiller number were recorded for both pollen-donor and –recipient plants (Table 2-1). Plant heights were represented by the height of the tallest tiller on each plant. Measurements were taken after panicles were removed for seed collection at the end of the growing season. Although seeds were not collected from pollen-source plants, for consistency panicles were removed prior to height measurement. Biomass measurements were taken as described in Baxter et al. (2014) with a few modifications. For each plant, the senesced above-ground biomass was harvested 12.7 cm above the soil, oven-dried at 43 °C for approximately 96 h, and the dry biomass data were taken for each plant. After harvest, tiller numbers were counted for each plant in the field. To ensure plants were grown within typical fertility ranges, soil samples were collected from the source area as well several pollen-receptor plots (east at a distance of 25, 50, 70 and 100 m, and west, north and south at a distance of 15 m). A detailed soil analysis was carried out at the University of Tennessee Soil, Plant and Pest Center in Nashville, Tennessee. Soil properties such as water pH, buffer value, the amount of salts and trace elements were also analyzed (Table 2-S1).

2.6.4 Pollen collection and screening

OFP pollen distribution measurements were taken by placing pollen traps adjacent to each pollen-receptor plot twice in September 2012 and three times in September 2013 for a total of five individual collections. Pollen traps were constructed by covering glass microscope slides (25 mm × 76 mm × 1 mm) with double-sided sticky adhesive tape (3 M136, 3 M Scotch Brand, St. Paul, Minn.) (Fonseca et al., 2002). Pollen traps were mounted horizontally onto vertical wooden stakes with a collection height of 1 m above the soil surface. For each sampling, the slides were exposed for six-hour period to collect pollen. OFP pollen frequency was tallied using an epifluorescence microscope (Olympus Reflected Fluorescence system BX51, Olympus Corporation, Shinjuku, Tokyo, Japan) with a TxRed filter set (Texas Red®/Cy3.5, EX-D560/40, BS-595DCLP, EM-D630/ 60, Olympus Corporation, Shinjuku, Tokyo, Japan). OFP-tagged pollen was imaged using a color digital camera (Olympus QColor 5) with Qcapture imaging software (Q Imaging Corp., Burnaby, Canada). We used Poisson regression analysis (generalized linear mixed model using SAS 9.4; SAS Institute Inc., Cary, N.C.) to examine the relationship of OFP pollen counts and distance from the pollen-source plot. At the time of pollen collection, meteorological data such as temperature, wind speed, humidity, and dew point were recorded using an Ambient weather station located at the Cumberland Forest Unit Headquarters in Oliver Springs, Tenn. (Table 2-S3).

2.6.5 Seed collection, germination and screening

At the end of the 2013 and 2014 growing seasons panicles were collected by hand from pollen-receptor plots and transported back to the laboratory; where seeds were cleaned, segregated by plot and stored at 4 °C for cold stratification. To estimate hybridization frequencies seeds were

germinated in an environmental-controlled growth room (16/8 h day/night and 24/22 ° day/night). Seeds were placed in a Petri dishes lined with Whatman® 2 filter paper (Whatman International Ltd., Maidstone, England) and water was added as needed. Once germinated, the resultant seedlings were screened for the presence of OFP through epifluorescent microscopy as described by Burris et al. (Burris et al., 2009). One-to-two weeks post-germination seedlings were transferred to potting mix (Fafard® Peat-Lite Mix, Fafard, Inc., Anderson, S.C.) in an environmental controlled growth room (16/8 h day/night and 24/22 ° day/night). To further verify transgenic progeny seedlings were PCR-screened using primers (forward primer: TTTCAAAGCAAAGTGGGGTC; reverse primer: CACCATCTTCAAAGGTCATG) designed to amplify a 302 bp fragment of the pporRFP gene. Using a standard protocol genomic DNA was extracted from leaves (Stewart and Via, 1993) and PCR was performed using conditions described by Mann et al. (Mann et al., 2012). Poisson regression analysis was performed as previously described to evaluate the relationship between distance and transgenic progeny per pollen-recipient plant while taking tiller number per plant into account. For pollen-recipient plots containing two plants, the average number of transgenic progeny was calculated on a per plant basis. The tallies of F_1 germinated seedlings, transgenic F_1 progeny, and tiller tallies were combined for both field seasons.

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Appendix

Tables and figures

Table 2-1. Growth characteristics of pollen-source and pollen-receptor switchgrass plants grown under field conditions.

Field plot	Tiller number	Height (cm)	Above-ground biomass (g)
Pollen source	44 ± 13.0	107.5 ± 11.0	26
North 10	29 ± 13.0	128 ± 389	10
North 20	27 ± 10.5	142.9 ± 22.5	78
South 10	18 ± 8.4	170.5 ± 7.5	52
South 20	10 ± 2.5	103.2 ± 14.4	24
South 30	24 ± 5.0	132.7 ± 21.3	96
West 10	19 ± 10.9	182.2 ± 4.1	81
West 20	7 ± 0.5	90.8 ± 13.0	14
West 30	21 ± 6.5	95.9 ± 15.6	48
East 10	24 ± 1.5	134.6 ± 24.1	24
East 20	19 ± 0.5	90.2 ± 23.5	10
East 30	15 ± 0.5	96.2 ± 21.7	18
East 40	24 ± 8.0	74.0 ± 10.0	16
East 50	23 ± 7.5	152.4 ± 7.6	130
East 60	32 ± 9.4	167.3 ± 11.6	87
East 70	48 ± 0.5	121.9 ± 26.7	94
East 80	70 ± 44.3	208.6 ± 1.1	707
East 90	67 ± 40.3	189.9 ± 7.3	404
East 100	80 ± 33.5	146.1 ± 32.4	464

Numbers for tillers, height, and above ground biomass are average per plant (means ± SE). Tiller number and height were recorded for both the 2013 and 2014 seasons. Measures for above-ground biomass were from the 2014 field season.

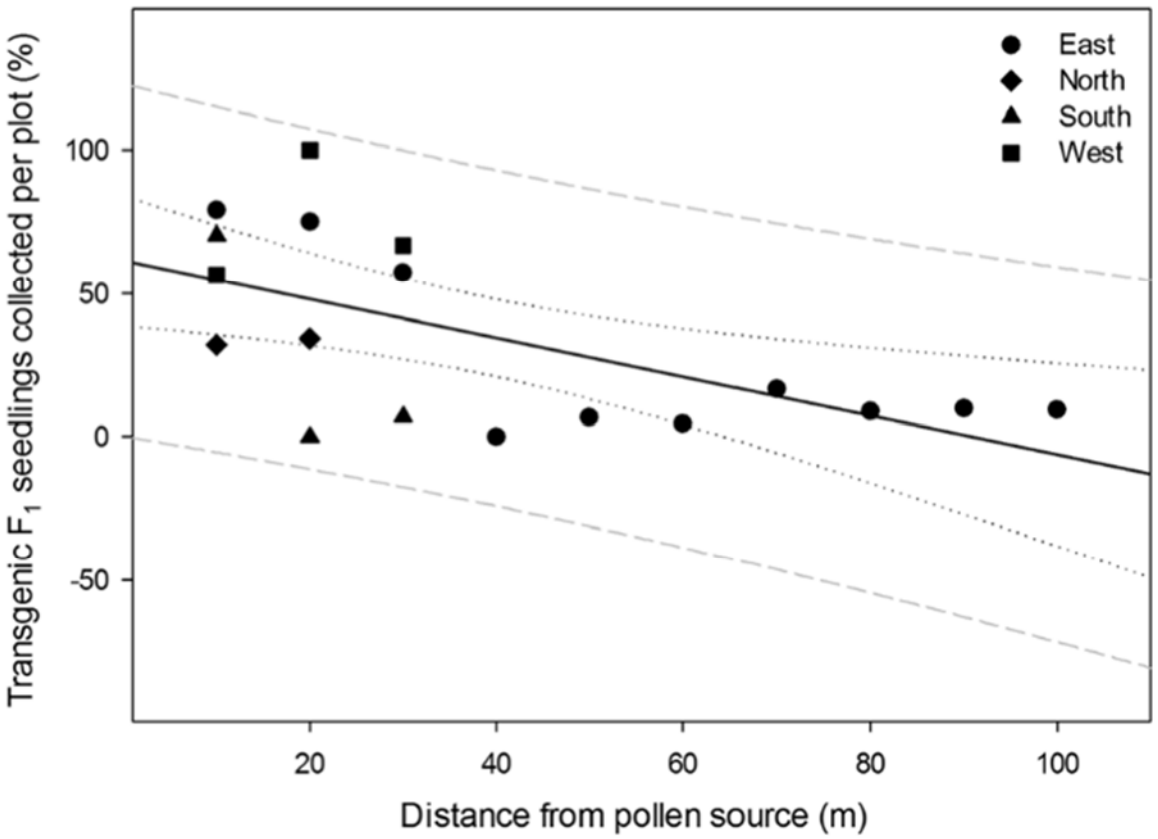


Figure 2-1. Relationship between transgenic F_1 switchgrass seedlings collected from individual pollen-recipient plots and distance from the transgenic switchgrass pollen-source plot (Poisson regression, $F_{1,15} = 12.98$, $P < 0.003$). Pollen-recipient plots were located at 10 m intervals from the pollen-source and planted in the four cardinal directions. Seed were collected from the field site at the end of the 2013 and 2014 growing seasons.

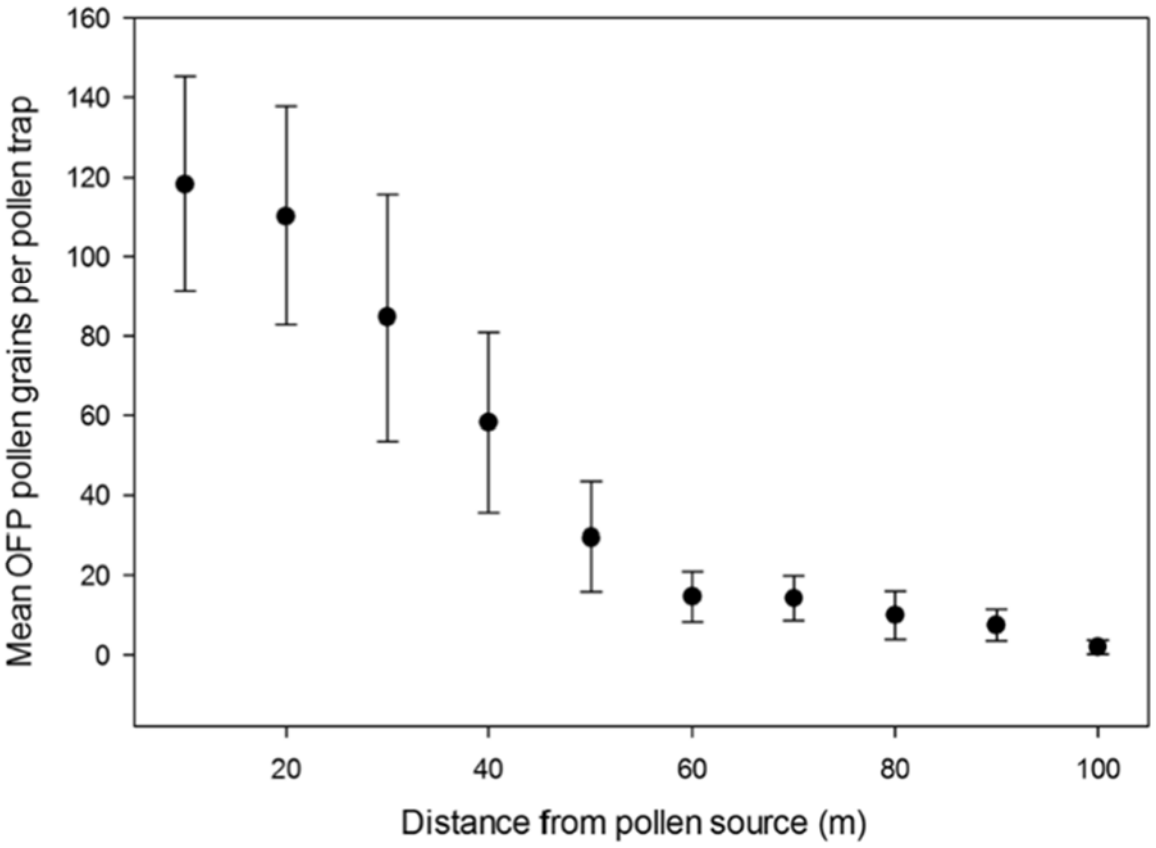


Figure 2-2. Average number of orange fluorescent protein (OFP)-tagged switchgrass pollen grains detected as a function of distance (Poisson regression, $F_{1,8} = 288.38$, $P < 0.0001$) under field conditions. Pollen grains were sampled from the eastward direction twice in 2012 and three times during the 2013 growing season (bars represent ± standard error of mean).

Supplementary tables and figures

Table 2-S1. Soil analysis from field site collected from the pollen-source plot and seven pollen-recipient plots.

Soil parameters	Transgenic source plot	North (15 m)	West (15 m)	South (15 m)	East (25 m)	East (50 m)	East (75 m)	East (100 m)
Water pH	5.3	5.2	4.8	4.9	5.1	5.1	5.2	5.1
Buffer value	7.4	7.4	7.1	7.3	7.2	7.2	7.3	7.3
Phosphorus*	5	7	6	5	6	5	10	11
Potassium*	16	53	41	22	29	38	43	70
Calcium*	282	482	144	104	286	410	379	532
Magnesium*	40	84	28	17	61	70	67	70
Zinc*	1.3	4.5	1.3	0.9	2.2	3.9	2.4	4
Copper*	0.6	1.4	0.8	0.4	0.5	0.4	0.5	1.3
Iron*	28	62	38	28	22	22	36	65
Manganese*	7	16	7	8	9 S	8	11	12
Boron*	0.2	0.3	0.2	0.2	0.3	0.3	0.3	0.3
Sodium*	15	16	13	10	14	13	14	15

*Nutrient values are in ppm.

Table 2-S2. PCR progeny screen of F_1 seedlings collected from pollen-recipient plots in the field.

Field plot	**Total germinated F_1 seedlings**	**Total *pporRFP* PCR positive F_1 seedlings**	**Transgenic F_1 seedlings (%)**
North 10	50	16	32.0
North 20	38	13	34.2
South 10	24	17	70.2
South 20	0	0	0.0
South 30	44	3	6.8
West 10	31	18	56.5
West 20	1	1	100.0
West 30	3	2	66.7
East 10	48	38	79.2
East 20	4	3	75.0
East 30	7	4	57.1
East 40	1	0	0.0
East 50	52	4	6.7
East 60	46	2	4.4
East 70	24	4	16.7
East 80	68	6	8.8
East 90	97	10	9.8
East 100	108	10	9.3

Table 2-S3. Mean meteorological data collected for the growing seasons of 2012 and 2013 at Oliver Springs, Tennessee.

Weather	**Mean**
Dew point (Celsius)	15.7
Wind speed (km/h)	5.2
Daily low temperature (Celsius)	18.3
Daily high temperature (Celsius)	25
Precipitation (cm)	7.9
Humidity - morning (percentage)	90
Humidity - afternoon (percentage)	57
Humidity recorded (percentage)	52.7
Sunshine (percentage)	62
Cloudy days - clear of clouds (percentage)	35
Cloud days - partly cloudy (percentage)	25
Cloudy days - cloudy days (percentage)	40
Cloudy days - days with precipitation (percentage)	30

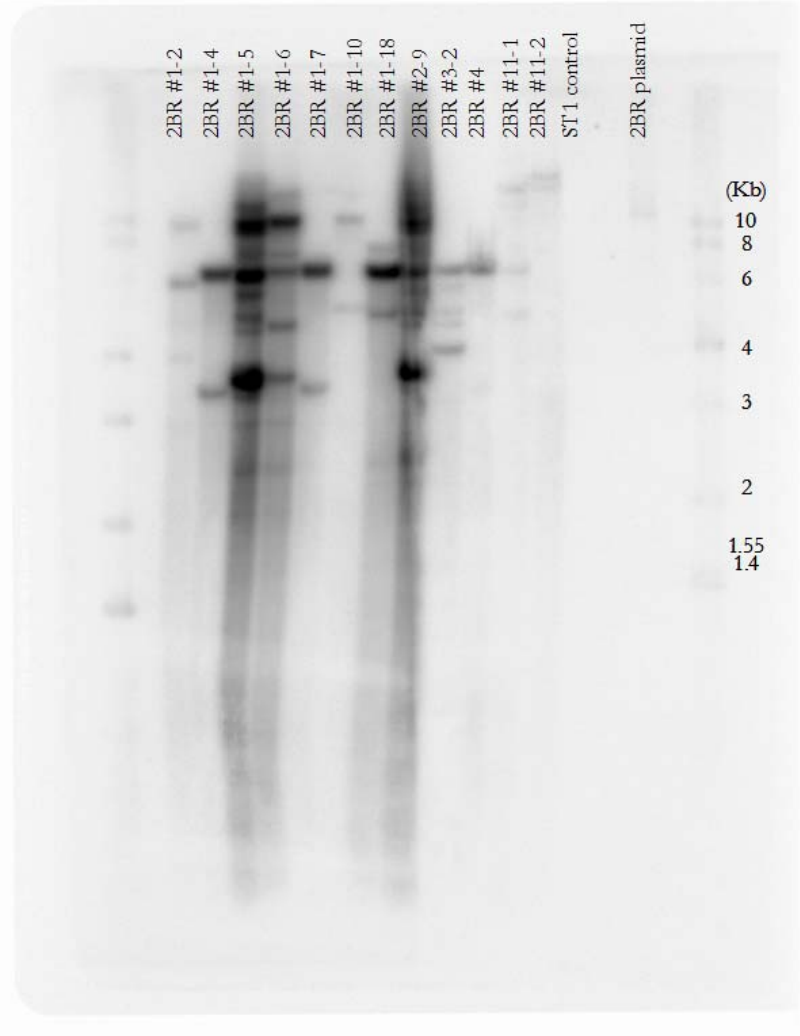


Figure 2-S1. Southern blot analysis of T_0 transgenic switchgrass events containing stable integration of the T-DNA. Blot was probed with ^{32}P-labeled pporRFP gene fragment. Lanes labeled with '2BR' contain transgenic line genomic DNA samples, lane 'ST1 control" is the nontransgenic control genomic DNA sample, lane '2BR plasmid' contains the plasmid positive control. Genomic DNA was digested *Bam*HI genomic DNA (10 μg) from each plant sample.

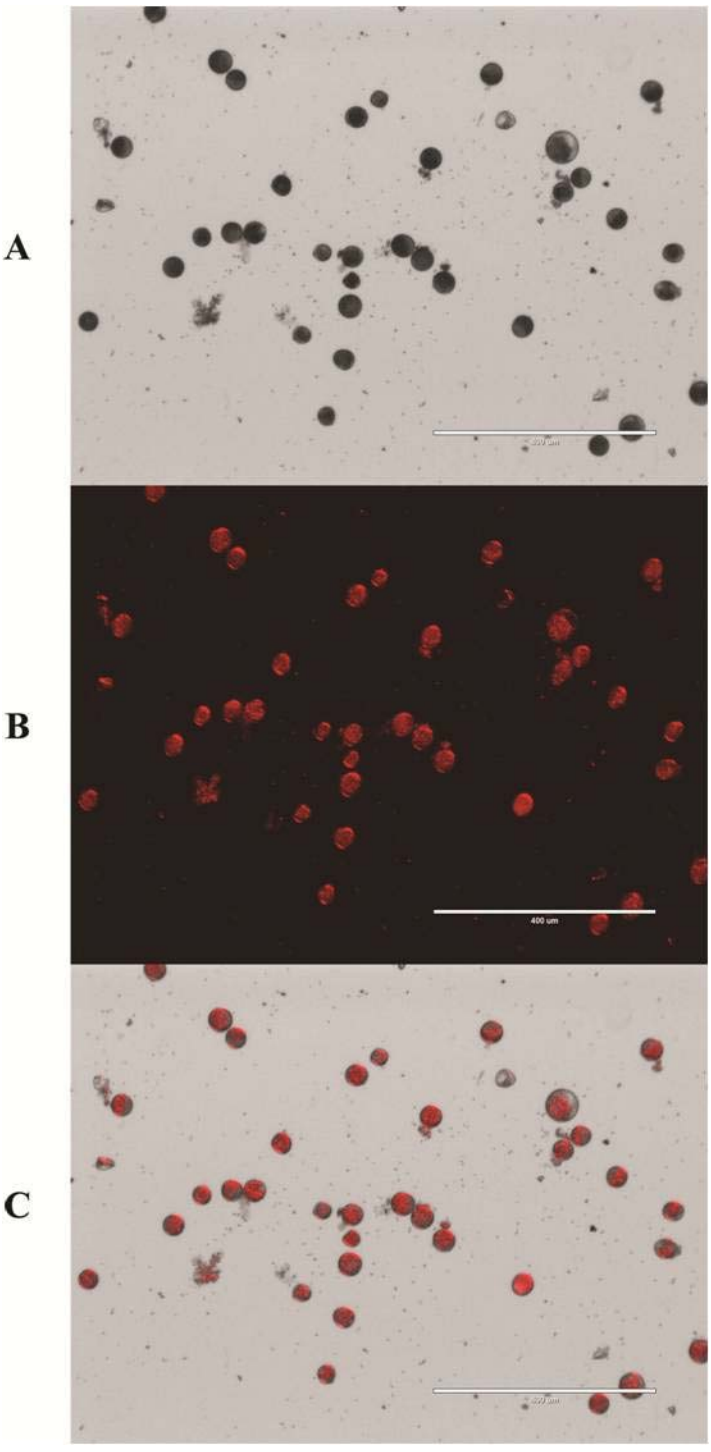


Figure 2-S2. Sample of orange fluorescent protein (OFP)-tagged switchgrass pollen from source plants. A), white light, B), TxRed filter, C), merged image. The scale bar is 400 µm.

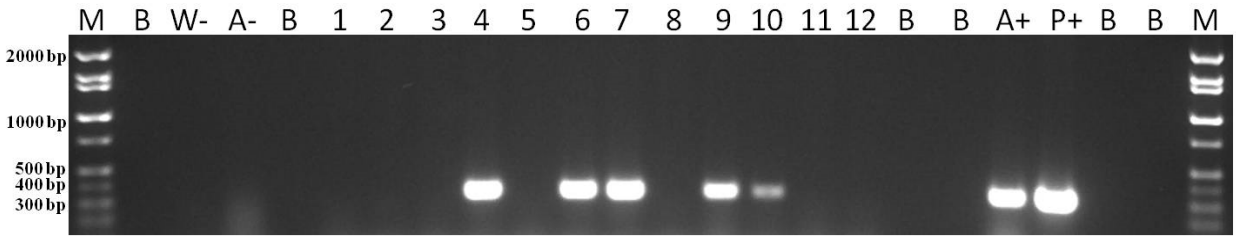


Figure 2-S3. A sample gel from PCR analysis for the *pporRFP* (OFP) gene that was performed on F_1 switchgrass progeny (lanes 1–12) collected from field study pollen-recipient plots. A water only sample (W) and a nontransgenic pollen-recipient parental type (A-; Alamo II) were used a negative control. A transgenic parental type sample (A+; line 2–9) and a plasmid with the *pporRFP* gene (P+) were used as positive control. A DNA size marker (M) was used to confirm expected band size (302 bp), and blank lanes (B) were used to separate sample types.

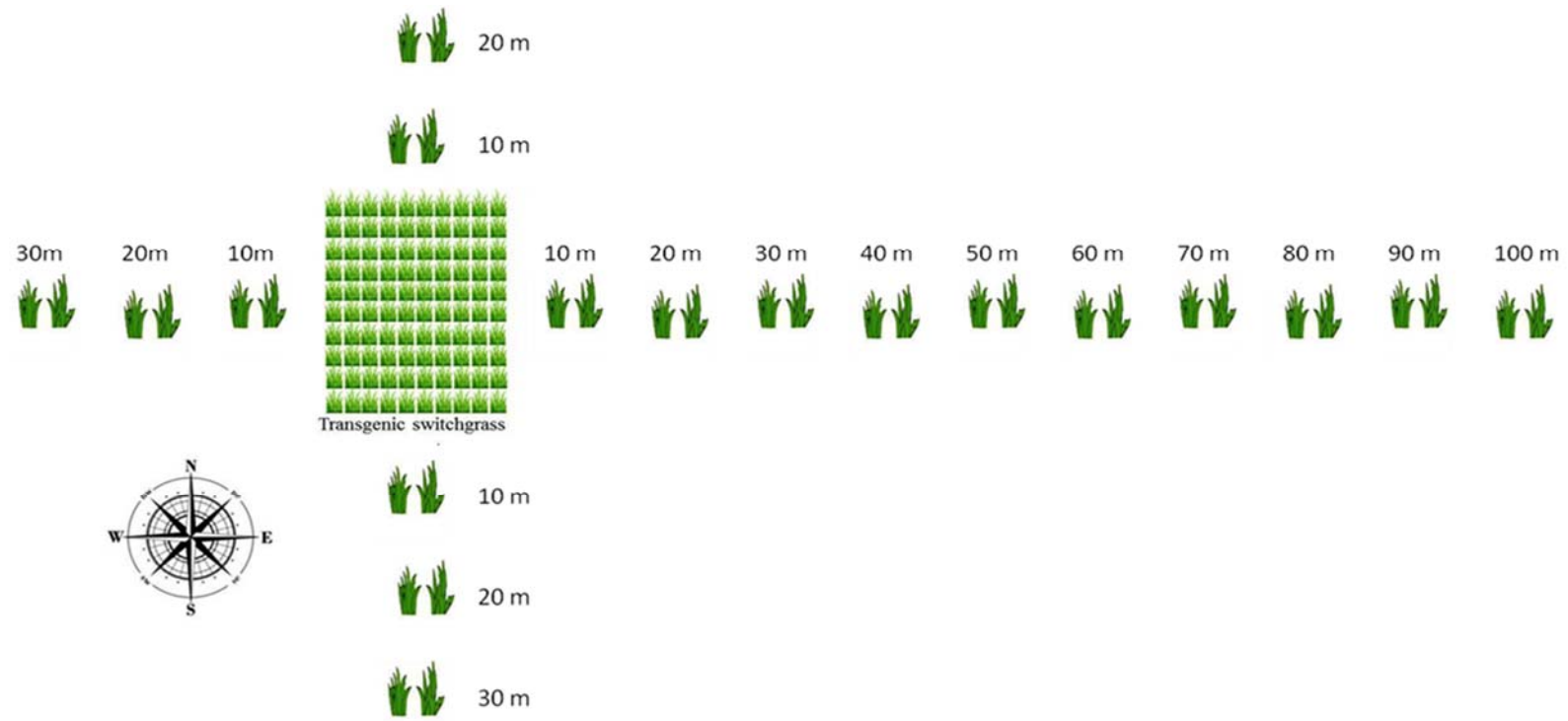


Figure 2-S4. Experimental layout of field study located in Oliver Springs, Tennessee, U.S.A. Transgenic switchgrass (cultivar 'Alamo', clone ST1) served as a pollen-source (10 rows × 10 plants) while non-transgenic switchgrass (cultivar 'Alamo', clone Alamo 2) plants were placed in pollen-recipient plots planted at 10 m intervals.

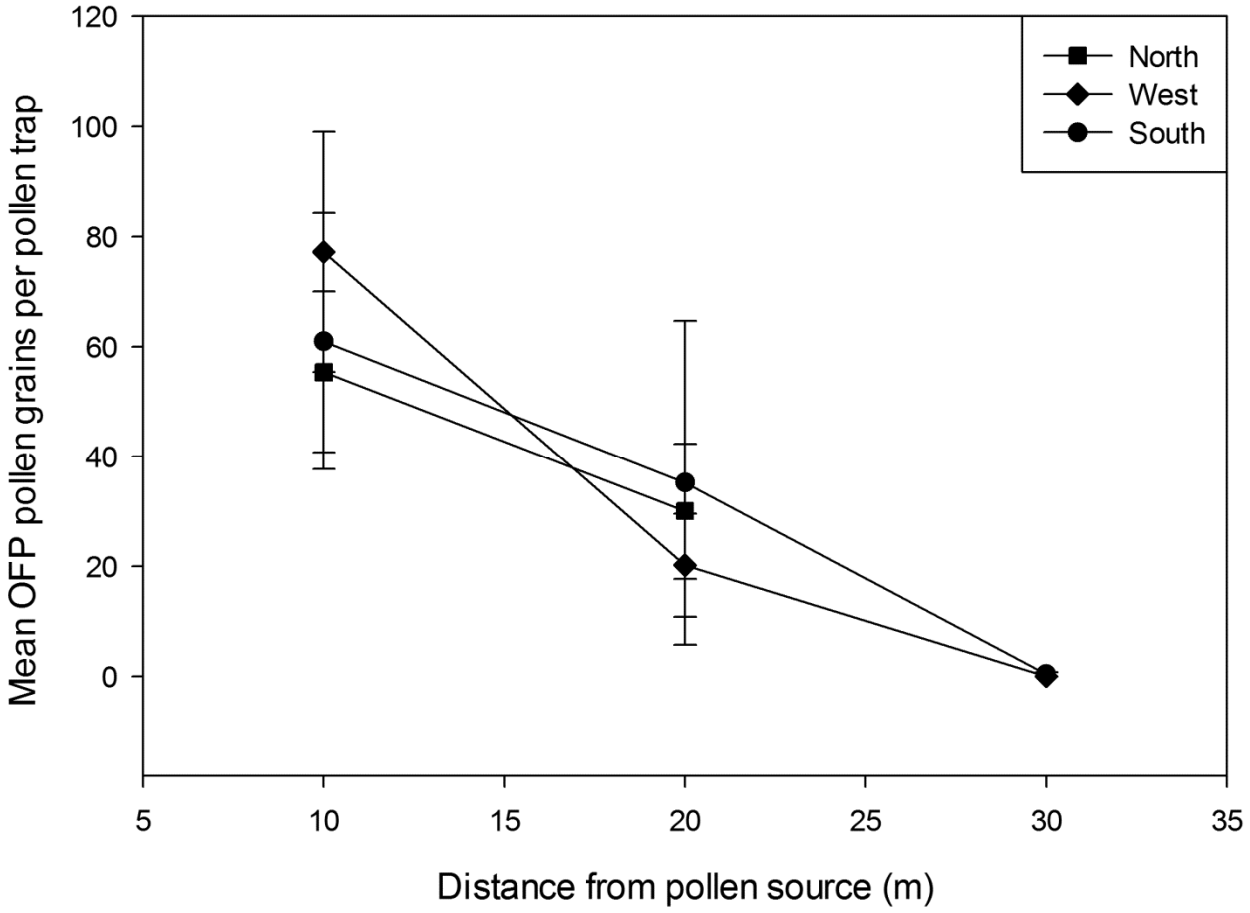


Figure 2-S5. Average number of orange fluorescent protein (OFP)-tagged switchgrass pollen grains detected as a function of distance in the north, south, and east directions (East, $R^2 = 0.953$; North, $R^2 = 0.990$; West, $R^2 = 0.930$; and South $R^2 = 0.993$). Pollen samples were collected in the field during the 2012 and 2013 growing seasons.

Chapter 3: Gene use restriction technologies for transgenic plant bioconfinement

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3.1 Abstract

The advances of modern plant technologies, especially genetically modified crops, are considered to be a substantial benefit to agriculture and society. However, so-called transgene escape remains and is of environmental and regulatory concern. Genetic use restriction technologies (GURTs) provide a possible solution to prevent transgene dispersal. Although GURTs were originally developed as a way for intellectual property protection (IPP), we believe their maximum benefit could be in the prevention of gene flow, that is, bioconfinement. This review describes the underlying signal transduction and components necessary to implement any GURT system. Furthermore, we review the similarities and differences between IPP- and bioconfinement oriented GURTs, discuss the GURTs' design for impeding transgene escape and summarize recent advances. Lastly, we go beyond the state of the science to speculate on regulatory and ecological effects of implementing GURTs for bioconfinement.

3.2 Introduction

Transgenic crops have become an integral part of modern agriculture and have been increasingly adopted worldwide (James, 2011). While the number of transgenic plants increases, so does the

possibility of adventitious presence of transgenes in the environment. Pollen- and seed-mediated gene flow from transgenic plants to nontransgenics has occurred at a low level for a few species, but with little measurable effect (Warwick et al., 2009). It is natural for crops to exchange genes with their wild and weedy relatives, but regulators have focused, in particular, on gene flow–associated risks for transgenic crops. However, just because hybridization and introgression could occur, it does not follow that transgenes necessarily give a selective advantage to their weedy relatives (Gressel and Al-Ahmad, 2012). Indeed, the introgression of endogenous crop genes to wild relatives is relatively rare for most crops in most places (Ellstrand et al., 2013). Even though ecological risk is generally associated with a trait, environment and mating system of the crop and wild relative, there is an apparent benefit in preventing gene flow altogether (Stewart et al., 2003). This would seem to be especially the case with transgenic perennial crops (Kausch et al., 2010; Kwit and Stewart, 2012; Moon et al., 2010a), and when transgenes and their conferred traits, such as drought, salt or cold tolerance, are not completely understood and fitness enhancing (Warwick et al., 2009). Crops producing pharmaceutical products may require bioconfinement by regulators for commercialization (Moon et al., 2010b; Stewart, 2008). Thus, technologies to reduce or eliminate gene flow would be beneficial from an ecological risk and regulatory standpoint.

The biological confinement (bioconfinement) of transgenes has been a topic of vigorous debate since the inception of plant biotechnology. Currently, no bioconfined transgenic plants have been commercialized wherein pollen- and seed-mediated gene flow is completely controlled. Many technologies have been proposed (Daniell, 2002; Gressel, 1999; Moon et al., 2011), including strategies for male sterility (Mariani et al., 1990), maternal inheritance (Daniell et al., 1998;

Iamtham and Day, 2000; Ruf et al., 2001), transgenic mitigation (Al-Ahmad et al., 2004, 2006), transgene excision (Luo et al., 2007; Moon et al., 2010b, 2011) and seed-based methods (Gressel, 2010a; Oliver et al., 1998, 1999a,b). Additionally, although the naturally existing bioconfinement systems, such as apomixis, ploidy barriers and genomic incompatibility, are only applicable in a limited number of species, which are less manageable for genetic engineering (Daniell, 2002; Gressel, 1999; Kausch et al., 2010), they have attracted intensive research attentions with exciting advances (Hanna, 1981; Johnson et al., 2006; Kannan et al., 2012; Sandhu et al., 2009, 2010). Each of these strategies has advantages and drawbacks. For example, one of the first technologies proposed and shown to be effective was a seed-based method (above) originally called the 'Technology Protection System' that later became known as 'GeneSafe Technologies' (Oliver et al., 1998, 1999a,b), which infamously became widely known as 'Terminator'. The GeneSafe invention rendered seeds that were not capable of germination (Oliver and Hake, 2012), thus eliminating the possibility of transgene flow. The critics of GeneSafe, who widely embraced the 'Terminator' label, argued that farmers in developing countries would be at a disadvantage because it would have prevented them from saving viable seed for planting the following season. The GeneSafe critics also argued that fields growing nontransgenic crops of the same species nearby would suffer viable seed loss from cross-pollination with GeneSafe plants. Because of public pressure and other reasons, this technology has never been commercially deployed even though GeneSafe is a good candidate for preventing gene flow.

Collectively, these bioconfinement technologies have become known as gene use restriction technologies (GURTs); the name has been adopted in a report to the Commission on Genetic

Resources for Food and Agriculture of the Food and Agriculture Organization of the United Nations in 2001 (Oliver and Hake, 2012). We believe that 'GURTs' might not be the best label because the implication of purpose is mainly to restrict the use of intellectual property or traits owned by agribusinesses (Oliver and Hake, 2012). Of course, GURTs can be used to protect intellectual property, but they can also be invaluable tools in the bioconfinement of transgenes, which we believe might eventually be a more valuable purpose.

In this review, we seek to survey the various GURTs that have been developed and examine the state of the technology for use in bioconfinement. Because of the proprietary nature of GURTs, there might be some technologies that have been invented in companies, but not found in the literature; there might be technology gaps in the review. Nonetheless, we discuss GURT components, the benefits of GURTs as the technology relates to intellectual property protection and bioconfinement, and the future outlook of GURTs from regulatory and commercialization perspectives.

3.3 Gene use restriction technologies defined

Gene use restriction technologies have been categorized into two classes: V-GURTs (variety-related GURTs) and T-GURTs (trait-related GURTs) (Hills et al., 2007; Van Acker et al., 2007; Visser et al., 2001). V-GURTs are designed to restrict the use of all genetic materials contained in an entire plant variety. Prior to being sold to growers, the seeds of V-GURTs are activated by the seed company. The seeds can germinate, and the plants grow and reproduce normally, but their offspring will be sterile (see below for details). Thus, farmers could not save seed from year-to-year to replant. In contrast, T-GURTs only restrict the use of particular traits conferred

by a transgene, but seeds are fertile. Growers could replant seed from the previous harvest, but they would not contain the transgenic trait.

3.4 Components of GURTs

For all GURTs, there are three indispensable necessary characteristics: controlled functionality, spatiotemporal specificity and controlled transgene expression. To achieve these, a signal cascade is required (Figure 3-1) that is generally composed of the following four genetic components or modules: the target gene, the promoter associated with the target gene, the trait switch and the genetic switch.

3.4.1 The target gene

For all GURTs, the target gene renders the host plant to have a specific trait such as lethality or an agronomic trait (‘gene 3’ in Figure 3-1). In theory, there is any number of genes that could be used as the target, even those whose gene product is simply RNA. The nature of the target gene determines a GURT’s functionality. If a gene’s product interferes with fundamental biological process (es) and therefore causes death of the host cell, it serves as a disrupter gene as discussed later. Because the entire genome of the host cell will be eliminated because of cell disruption, the GURTs utilizing these disrupter genes are classified as V-GURTs. In contrast, if the expression of the target gene results in particular traits (such as pest resistance or removal of specific genomic fragment as detailed below), but not lethality, it would be a T-GURT application.

However, because the expression of the target gene has to be tightly controlled in order to meet the requirement of original IPP designs or derived bioconfinement applications, other gene regulation elements (see below) are necessary.

3.4.2 Promoter of the target gene

A promoter ('promoter 3' in Figure 3-1) regulates the expression of the target gene in a spatiotemporal pattern, that is, where and when it should be conditionally expressed. To prevent unwanted pleiotropic effects, that is, damage to the plant from possible leakage of promoter activity especially in the case of V-GURTs, a 'blocking sequence' is often used to physically separate the promoter and its target gene. Thus, the interaction between the target gene and its promoter can be set by default 'on' (without blocking sequence) or 'off' (with blocking sequence) (Figure 3-1e). In order to deactivate or activate the desired trait, additional genetic elements, including the trait switch gene and the genetic switch components, are required.

3.4.3 Trait switch gene

This group of genetic elements has been previously termed 'trait activator gene' because in most cases it is used to activate the expression of the target gene. The trait switch gene ('gene 2' in Figure 3-1) often encodes for a recombination enzyme, such as a site-specific recombinase, that physically deletes DNA sequences between specific recognition sites (Wang et al., 2011). In the cases that the target gene is set by default 'off' (i.e. there is a blocking sequence between target gene and its promoter), these activator genes' products do act as activators by removing the blocking sequence (Figure 3-1e,f). However, there are two proposed designs in which the target gene is instead deactivated by the switch genes (Figure 3-1e,f). One is when the recombinase

deletes part or all of the target gene or its promoter (or both) to deactivate the target gene (Figure 3-1e,f). The other common design uses repressor molecules (either RNA or protein) to block the target gene's transcription/translation and therefore also function as a deactivator (Figure 3-1e,f). The consequence of the trait switch gene's activation would be either deletion of certain genomic fragment (upper two levels of Figure 3-1e,f) or repression of the target gene (bottom level of Figure 3-1e,f), representing permanent switch (also called physical switch) or reversible regulation, respectively. Because of the obvious advantage of physical switch over reversible regulation in terms of absolutely tight control, the former is commonly preferable in all GURT designs and recombinases as the trait switch genes.

The expression of the trait switch gene is driven by a directly associated promoter ('promoter 2' in Figure 3-1), which reacts to signals from the genetic switch (see below). Regardless of its role as 'activator' or 'deactivator', the trait switch gene stands between the physical switch and the genetic switch, that is, receives input from genetic switch followed by releasing output to physical switch (Figure 3-1).

3.4.4 Genetic switch

The genetic switch component is made up of an 'enabler' gene ('gene 1' in Figure 3-1) and its promoter ('promoter 1' in Figure 3-1). Through this component, the application of the exogenous inducer could either activate or repress the expression of the enabler gene that directly controls the activity of the trait switch gene component and therefore downstream signal transduction (Figure 3-1). Thus, as a gateway that converts exogenous input (inducer) to biological signal (the trait switch gene or more directly the target gene itself; see below), the genetic switch should

further include the promoter of the trait switch gene ('promoter 2' in Figure 3-1) as a subcomponent, which would be apparently indispensable for all GURT designs. However, to simplify discussion and diagrams (see below and Figures 3-1 and 3-2), the promoter of the trait switch gene will still be put into the trait switch component, and the genetic switch component will be discussed as dispensable throughout the manuscript.

3.4.5 Functionality of GURTs: putting all the components together

The signal cascade shown in Figure 3-1 represents the original V-GURT GeneSafe design (Oliver et al., 1998, 1999a,b) (Figure 3-2). The application of the inducer serves as input and the switch of the target gene's expression status as output. The three components, the genetic switch, the trait switch gene and the promoter of the target gene, transduce the signals from input to output (Figure 3-2a). By rearranging the genetic elements of them, the GURT system could be modified to fit other purposes. For example, the inducer-responsive promoter ('promoter 1' in Figure 3-1 and 'inducible promoter' in Figure 3-2), which originally belongs to the genetic switch (Oliver et al., 1998, 1999a,b), could replace the promoter of the trait switch gene (Figure 3-2b). Thus, an inducer treatment would more directly regulate its expression compared to the original design (Figure 3-2a,b). However, based on logical reasoning rather than empirical evidence, the cost could probably be weaker induction of the trait switch gene because of the absence of signal amplification. Therefore, this design would be considered to fit the cases that require rapid but weak responses. In these two designs (Figure 3-2a,b), the dual switch provides controllability of the GURT system by breeders/companies via the inducible promoter, as well as spatiotemporal specificity of the target gene's expression via the natural signal responsive promoter.

The design can be further simplified. As shown in a single switch design (Figure 3-2c), the inducible or natural signal-responsive promoter exclusively controls the target gene's expression so that the system can solely respond to exogenous inducers or developmental signals instead of both. This design has been widely used for bioproduction in plants, and recent advances have been reviewed by Corrado and Karali (2009).

Instead of placing all the components in one plant, the controllability could be realized by a hybridization event, that is, in trans (Oliver et al., 1998, 1999a,b). In this case, the target gene and its promoter, as well as the blocking sequence in most cases, are constructed in one plant ('maintainer' line) and the trait switch gene is constitutively expressed in another ('inducer' line or 'activator' line) so that the GURT system would be triggered for the first time in F_1 hybrid plants (Figure 3-2d). This design also contains a dual switch.

Based on these four fundamental designs and the fact that every single component in a GURT design is exchangeable and the outcome of different combinations of these components varies considerably, GURTs could be further modified to fit particular purposes and provide value beyond simply protecting intellectual property.

3.5 Benefits of GURTs

3.5.1 Intellectual property protection

The original aim of GURTs was for protection from patent infringement from saving seed (Oliver et al., 1998, 1999a,b). A common provision in seed sales agreements to farmers disallows saving seed. Even though many of today's crop varieties are hybrids for which saving

and replanting seed would not be valuable, there was an initial very intense public relations drive made by nongovernmental organizations to protest GURTs, which largely led companies to forego commercialization of the technology. No matter which side GURTs are viewed from, robust IPP would stimulate private research and development in plant breeding, as proven by the activities to create hybrid varieties (Goeschl and Swanson, 2003; Lence et al., 2005). There is nothing to be gained from saving and replanting inbred GURT-protected lines and hybrids. However, farmers should profit in the long term from improvements in innovation because increased breeding efforts will, in turn, yield more productive varieties and unique novel traits. Furthermore, these innovations might help to enhance genetic diversity in many important crops, thereby providing long-term economic and environmental benefits (Van Acker et al., 2007).

3.5.2 Bioconfinement

The benefits and risks of growing transgenic crops have been discussed over the past three decades (Conner et al., 2003; Lu and Yang, 2009; Nap et al., 2003; Stewart et al., 2003; Warwick et al., 2009). Gene flow continues to rank near or at the top of the list among debated risks. Gene flow is the transfer of genes from one population to another. In the context of biosafety of transgenic crops, the movement of transgenes from crop to wild relative and/or weedy plants (both intra- and interspecies and also known as transgene escape) is of continued concern (Conner et al., 2003; Daniell, 2002; Nap et al., 2003; Stewart et al., 2003; Warwick et al., 2009). Pollen, seed or vegetative propagules can contribute to gene flow (Husken et al., 2010; Warwick et al., 2009), with pollen- and seed-borne gene movement of primary concern (Daniell, 2002; Ellstrand, 2003; Heuberger et al., 2010; Hills et al., 2007; Husken et al., 2010; Lu and Yang, 2009). It has been proposed that using GURTs would greatly impede transgene escape (Gressel,

2010b; Hills et al., 2007; Kausch et al., 2010). Briefly, the idea is to apply a GURT system together with the targeted transgene, so the latter will be eliminated once the GURT is activated (see below and Figure 3-3). The definitions of V- and T-GURTs for bioconfinement would be slightly different from those for IPP. They refer to the technologies that restrict possible transgene dispersal mediated by sexual reproduction from host plants to their relatives, via elimination of the entire genome (V-GURTs) or removal of the transgene from the genome (T-GURTs). On the other hand, all GURTs, regardless of the aims, are based on similar molecular mechanism as discussed above. Below, a detailed comparison will be discussed.

3.5.3 Comparison of IPP- and bioconfinement-oriented GURTs

All plants containing GURTs have to be grown under two distinct conditions: (i) for research, breeding, seed bulking, (ii) in commercial fields. In the first, the GURTs are 'off' in which plants reproduce normally, and in the second, the GURTs are 'on,' meaning that the particular trait is protected (IPP GURTs) or gene flow is restricted (bioconfinement GURTs). It is important to have a switchable system so that during breeding, for example, the GURT system is inactivated for improving plant genetics.

The target tissue (determined by the natural signal-responsive promoter shown in Figure 3-2) of GURTs aiming for IPP could be the seed for V-GURTs or any part of the plants for T-GURTs. In contrast, the GURTs for bioconfinement are mainly focused on reproductive parts of a plant such as pollen, seed and floral organs (Gressel, 2010a; Hills et al., 2007; Kausch et al., 2010). In many cases, the supposed outcomes of IPP and bioconfinement GURTs might be the same, barring some caveats noted here. In all V-GURTs, the activation of the system should result in

the elimination of an entire genome of the target cells without altering the genetic material in nontarget tissues. By contrast, IPP plants that employ T-GURTs should express the desired trait but not change genome sequence in both target and nontarget tissues, whereas bioconfinement T-GURTs generate nontransgenic genome only within the target cells. When doing so, the efficiency required for efficacy varies. In the case of IPP V-GURTs, there is no need to approach 100% efficiency, because greatly reduced germination rate is enough to force farmers to continually buy seeds from companies. The requirement for IPP T-GURTs would be higher, depending on the particular nature of the desired traits. However, the efficiency requirement for any bioconfinement GURTs should approach 100%.

3.6 Designing GURTs for bioconfinement

3.6.1 Host plants and system design

The major similarities and differences between IPP and bioconfinement GURTs are notable when designing a strategy (Table 3-1). Both V- and T-GURTs could be targeted to reproductive tissues, most typically pollen and seed (or embryo) (Table 3-1). It is important to tailor the GURT to the target species and its reproductive system, as well as that of wild relatives that could be gene flow recipients. For those crops that are primarily self-pollinated such as wheat, rice and cotton, the best choice would be seed-specific GURTs because of the relative lower probability of pollen-mediated transgene escape. In contrast, the pollen-specific GURTs are more suitable for plants that primarily cross-pollinate (Hills et al., 2007; Kausch et al., 2010; Van Acker et al., 2007; Visser et al., 2001). In species that are primarily farmed for vegetative organs, a mechanism has been proposed to ablate the entire reproductive organs (Kausch et al., 2010). Additionally, because longer-lived and more fecund species will likely have higher gene escape

frequency, the perennial plants that produce abundant pollen and seeds might be in the worst-case scenarios. An important design feature is the choice between V-GURTs and T-GURTs. In cases in which seeds are target for harvest regardless of their ability to germinate, T-GURTs may not work because excision of the transgene will result in the loss of the desired trait. One example is in the case of plants engineered to produce recombinant pharmaceutical and industrial proteins or other biomolecules. In these so-called biopharming plants, the products for harvesting are typically accumulated and stored in endosperm tissue of a seed (Mascia and Flavell, 2004; Obembe et al., 2011). Thus, application of V-GURTs that disable only embryos will eliminate transgene escape without compromising production. In contrast, T-GURTs are preferred when the goal is to produce nontransgenic seed or pollen by excising transgenes from the genome in order to not interrupt natural food chains (Kwit et al., 2011; Moon et al., 2010b).

It is also possible to further improve the bioconfinement efficiency by combining GURTs with other bioconfinement systems. It has been demonstrated that the pollen-mediated transgene flow between transgenic apomictic tetraploid and sexual diploid bahiagrass could be as low as 0.03%, compared with 0.17% between transgenic apomictic tetraploids and nontransgenic apomictic tetraploids (Sandhu et al., 2010). Because the viability of pollen from paternal plants is unaffected (Quarin, 1999; Sandhu et al., 2010), additional application of pollen-targeted GURTs is probably able to further confine the gene flow mediated by pollen to an acceptable level for both public and regulatory sectors.

The linkage of V- or T-GURTs to the target transgene might be variable depending on desired outcome. V-GURTs function at any genetic distance from the trait (Figure 3-3a), whereas a T-

GURT DNA construct has to be genetically linked to the target gene (Figure 3-3b). Thus, V-GURTs could be easily introduced into any existing varieties, which might already contain multiple transgenes in the genome, without greatly increasing the cost. By contrast, implementation of a T-GURT system requires that all transgenes, including T-GURTs itself, be packaged into one contiguous DNA insertion fragment. Because of the limitations of currently available DNA engineering and delivery methods (Ananiev et al., 2009; Chen et al., 2010; Dhar et al., 2011; Gelvin, 2003; Hamilton et al., 1996; Liu et al., 1999; Yu et al., 2007), T-GURTs are only able to restrict the movement of a few transgenes that are directly linked. Thus, T-GURTs could be costlier to implement than V-GURTs.

3.6.2 Choosing appropriate components

An ideal GURT system should have no unexpected phenotypic effects when off or on (Table 3-1). Because a physical switch (blocking sequence or DNA fragment between excision sites in Figure 3-1) is apparently the best way to prevent unwanted gene expression, both V- and T-GURTs for bioconfinement contain it where an excisor (recombinase in Figure 3-1) is required for deletion upon GURTs' activation (Figure 3-3). In the context of V-GURTs-based bioconfinement system, the following three factors are critical for efficacy: (i) the ability of the disrupter to ablate target cell, (ii) activity strength of the excisor gene to remove the blocking sequence, (iii) the spatiotemporal specificity that the promoter of the disrupter gene provides (Figure 3-3a). In contrast, when designing a feasible T-GURT system (Figure 3-3b), only the latter two variants play key roles. Because of the importance of these three components in a bioconfinement GURT system, we focus on them for the remainder of this section.

3.6.3 Disrupter gene

All disrupter genes used to date in V-GURTs are those coding for cytotoxins. These include nucleases such as barnase and ribonuclease A, which degrade either DNA or RNA, and catalytic lethal proteins such as diphtheria toxin and ribosomal inhibitor protein that prohibit RNA or protein synthesis (Burgess et al., 2002; Gils et al., 2008; Mariani et al., 1990; Moon et al., 2010a; Oliver et al., 1998, 1999a,b; Zhang et al., 2012). Although there is no evidence to date that the products of these genes are toxic to human and livestock, the acceptance by public might be questionable (Conner et al., 2003; Gressel, 2010b; Lu and Yang, 2009; Nap et al., 2003; Stewart et al., 2003; Warwick et al., 2009). Because of these concerns, certain cytotoxins should probably be expressed in nonfood crops or not expressed in tissues that are used for food or feed.

3.6.4 Excisor gene

The requirements for an ideal excisor include its (or its orthologs') absence within the host plant, specificity of recognition of target excision sites, the uniqueness of excision sites within the host plant's genome and efficient excision. There are a few recombinases, combined with corresponding excision or recombination sites, that have been tested in different plant species, which include CRE-*loxP* (Dale and Ow, 1990; Luo et al., 2007; Zuo et al., 2001), FLP-*FRT* (Akbudak and Srivastava, 2011; Lloyd and Davis, 1994; Luo et al., 2007; Nandy and Srivastava, 2011; Rao et al., 2010), R-*RS* (Khan et al., 2011; Onouchi et al., 1995), Bxb1-*att* (Blechl et al., 2012; Thomson et al., 2012; Yau et al., 2011), ParA-*MRS* (Thomson et al., 2009; Zhou et al., 2012), PhiC31-*att* (Kempe et al., 2010; Thomson et al., 2010) and CinH-*RS2* (Moon et al., 2011; Zhou et al., 2012) systems. In addition, recently developed genome-editing technologies such as zinc finger nucleases (ZFNs) and transcriptional activator–like effectors (TALEs) fused with

nucleases (TALENs) also have potential to be used as excisor genes (Bogdanove and Voytas, 2011; Petolino et al., 2010; Tzfira et al., 2012).

Among these, a combination of CRE-*loxP* and FLP-*FRT* systems demonstrated 100% excision efficiency when tested in tobacco pollen and seed in many, but not all, transgenic events (Luo et al., 2007). CinH-*RS2* system has also been tested in tobacco pollen, and more than 99% excision events were observed (Moon et al., 2011). These results suggested that both systems are powerful candidates as acceptable excisors in GURT-based bioconfinement systems. However, field data are required to determine whether such systems would be suitable for commercial applications. Secondly, and also importantly, their efficiency in crop species is yet unknown. Based on the fact that the CRE-*loxP* system showed a wide range of efficiency in several plant species (Sreekala et al., 2005; Zhang et al., 2006; Zuo et al., 2001), it is reasonable to conclude that host plant's genome could affect transgene excision efficiency within a recombination system.

3.6.5 Promoter

Regardless of whether a V-GURT or T-GURT system is chosen, a promoter that provides spatiotemporal specificity is required (Figure 3-3). Although there have been many efforts to find candidate promoters whose activities are exclusively restricted within seed (Coussens et al., 2012; Fauteux and Stromvik, 2009; Furtado et al., 2008; Galau et al., 1992; Hood et al., 2003; Huang et al., 2011a; Hughes and Galau, 1991; Kawakatsu et al., 2008; Kuwano et al., 2011; Qu le and Takaiwa, 2004; Qu le et al., 2008; Streatfield et al., 2010; Woodard et al., 2003) or pollen/anthers (Anand and Tyagi, 2010; Cook and Thilmony, 2012; Gupta et al., 2007; Huang et

al., 2011a,b; Jeon et al., 1999; Kato et al., 2010; Khurana et al., 2012; Mlynarova et al., 2006; Park et al., 2006; Swapna et al., 2011; Twell et al., 1989; Xu et al., 1999; Zhang et al., 2012), most tissue-specific promoters characterized to date have been suboptimal for GURTs. Both high expression and sharp tissue specificity are needed; system tuning is needed.

3.6.6 Tuning GURTs

The regulation of gene expression is not limited to its promoter, but also extends to its introns and 3′-untranslated regions (3′-UTRs). In many cases, the introns of a gene could act as enhancers or alternative promoters to affect its spatiotemporal expression pattern, or elevate mRNA accumulation to increase expression strength, or even facilitate expression in heterologous species (Bartlett et al., 2009; Giani et al., 2009; Liu and Liu, 2008; Rose, 2008; Yang et al., 2011). In addition, 3′-UTR also plays an important role in regulating gene expression at both the transcriptional and post-transcriptional levels by affecting mRNA accumulation, stability and translation efficiency (Bashirullah et al., 2001; Fabian et al., 2010).

Other methods and DNA elements have been explored as gene expression regulators. For example, the disrupter protein barnase could be split into two separate but complementary peptides and synthesized under the control of two separate promoters, and thus, only cells that contained both would be ablated (Burgess et al., 2002; Kempe et al., 2009). By carefully selecting their promoters, it is very possible to precisely restrict the functional barnase complex within desired tissue. Recently, Zhang et al. (2012) demonstrated that mutant versions of barnase expressed in transgenic tobacco, pine and *Eucalyptus* (spp.) had various activities. One of these

mutants, H102E, exhibited enough ability to ablate pollen with no obvious off-target effects. These results suggest that there is room to tune GURT components to increase efficiency.

3.7 Perspectives and future outlooks

Clearly, GURTs have utility for both technology/intellectual property (IP) protection and limiting gene flow, that is, for environmental and regulatory reasons. These latter reasons are probably the most imminent in the case of next-generation biofuel feedstock crops and the case of other crops in which there are special concerns about gene flow and introgression (Gressel, 2010a,b; Kausch et al., 2010; Kwit and Stewart, 2012; Stewart, 2007; Strauss et al., 2010). The concerns can be broken down into two general categories. First, scientists, regulators and the public are concerned about preventing gene flow in crops where hybridization, and especially introgression, could jeopardize the integrity of managed or unmanaged ecosystems (Kwit et al., 2011; Stewart et al., 2003). The second concern, admixture of transgenic grain containing pharmaceutical or other products into the food and feed supplies, is held mainly by consumers and regulators (Kausch et al., 2010; Kwit and Stewart, 2012; Stewart, 2007). The commonality here is the ubiquitous governmental regulation of transgenic plants, which seems to be getting more stringent throughout the world. This stringency is paradoxical given the environmental and food safety record of transgenic plants as a whole.

Thus, we see a push–pull dynamic emerging with regard to research and development with the goal of eventual commercialization of GURTs. The push to improve and commercialize GURTs would have a tangible regulatory and IP benefit. We envisage the pull coming from consumers and regulators, thus begging the question: Why are GURTS not currently implemented in

commercial transgenic crops? Agricultural companies have been chided for even considering GURT deployment for an IP benefit, even though it is completely reasonable for companies to diligently protect their IP. This situation could well limit the push for companies to seriously consider GURTs for ecological reasons. In addition, companies could well be weighing implementation benefits versus the potential for regulators to expect GURTs in all crops in future; that is, that GURTs could become a regulatory requirement even when it is not necessary. An additional factor affecting push and pull is that it might be quite expensive for companies to deploy GURTs given the large number of discrete units of DNA that would need to be licensed. One solution is the invention of a complete system *de novo* that could be in-licensed as a whole. A final factor affecting the regulatory pull is the tendency for regulators to desire streamlined constructs and the insertion of minimal DNA into plant genomes via biotechnology. Again, paradoxically, by installing a GURT system into a transgenic plant (more DNA), it increases the likelihood that less DNA of interest will be released from a transgenic field. In addition, it is also unknown how much testing would be required for regulatory approval. Would it be prohibitively expensive for companies to test the ecological and food safety effects of each GURT component? One last consideration revolves around nontransgenic gene flow from crops to wild and weedy relatives. There is some ecological and agricultural concern about crop genes increasing weediness of wild relatives and disrupting natural ecosystems (Warwick et al., 2009). GURTs could also limit introgression of crop alleles into their wild and weedy relatives.

Thus, we see that there is a tipping point that involves complexities of economics, government regulation and consumer affairs; these go beyond the science and technology of GURTs. That said, the science plays a role in the tipping point of adoption too. The key is the creation of an

extremely efficient GURT system that is environmentally robust and deployable in a number of crops. Synthetic biology will likely play an important role in the discovery, creation and implementation of key components for GURTs. Whereas plant synthetic biology lags behind that for microbial and biomedical applications, it is poised to revolutionize agriculture (Liu et al., 2013). Specifically, bioinformatic and synthetic biology tools should greatly improve our ability to make very strong specifically inducible promoters to regulate key GURT components. These tissue-specific inducible promoters would likely be the key to creating effective ecological GURTs. In addition to designing promoters, synthetic biology should be able to improve the efficiency of site-specific recombination systems and also employ new tools such as TALENs to produce very effective excision systems (Liu et al., 2013). Thus, we envisage synthetic biology as the key to develop gene circuits and other components to push the efficiency of GURTs to approach the 100% efficacy that could be required for bioconfinement purposes.

In conclusion, GURTs hit the scene of plant biotechnology with a bang in the late 1990s and early 2000s, but their potential has never been reached. We envisage plant synthetic biology and new crops and products as driving the research and development of GURTS towards implementation into a commercial crop pipeline within the next 10 years.

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Appendix

Tables and figures

Table 3-1. Comparison of GURTs aiming to intellectual property protection and bioconfinement.

	Intellectual property protection	Bioconfinement
GURTs' status	Off in research and breeding and on commercial in field (with exceptions, see text)	
Target tissue	Seed (V-GURTs); Any tissue (T-GURTs)	Reproductive tissues (commonly seed and pollen; both V- and T-GURTs)
Desired effects on target tissue	Genome eliminated (V-GURTs); Trait expressed while the genome is unchanged (T-GURTs)	Genome eliminated (V-GURTs); Nontransgenic genome generated (T-GURTs)
Desired effects on nontarget tissue	Genome unchanged* (all GURTs)	
Required efficiency	100% not necessary (V-GURTs); Higher, the better, need to be 100% (T-GURTs)	Approaching 100% (both V- and T-GURTs) but no

*A blocking sequence would be deleted from the genome in some cases, but other parts of the transgene retained.

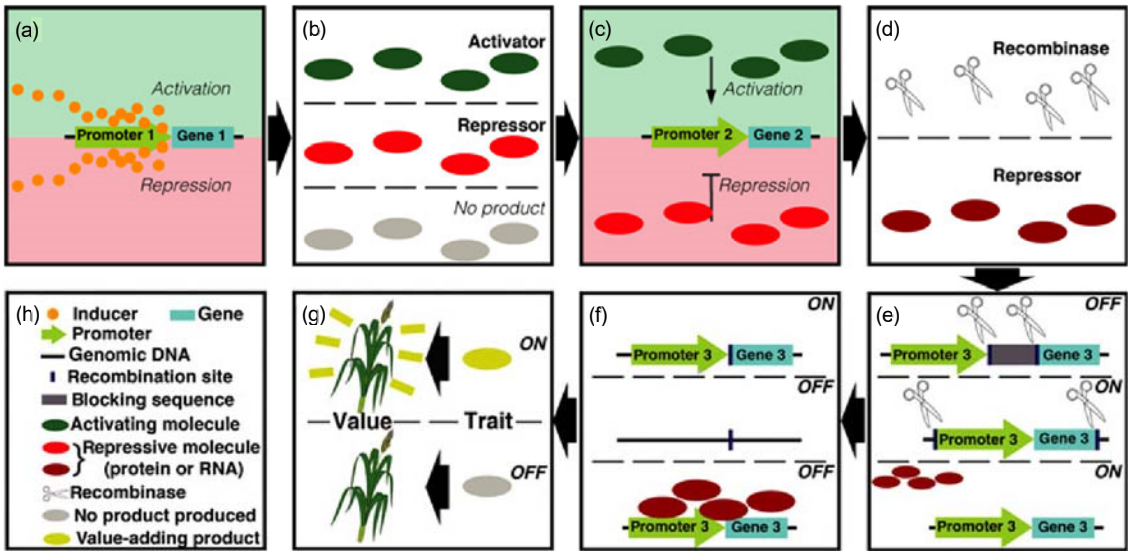


Figure 3-1. A schematic diagram of signal cascade of GURTs. (a) A promoter (promoter 1), whose activity is either activated or repressed by exogenously applied inducers, drives expression of the enabler gene (gene 1). (b) Output of (a) serves as input of (c). The product of the enabler gene in (a) could act as either activator (upper level) or repressor (middle level). Alternatively, there is no product generated at all (lower level) if the enabler gene is repressed in (a). (c) In response to the input from (b), the promoter 2 that drives expression of the trait switch gene (gene 2) is either activated repressed. (d) Output of (c) serves as input of (e). The products could be either recombinases or repressor molecules. (e) Depending on the presence of the 'blocking sequence', the target gene (gene 3) could be set by default on or off. (f) The recombinases generated in (d) specifically recognize the recombination sites and remove the sequence in between, resulting in switching on or off the target gene (upper and middle levels, respectively). Alternatively, the repressors block the transcription/translation of the target gene (lower level). (g) The product of the target gene (trait) renders the host plant to express specific agronomic trait(s). (h) A list of symbols used in this figure.

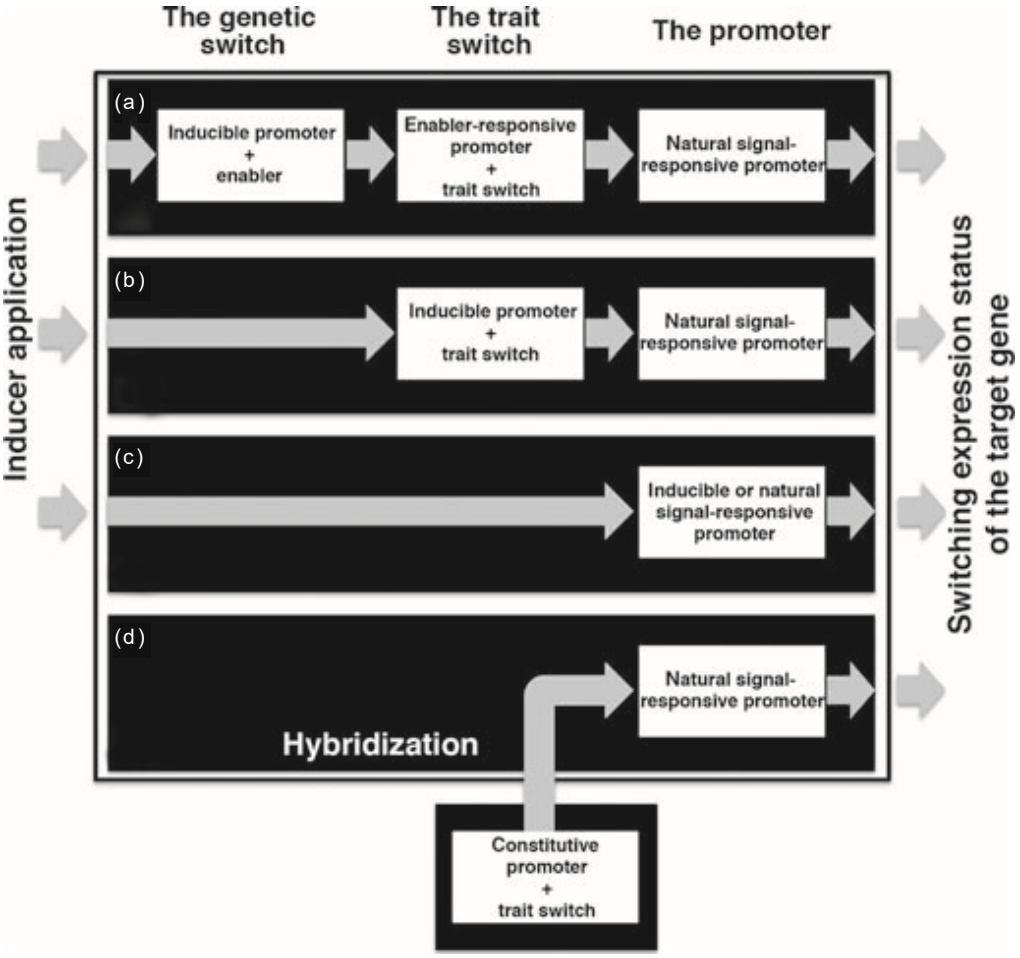


Figure 3-2. A schematic diagram describing fundamental designs of GURTs. Each black box indicates a genome containing components shown in white boxes. The input could be either inducer application ((a)-(c)) or hybridization event (d) that initiates the signal transduction indicated by arrows regardless of positive or negative effects leading to the toggling of the expression status of the target gene. (a) Typical GURT system containing all components (indicated above the frame) discussed in the text and Figure 3-1. (b) GURTs in which the genetic switch component is omitted. (c) GURTs in which the genetic switch and the trait switch components are omitted. (d) GURTs in which the target gene's expression is switched only when hybridized with the inducer line constitutively expressing the trait switch gene.

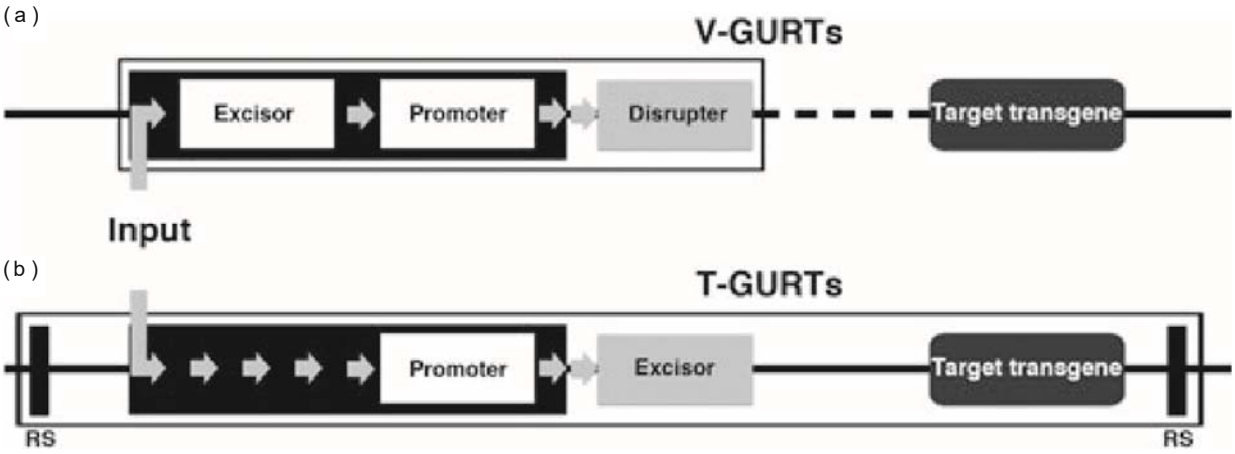


Figure 3-3. Schematic diagrams showing V- and T-GURTs for bioconfinement. All components belonging to a GURT design are framed. Arrows indicate the signal transductions initiated by input. The outputs are activation of disrupter gene in V-GURTs (a) or excisor gene in T-GURTs (b). The key components (see text for details) are represented by white or grey boxes. The round cornered rectangles indicate the target transgene(s) that should be eliminated upon activation of the GURT system. There would necessarily be two recognition sites (RS) in each design, but those in V-GURTs (a) are not shown to simplify the black box. Lines, with dashed line indicating any genetic distance, represent the genomic DNA. V-GURTs allow confining the target transgenes located on any chromosome(s) within a genome. T-GURTs only allow confining the target transgenes located within the T-GURTs itself.

Chapter 4: Engineered selective plant male sterility through pollen-specific expression of the *Eco*RI restriction endonuclease

A version of this chapter has been published in the Plant Biotechnology Journal with the following authors: Reginald J. Millwood, Hong S. Moon, Charleson R. Poovaiah, Balasubramaniam Muthukumar, John Hollis Rice, Jason M. Abercrombie, Laura L. Abercrombie, William Derek Green and Charles Neal Stewart Jr.. Reginald J. Millwood performed plant transformation, collected data, performed data analyses, and drafted and revised the manuscript. Hong S. Moon assisted in vector construction, plant transformation, and assisted in the manuscript draft. Charleson R. Poovaiah assisted in qRT-PCR analysis. Balasubramaniam Muthukumar assisted in Southern blot analysis. John Hollis Rice assisted in nucleic acid extraction and greenhouse experiments. Jason M. Abercrombie participated in transformation vector design and the manuscript draft. Laura L. Abercrombie performed vector construction. William Derek Green helped establish the field experiment and collected field data. C. Neal Stewart Jr. supervised the study and participated in manuscript drafts and revisions. Journal article citation: Millwood, R. J., Moon, H. S., Poovaiah, C. R., Muthukumar, B., Rice, J. H., Abercrombie, J. M., Abercrombie, L.L., Green, W.D. and Stewart, C.N. Jr. (2016) Engineered selective plant male sterility through pollen-specific expression of the *Eco*RI restriction endonuclease. Plant Biotechnol. J. 14, 1281-1290. doi: 10.1111/pbi.12493

4.1 Abstract

Unintended gene flow from transgenic plants via pollen, seed and vegetative propagation is a regulatory concern because of potential admixture in food and crop systems, as well as hybridization and introgression to wild and weedy relatives. Bioconfinement of transgenic pollen would help address some of these concerns and enable transgenic plant production for several crops where gene flow is an issue. Here, we demonstrate the expression of the restriction

endonuclease *Eco*RI under the control of the tomato pollen-specific LAT52 promoter is an effective method for generating selective male sterility in *Nicotiana tabacum* (tobacco). Of nine transgenic events recovered, four events had very high bioconfinement with tightly controlled *Eco*RI expression in pollen and negligible-to-no expression other plant tissues. Transgenic plants had normal morphology wherein vegetative growth and reproductivity were similar to nontransgenic controls. In glasshouse experiments, transgenic lines were hand-crossed to both male-sterile and emasculated nontransgenic tobacco varieties. Progeny analysis of 16,000–40,000 seeds per transgenic line demonstrated five lines approached (>99.7%) or attained 100% bioconfinement for one or more generations. Bioconfinement was again demonstrated at or near 100% under field conditions where four transgenic lines were grown in close proximity to male-sterile tobacco, and 900–2,100 seeds per male-sterile line were analyzed for transgenes. Based upon these results, we conclude *Eco*RI-driven selective male sterility holds practical potential as a safe and reliable transgene bioconfinement strategy. Given the mechanism of male sterility, this method could be applicable to any plant species.

4.2 Introduction

Confinement of transgenes in crops is an important regulatory goal, yet we know of no method that completely eliminates gene flow. Although no US regulatory agency has set a threshold for the adventitious presence of genetically engineered products, the EU has set a limit at or below 0.9% (Regulation (EC) No 1830/ 2003). Practically speaking, for any crop of interest in this regard, a transgene confinement tool would likely have to far exceed 99.1% efficiency. Indeed, this would particularly apply to the next generation of genetically engineered crops that possess novel fitness-enhancing traits and taxonomic reasons to confine them. Types of transgenes would

include those whose products are responsible for abiotic stress tolerance (Warwick et al., 2009), pharmaceuticals (Stewart, 2008) and altered cell walls for bioenergy (Kausch et al., 2010). One such new crop is switchgrass, *Panicum virgatum* L., the lignocellulosic bioenergy feedstock. Wild populations of switchgrass grow in the same geography as crop switchgrass, and the ramifications of gene flow from one to the other is unknown (Kwit and Stewart, 2012). The genetic diversity and ecological concerns might be exacerbated in cases such as switchgrass where the crop is grown in its geographic centre of diversity (Stewart, 2007). The lack of predictive invasiveness models to assess ecological impacts and interactions associated with these new transgene–crop combinations (Warwick et al., 2009) has in part been a driver for confinement technologies. Dependable and robust transgene confinement systems that are readily deployable would reduce environmental risks and alleviate many of concerns that associated with genetic engineering of switchgrass and other perennials such as trees and turfgrass.

There are many potential transgene confinement strategies, which include physical, chemical and biological methods. Physical confinement, such as fences, setting isolation distances and manually removing flowers, could conceivably suppress unintended transgene flow to sexually compatible plants in the environment, as would chemical sprays that alter flowering and gamete production. Extreme physical confinement (reviewed by Dunwell and Ford, 2005) may be effective in certain cases. For example, growing a pharmaceutical-producing crop, such as rice, far outside its normal production area would likely prevent admixture (Stewart, 2008), but might be impractical for most cropping goals. Chemical treatment to induce delayed flowering or

pollen ablation (e.g. Efisue et al., 2010) would likely be too costly and user-dependent (Kausch et al., 2010).

Biological transgene confinement (bioconfinement) using genetic engineering or genome editing techniques has the potential to eliminate pollen and/or embryo development (Liu et al., 2013). Technologies for pollen or embryo ablation have been assessed for many years (DeBlock et al., 1997; Elorriaga et al., 2014; Liu and Liu, 2008; Liu et al., 2008; Mariani et al., 1990; Thorsness et al., 1991; Yui et al., 2003; reviewed in Ding et al., 2014; Kausch et al., 2010; Li et al., 2004). Gene use restriction technologies (GURTs) could be useful both for bioconfinement and as tools for plant breeding (Kuvshinov et al., 2001; Lin et al., 2010; Liu et al., 2012; Oliver et al., 1998; reviewed in Hills et al., 2007; Oliver and Hake, 2012; Sang et al., 2013). In addition, inducible and/or cell-specific transgene excision (Dale and Ow, 1991; Luo et al., 2000, 2007; Moon et al., 2011; reviewed in Li, 2012; Moon et al., 2010) has been shown to be suboptimally effective at transgene removal. In many cases, transgene removal has not been field-verified, and in no instances have rates exceeded 99% efficacy in the field. Finally, transgenic alteration of developmental gene expression can be used to prevent flowering (Kim et al., 2007; reviewed in Kwit et al., 2011). One example is found in varying the expression of microRNA156, which results in delayed- or no flowering (Chuck et al., 2007, 2011; Fu et al., 2012; Xie et al., 2006). The obvious limitation of eliminating flower production is that the transgenic plants could not be used for downstream breeding and seed production.

Although there seems to be many potential tools for bioconfinement, there are few-to-none that are both highly efficient and deployable in a wide range of plant species. To that end, we

produced transgenic plants that utilize pollen-specific expression of *Eco*RI to test restriction enzyme-mediated gamete ablation as a potential bioconfinement technology in a model system.

4.3 Results

4.3.1 Transgenic plants: survival and *Eco*RI inheritance and expression

Initially, nine putative independent transgenic tobacco (*Nicotiana tabacum* cv. Xanthi) events were generated that harboured the *Eco*RI construct (Figure 4-1a); lines were downselected as described below. Southern blot analysis revealed that transgenic lines E1, E2, E3 and E4 were positive for the hygromycin gene, and lines E7, E8 and E9 had faint bands of high molecular weight (Figure 4-S1). No bands were observed in the blot for the nontransgenic control tobacco. Line E6 did not survive when grown in the glasshouse, and line E5 apparently did not express the transgene; these two lines were excluded in subsequent analyses. All remaining T_0 *Eco*RI events produced were self-pollinated to produce T_1- and subsequently T_2-progeny seeds. Each T_0 event was expected to be hemizygous at each insertion locus, and the maintenance of hemizygotes and homozygous nulls would be expected for each generation if the transgene product ablated pollen. If *Eco*RI effectively ablated pollen, the transmission of the *hpt* gene (hygromycin phosphotransferase, conferring resistance to hygromycin) would be prevented, and the expected segregation ratio for a single-copy selfed line would be 1 : 1 transgenic : nontransgenic). However, transgenic progeny percentage could be lower if the female gametophyte or seed development is affected by *Eco*RI expression or higher if the transgene copy number was greater than one.

To test for the predicted segregation ratio, progeny analysis on selective media showed that T_2 generation progeny significantly deviated from the expected 1 : 1 ratio of hygromycin-resistant (hmR) to hygromycin-susceptible (hmS) individuals with the exception of line E3 (Table 4-S1). The transmission of hmR was reduced through the female for lines E1, E3 and E4 as determined by significantly fewer hmR individuals (≤1 : 1) than predicted. Further, lines E7, E8 and E9 demonstrated increased hmR in the populations as the observed ratios were ≥1 : 1 for hmR to hmS. All nontransgenic seedlings died when grown on selective media (Table 4-S1), and all seedlings survived when grown on nonselective media (data not shown).

In pollen, the *Eco*RI transcript was present in all transgenic lines with none detected in nontransgenic control plants (Figures 4-2a and 4-S2). Line E1 had the highest *Eco*RI transcript abundance than other transgenic lines (Figure 4-2a), and line E9 had the lowest transcript abundance (Figure 4-S2). Lines E7, E8 and E9 were eliminated from further tissue-specific *Eco*RI transcript analysis owing to progeny analysis results from crossing experiments. For all other tissues analyzed, there was negligible *Eco*RI transcript detected (Figure 4-2b). The *Eco*RI transcript abundance was <0.5% of the lowest signal observed in pollen. However, transcript abundance in stems from line E3 was significantly higher than all others (Figure 4-2b). In the case of line E3, there was large variation in measurements among biological replicates (replicate 1 = 0.01, replicate 2 = 0.14, and replicate 3 = 0.00), but no morphological variation was observed.

4.3.2 Plant morphology

Transgenic plants were morphologically indiscernible from nontransgenic control plants (Figure 4-3), with the exception of less biomass produced by transgenic lines E1, E4 and E7 ($P \leq 0.01$) (Table 4-1). The biomass production and stem circumference of lines E1 and E4 were significantly lower than all others (Table 4-1). Transgenic plant height ($P = 0.66$), internode length ($P = 0.10$) and number of flowers per inflorescence ($P = 0.50$) were not significantly different among transgenic lines and the control (Table 4-1).

Pollen counts were similar among transgenic lines and the nontransgenic control (Figure4-S3). Several glasshouse-grown transgenic lines produced significantly fewer seeds per pod compared with the control (Figure 4-4a, $P = 0.03$), with no differences evident among lines E4, E7 and the control. When grown under field conditions, the mean seed production was apparently lower for lines E1 and E4, but no significant differences were detected among lines and the control (Figure 4-4b).

4.3.3 Bioconfinement

When hand-crossed with male-sterile stamenless tobacco (cv. 'MS TN90') in the glasshouse, all transgenic lines we assayed produced F_1 seed, and germination was apparently normal in the glasshouse and field crosses (Table 4-2). The crosses using lines E1, E2 and E3 did not produce any transgenic progeny in the glasshouse, resulting in 100% efficient bioconfinement, whereas other lines produced at least one transgenic seed (Table4-2). Line E4 produced the most transgenic progeny followed by lines E8 and E7; however, all progeny produced from transgenic lines were >99% transgene-free. All transgenic progeny that survived hygromycin selection

(Figure 4-S4) appeared to be morphologically normal. Additionally, all transgenic lines had decreased pollen germination compared with the nontransgenic control ($P \leq 0.01$; Table 4-2). Line E4 had the highest rate of pollen germination among all the transgenic lines.

All open-pollinated field experiments resulted in F_1 seed collected from 'MS TN90'. As hand-crossing was not performed in the field, the total seed number screened for transgenicity was noticeably lower than crosses performed in the glasshouse experiments (Table 4-2). Of the 13 733 F_1 progeny seedlings screened on hygromycin media, a single surviving transgenic F_1 progeny was detected from line E2. Seeds produced in plots from lines E1, E2 and E4 were 100% transgene-free (Table 4-2). When compared with controls, pollen germination means were slightly lower for field-grown transgenic lines, but no significant differences were detected ($P = 0.15$; Table 4-2).

Similar results were observed when transgenic lines were crossed to emasculated nontransgenic tobacco (cv. 'Xanthi') over multiple generations. Lines E1, E2 and E3 produced no transgenic F_1 progeny, again, resulting in 100% bioconfinement (Table 4-S2). Lines E4, E7, E8 and E9 produced transgenic progeny, but all F_1 populations were 99% transgene-free (Table 4-S2). Transgenic F_1 individual plants from lines E4, E7, E8 and E9 were again backcrossed to emasculated 'Xanthi' to produce BC_1F_1 progeny. Bioconfinement efficiencies were recorded for each plant within a transgenic line and ranged from 99% to 100% in all BC_1F_1 progeny populations (Table 4-S2). Backcrosses performed with F_1 individuals from line E7 produced no BC_1F_1 seed.

4.4 Discussion

4.4.1 Pollen-expressed *Eco*RI as a bioconfinement tool

We know of no report in which restriction endonuclease genes have been expressed in plants for any purpose. However, *Eco*RI has been shown effective to ablate cells in other organisms. When *Eco*RI was transformed and expressed in yeast (*Saccharomyces cerevisiae*; Barnes and Rine, 1985; Donahue et al., 2002) and Chinese hamster (*Cricetulus griseus*) ovary cells (Morgan et al., 1988), double-strand DNA breaks were produced and cells had aberrant growth. Additionally, when sunflower and maize leaf cells were exposed to exogenous purified *Eco*RI enzyme through tissue abrasion, genomic DNA damage was observed (Gholizadeh and Kohnehrouz, 2009). Here, we report the first targeted expression of any restriction endonuclease in plants, in this case, pollen, resulting in transgene bioconfinement via selective male sterility. Bioconfinement efficiencies of over 99% and up to 100% were observed in all *Eco*RI lines and stable over multiple generations. Thus, pollen-specific *Eco*RI expression was demonstrated to be an effective biocontainment strategy in tobacco.

Restriction endonucleases are well-characterized defense mechanisms in bacteria (Wilson and Murray, 1991), in three classes (I, II, and III) based on their enzymology and cofactor requirements (Wilson, 1988). Among type II restriction endonucleases, which uniquely consist of separate endonuclease and methylase enzymes, *Eco*RI is likely the best characterized. *Eco*RI recognizes the nucleotide sequence 5′-GAATTC-3′, requires Mg^{2+}, functions as a homodimer and creates a double-strand break at the site resulting in a staggered-end four nucleotide overhang (Wilson, 1988). *Eco*RI has increasing enzymatic activity from 0 to 40 °C with a Q_{10} of 1.7 (Pohl et al., 1982), offering broad endonuclease activity across the physiological conditions

of plants. It is conceivable that other enzymes with various temperature maxima could be more appropriate for certain species of plants. For example, *Hind*III has maximum activity at 25 °C (Pohl et al., 1982), which could be a better choice as a bioconfinement ablation agent in cool-season crops. *Eco*RI or *Hind*III would be expected to cut DNA up to 1.8×10^7 times in a 4.4 Gb plant genome such as tobacco or 3×10^6 times in the 730 Mb *Sorghum bicolor* genome, given that a 6-base cutter would randomly appear every 4096 bases (4^6). Even though these very high frequencies would likely not occur because of inefficiencies and DNA repair, it is conceivable that high expression of a restriction endonuclease is sufficient to ablate targeted plant cells given the large number of potential targets.

4.4.2 Comparison of LAT52-*Eco*RI-mediated male sterility with other methods

Alternative strategies for engineered male sterility in plants have been published that also use cytotoxic genes to induce reproductive tissue ablation [e.g. diphtheria toxin A chain DT-A (Thorsness et al., 1991); bacterial *Barnase* from *Bacillus amyloliquefaciens*, *Barnase* (Mariani et al., 1990); ribosomal inactivating protein (*RIP*, Cho et al., 2001); stilbene synthase gene (*STS*, Höfig et al., 2006); and artificial microRNA genes used to suppress transcription factors (*amiRNA*, Toppino et al., 2011)]. The most widely studied of this group are *DT-A* and *Barnase*, and these genes offer the best comparisons to results presented here.

DT-A is well-characterized cytotoxic protein (Pappenheimer, 1977) that has been used for decades in developmental biology studies because of its utility to ablate cells. In early experiments with plants, DT-A was observed to be highly toxic to cells and caused pollen ablation in tobacco (Kim et al., 1998; Thorsness et al., 1991; Twell 1995), *Arabidopsis*

(*Arabidopsis thaliana* (L.) Heynh.); Nilsson et al., 1998; Thorsness et al., 1993) and canola (*Brassica napus* L.; Kandasamy et al., 1993). Of particular relevance to our experiments, Twell (1995) used the LAT52 promoter to control expression of the *DT-A* gene in tobacco pollen, and when crossed to nontransgenic tobacco, zero transgenic progeny were observed out of 1,469 plants analyzed. In another study, transgenic tobacco containing *DT-A* driven by a putative pectin esterase pollen-specific promoter from Chinese cabbage (*Brassica campestris* L. ssp. *pekinensis*) was observed to produce 50% aborted pollen and 50% normal pollen as expected (Kim et al., 1998). In subsequent studies, the *DT-A* gene was used to engineer male sterility for the purpose of transgene bioconfinement (Liu and Liu, 2008; Liu et al., 2008; Skinner et al., 2003; Yang et al., 2010, 2011). Although each of these studies reported a high degree of reproductive tissue ablation, only one study reported outcrossing results. Liu et al. (2008) crossed three pollen-specific DT-A and three chimerically (pollen-, carpel- and stigma-specific) expressed DT-A *Arabidopsis* lines to nontransgenic plants, and bioconfinement efficiencies were 42% and 100%, respectively. While the DT-A system is efficient, there might be concerns about effects on pollinators and human health associated with the widespread usage of a diphtheria toxin gene in plants. Although eukaryotic organisms cannot actively transport DT-A inside cells in the absence of the diphtheria toxin B chain (Thorsness et al., 1991; referenced therein Collier, 1977; Pappenheimer and Gill, 1972), one DT-A molecule is enough to induce cell death (Yamaizumi et al., 1978).

Another method to achieve genic male sterility is the use of cytotoxic RNase genes (*Barnase* and *RNAse-T1* from *Aspergillus oryzae*) expressed in tapetum cells, the layer of nutritive cells surrounding the pollen sac, which results in the selective destruction of the tapetum during pollen

formation and leads to pollenless male-sterile plants (Mariani et al., 1990). In these early studies, 92% of *Barnase* and 10% of RNase T_1 tobacco transformants failed to shed or produce viable pollen. Additionally, a single copy of the *Barnase* gene was sufficient to achieve male sterility tobacco, whereas four gene copies were needed for *RNase T1*. In subsequent experiments, *Barnase* expression was shown to induce male sterility in *Arabidopsis* (Luo et al., 2006; Roque et al., 2007), canola (Mariani et al., 1990; Roque et al., 2007), creeping bent grass (*Agrostis stolonifera* L.; Luo et al., 2006), hybrid poplar trees (P*opulus tremula* × *Populus alba*; Wei et al., 2006; *Populus tremula* × *Populus tremuloides*; Elorriaga et al., 2014), the ornamental plant Kalanchoe (*Kalanchoe blossfeldiana*; Garcıa-Sogo et al., 2010), rice (*Oryza sativa* L. ssp. *indica*; Luo et al., 2006), tobacco (Custers et al., 1997; Deblock and Debrouwer, 1993; Mariani et al., 1990; Roque et al., 2007; Zhan et al., 1996), tomato (*Solanum lycopersicon*; Burgess et al., 2002; Roque et al., 2007) and wheat (*Triticum aestivum*; DeBlock et al., 1997). Although this method has shown promise in a wide variety of plant species, the efficacy of the system has been questioned because tapetal cell lysis can be inconsistent, which leads to partial male fertility. Moreover, ablating all pollen in a plant could raise concerns about negative impacts on pollen-feeding insects (Cook et al., 2004), whereas our system ablates transgenic pollen only. Promoter specificity and subcellular localization

Tightly controlled targeted expression of an ablation gene is crucial to a bioconfinement system. First, promoter specificity is also the key to limit nontarget tissue damage to the body of the plant. Reduced biomass productivity has been reported for both DT-A (Nilsson et al., 1998; Skinner et al., 2003) and *Barnase* systems (Kobayashi et al., 2006; Wei et al., 2007). Several candidate anther- and pollen-specific promoters have been characterized for many species

(reviewed by Khurana et al., 2012), and methods for synthetic promoter construction have been developed (Liu et al., 2013; Liu et al., 2014) that could address this issue. Second, it is important that DNA damage is confined to cells of interest, so that there are no off-effects to the rest of the plant body or environment. Targeting pollen cells in the development stage would limit gratuitous spread of restriction endonuclease in the environment. Therefore, promoter choice is very important.

The LAT52 promoter is appropriate to drive the expression of cytotoxic genes in tobacco for bioconfinement because activity is tightly controlled for pollen in tomato and *Arabidopsis* (Twell et al., 1990) during late pollen development (Twell, 1995). These results were confirmed in our experiments as there was no evidence of off-target effects on the growth of *Eco*RI lines compared with nontransgenic controls, except in two lines with decreased biomass in field experiments, which were also the highest expressers. Nonetheless, five lines had over 99.7% bioconfinement, so the current system requires a 'sweet spot' of expression that results in ablated transgenic pollen but no plant body growth penalty. Also, in previous experiments, LAT52 activity was thought to occur in the postmeiotic development of the female gametophyte or in the early stages of seed development, because low numbers of transgenic progeny were observed in segregation analysis (Twell, 1995). This was observed in our experiments as well.

Given that we did not use a nuclear localization signal, we are unsure of the mechanism of nuclear targeting of *Eco*RI in plant cells, which apparently occurred. A survey of tobacco genomic sequence (96.6 Mb total surveyed; Tobacco Genome Initiative, North Carolina State University) revealed *Eco*RI recognition sites (34,152 total sites) occur at a 2.8 kb frequency (data

not shown), which is higher than would be expected on average (see Discussion section prior). Therefore, we can speculate that very high expression in pollen cells allows sufficient *Eco*RI protein to damage the pollen nuclear genome. Furthermore, we also speculate that a modified *Eco*RI gene with a nuclear localization signal might increase off-target effects in the plant body owing to the increase in efficiency of nuclear transport of the nuclease. Nonetheless, it is possible that the mitochondrial genome is the actual target within pollen. When using other pollen-specific promoters, such as the maize Zm13 promoter, driving nuclear targeted *Eco*RI, we have noticed decreased *Agrobacterium tumefaciens*-mediated transformation rates that seem to be accompanied by decreased *Agrobacterium* survivorship and growth in media (data not shown), which indicates potential leakiness of expression in bacteria using certain plant promoters. Therefore, there appears to be a fine line between sufficient expression and nuclear targeting. However, *Escherichia coli* has been documented to express visual marker genes controlled by plant tissue-specific promoters (Jopcik et al., 2013), and perhaps this is the case for *A. tumefaciens*. We have also experienced repeated failed experiments for *A. tumefaciens* transformation with plasmids containing pollen-specific promoters driving the expression of the *Eco*RI gene (data not shown). Further experiments need to be performed to determine whether either or both of these factors influence plant transformation rates when using *Eco*RI expression cassettes.

4.5 Conclusions

The targeted expression of restriction enzymes such as *Eco*RI holds great potential as bioconfinement tools in transgenic crops. Given that it uses a novel mechanism, in plants anyway, to ablate pollen, additional research is needed to understand potential off-effects and

other regulatory and governance concerns. In addition, further research is needed to identify additional tissues for targeting (e.g. various female or embryo/seed tissues) to produce an effective and robust total bioconfinement system (Sang et al., 2013; Stewart et al., 2003). It is unknown whether a restriction enzyme-based system would be commercially viable. Nonetheless, we envisage leveraging restriction endonuclease-mediated ablation in a compact multigenic synthetic circuit that would allow sterility to be toggled off as needed for breeding and on for field-deployed bioconfinement (Sang et al., 2013), but careful transgene tuning would be necessary for efficacy. Currently, no bioconfinement system has been commercialized in transgenic crops, but that is likely to change as new bioinformatic and synthetic biology tools advance our capability to increase efficacy and control of these systems (Liu et al., 2013).

4.6 Experimental procedures

4.6.1 Vector construction and plant transformation

A vector carrying a translational fusion of the *Eco*RI restriction endonuclease (the gene was kindly supplied by provided by Linda Jen-Jacobsen at the University of Pittsburgh) and *G3GFP* genes under the control of the pollen-specific LAT52 promoter (kindly supplied by Sheila McCormick at the University of California, Berkeley, USDA-ARS; Twell et al., 1990) was constructed with a R4pGWB550 Gateway binary vector (Nakagawa et al., 2008) using multisite Gateway cloning (Life Technologies, Carlsbad, CA). The expression vector also contains a hygromycin selection cassette. The resulting vector, pBC-LAT52-*Eco*RI (Figure 4-1a), was transformed into *A. tumefaciens* st. EHA105 using the freeze/ thaw method.

Tobacco cv. Xanthi was transformed by the Horsch et al. (1985) method whereby transgenic plants were selected using 50 mg/L hygromycin (Moon et al., 2011). Rooted plantlets were transplanted to pots containing potting mix, placed in an environmental plant growth chamber (16 h day length, 28/ 22 °C day/night) and acclimated for 2 weeks. T_0 plants were then grown in a glasshouse (16 h supplemental lighting, 27–30 °C). The inflorescences were covered with mesh pollination bags (DelStar Technologies, Inc., Alpharette, GA) to prevent cross-pollination, and plants were allowed to set seed. Each transgenic event was self-pollinated to the T_1 and T_2 generations. Transgenic individuals were selected at each generation by seed surface sterilization with 10% bleach and 70% EtOH (Moon et al., 2011) and placed on solidified MS medium supplemented with hygromycin (50 mg/L).

4.6.2 Polymerase chain reaction (PCR) and Southern blot analyses

Transgenic plants at various stages were PCR-screened for the *Eco*RI gene (Figure 4-1b). Genomic DNA was extracted from leaf tissue samples using an existing protocol (Stewart and Via, 1993). The *Eco*RI gene was amplified using primers with the following sequences: Forward: 50-ATGTCTAATAAAAAACAGTCAAATA-30; Reverse: 50-CTTCTTAGATGTAAGCTGTTC-30. The PCR conditions were as follows: 40 cycles of 94 °C for 1 min, 50 °C for 30 s and 72 °C for 1 min.

To confirm stable transgene integration and copy number Southern blot analysis was performed (Figure 4-S1). Genomic DNA extraction, gel electrophoresis, and blotting conditions were performed as described in Sambrook et al. (1989) and Muthukumar et al. (2013).

4.6.3 Quantitative reverse-transcriptase PCR (qRT-PCR)

Transcript levels of *Eco*RI were estimated in pollen, petals, leaves, ovaries and stems using qRT-PCR. Samples for RNA extraction were collected from three T_2 transgenic plants per event and a nontransgenic control. Pollen samples were collected from three flowers per plant at approximately 9 a.m. from freshly dehisced anthers. Flower petals and ovaries were taken from fully extended unopened flowers, and stigma, style and stamen were removed. Leaf samples were taken from young, unexpanded leaves. Stem samples were taken from cuttings of approximately 200 mg from internodes of lateral shoots. All samples were flash-frozen in liquid nitrogen and stored in 80 °C until extraction. RNA was extracted from samples using Tri Reagent (Molecular Research Center, Cincinnati, OH) following the manufacturer's protocol. Two hundred and fifty nanograms of total RNA was used for cDNA synthesis following a 1-h DNase treatment. Complementary DNA was synthesized from mRNA using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) according to the manufacturer's guidelines. The cDNA samples were diluted 10-fold, and 1 µL of diluted cDNA samples were used as the qPCR templates in a 10 µL reaction containing Power SYBR Green PCR master mix (Applied Biosystems) and 200 nM each of forward and reverse primers for *Eco*RI gene (forward AGGAGATGGGAGGGAGTGGGA, reverse CGCCCCAACACTCTGAGCGA) and L25 reference gene (GenBank L18908) forward and reverse primers (forward GGCTCCAGCTAAAGCTGATCCGT, reverse ACAGCCTTGGCAACCTTAGCTGC). The PCR conditions were 40 cycles of 95 °C for 10 s, 55 °C for 15 s and 72 °C for 30 s. The qRT-PCR data were analyzed by the standard curve method. Data were analyzed by analysis of variance (ANOVA) using mixed models, and

Fisher's LSD mean separation was performed (SAS 9.2 for Windows; SAS Institute Inc., Cary, NC).

4.6.4 Plant productivity

To quantify potential off-effects in transgenic lines, plants were grown to maturity in the glasshouse and compared with nontransgenic 'Xanthi' plants. Data collected were plant height at maturity, stem circumference at 5 cm above soil line, internode length between the first and second node above the 5 cm mark above potting mix line and number of flowers per inflorescence (three inflorescences were randomly chosen per plant). Plantlets from lines E1, E2, E3, E4, E7, E8 and E9 at the T_2 generation were initially grown on hygromycin-containing media, and nontransgenic control plantlets were grown on media without antibiotic. The resulting plants were then transplanted into 1-L pots and grown in a glasshouse (16/8 h day/night and 28/22 °C day/night). A randomized design was used, and four replicate plants were grown per line. Plants were grown to full maturity and the experiment was terminated when flowering was mostly complete and the majority of seed pods were mature. Individual plants were air-dried in mesh bags for 14 days. Once dried, total biomass weight was recorded and then seed pods were collected for each plant. Seed production was estimated by randomly collecting five mature seed pods per plant. Seed was collected from each pod, cleaned and weighed. To estimate the seed production per pod, first the total seed weight per pod was recorded, and then all seed numbers were derived after the 200 seed weight average was calculated for each pod. Two hundred seed weight averages were calculated by counting two independent batches of 200 seeds for each pod, recording the weight of each 200 seed batch, and averaging the two weights. If a pod contained <400 seeds, that number was recorded as total seed produced. Mean seed

production was determined per plant, and line and mean separation were performed as described above.

4.6.5 Glasshouse bioconfinement experiments

Transgenic pollen production and hybridization experiments were performed in a glasshouse (16 h day and 28–30 °C day). To determine the frequency of transgene inheritance in progeny, transgenic T_2 *Eco*RI tobacco lines (E1, E2, E3, E4, E5, E7, E8, E9) were used as pollen donors and hand-crossed to nontransgenic pollen receptor tobacco plants. Pollen receptor plants were a variety of male-sterile tobacco 'MS TN90'. Given that tobacco is highly self-pollinating, 'MS TN90' enabled facile crosses to be made. Thus, all pollination to 'MS TN90' must originate from the transgenic and nontransgenic 'Xanthi' plants used as pollen donors. Prior to crossing, seeds were surfaced sterilized as previously described, transgenic lines were selected on MS– hygromycin media (Figure 4-S4), and nontransgenic controls were grown on MS media with no antibiotics. Post-antibiotic selection, PCR analysis was performed to assay for the presence of the *Eco*RI gene. Two plants were randomly chosen for each line and transplanted into individual 3.5-L pots containing potting mix. Once plants began to flower, each pollen donor plant was paired with a single 'MS TN90' plant and hand-crossed over a 2-week period. *Eco*RI events and the controls were crossed with 'MS TN90' over a 2-week period. Pollen mesh bags were placed over 'MS TN90' plants between crossing periods to prevent adventitious cross-pollination with nonpaired pollen donor plants. When 'MS TN90' seed pods were mature, F_1 hybrid seeds were harvested and sorted by pollen donor type. To screen for transgenic individuals, seeds were surface-sterilized as previously described and placed on MS– hygromycin media in batches of 2,000 seeds per hybrid type, and a total of 40,000 seeds per hybrid seed type or the total amount

seeds produced, if <40,000. Additionally, seed germination percentage was estimated between 5 and 10 days after plating by counting the number of seed and the number of plants germinated for three plates per plant type. This method was repeated for each batch to account for variation in seed surface sterilization. Fourteen to 21 days after plating, the numbers of live (transgenic) and dying (nontransgenic) seedlings were recorded. Bioconfinement efficiency was calculated by dividing the number of nontransgenic germinated seed by the number of germinated seed.

A multigeneration hand-crossing experiment was also performed under glasshouse conditions as described above. Four transgenic T_2 *Eco*RI plants were selected from each line on MS–hygromycin media (E1, E2, E3, E4, E5, E7, E8, E9) and transplanted into individual 3.5-L pots containing potting mix. Transgenic individuals were used as pollen donors and paired each with an individual nontransgenic 'Xanthi' pollen recipient plant. As a negative control group, four 'Xanthi' pollen donor plants were paired with four 'Xanthi' pollen recipient plants. Ten flowers were emasculated by hand on each 'Xanthi' pollen recipient plant, and a single flower from a paired pollen donor plant was used to pollinate each emasculated flower. Pollen recipient plants were then covered with pollination bags to prevent unintended pollination. Upon maturation, seed pods were removed and threshed, and the resulting F_1 seeds were segregated per individual plant. Approximately 250 F_1 seeds (minimum of 1,000 seeds per line) from each individual cross were subsequently sterilized and screened on MS–hygromycin media as previously described. Transgenic lines E4, E7, E8 and E9 were the only lines to produce transgenic F_1 progeny. These progeny were backcrossed to nontransgenic emasculated 'Xanthi' in the same manner, and BC_1F_1 progeny were screened on MS–hygromycin media as previously described. All F_1 progeny from lines E7, E8 and E9 were used in the backcross experiment, and five individuals

(of 65 individuals) were randomly chosen from line E4 and used for crossing. Additionally, five nontransgenic ‘Xanthi’ individuals were designated as pollen donors and used as negative control crosses.

In addition, pollen germination frequency was examined to estimate the amount of viable pollen used in the crossing experiment. Pollen was collected from newly opened flowers, placed in 30 μL of BK medium (Brewbaker and Kwack, 1963) and incubated at 22 °C for 3 h, and pollen germination frequency was calculated as described in Hudson and Stewart (2004). Pollen was collected on three different days from one plant per plant type. In total, 5,600 pollen grains were analyzed, with 700 pollen grains screen per plant type. Statistical analyses including mean separation were performed as described in the previous section.

4.6.6 Field bioconfinement experiment

Transgenic pollen production and hybridization were studied in a field experiment in Knoxville, TN, USA, using ‘MS TN90’ as the female parent. Plantlets for the field experiment were grown as described above, and plants were hardened off in pots and glasshouse-grown for 1 month. Healthy plants were transplanted into the field in July 2012. Each plot contained two ‘MS TN90’ plants that were surrounded by 10 pollen donor T_2 transgenic or control plants in a randomized design wherein each transgenic line was replicated in four plots that each contained a single transgenic line. Each plot measured 3 m^2 with a 5 m distance between plots. Standard agronomic practices were followed in the field experiment. In October, 2012, the seed from each ‘MS TN90’ plant was collected, surface-sterilized and sown on MS–hygromycin media as above. The resulting progeny plantlets were scored as transgenic or nontransgenic as above to calculate

bioconfinement efficiency. A honeybee hive was located approximately 75 m of the experiment to ensure pollinators were present. Pollen germination frequency was examined as above. Pollen was randomly collected on five separate days in the morning hours. Samples were taken from 10 newly opened flowers per plot, and pollen was pooled into a single tube for each plot. Pollen germination frequency was observed, calculated and analyzed as described in previous section.

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Appendix

Tables and figures

Table 4-1. Growth characteristics of transgenic *Eco*RI tobacco plants grown under glasshouse conditions (means ± SE).

Line	N	Dry total biomass (g)	Plant height (cm)	Stem circumference 5 cm above soil level (cm)	Internode length (cm)	Flower number per inflorescence
Nontransgenic	5	541.2 ± 27.8^{a}	146.5 ± 0.8	5.3 ± 0.1^{a}	6.2 ± 0.4	28 ± 3
E1	5	394.4 ± 21.5^{c}	140.3 ± 1.0	4.6 ± 0.1^{c}	6.4 ± 0.5	28 ± 3
E2	5	529.3 ± 60.0^{ab}	146.0 ± 1.0	5.0 ± 0.04^{ab}	7.3 ± 0.6	31 ± 3
E3	5	528.0 ± 17.0^{ab}	145.7 ± 2.5	5.1 ± 0.1^{a}	7.7 ± 0.3	27 ± 2
E4	5	382.2 ± 31.0^{c}	148.4 ± 0.7	4.7 ± 0.0^{bc}	7.2 ± 0.8	27 ± 3
E7	5	434.6 ± 30.3^{bc}	145.9 ± 1.8	5.0 ± 0.1^{abc}	6.6 ± 0.5	26 ± 3
E8	5	449.6 ± 25.6^{abc}	143.5 ± 1.9	5.0 ± 0.0^{ab}	7.7 ± 0.5	32 ± 4
E9	5	516.4 ± 44.6^{ab}	137.4 ± 0.5	5.0 ± 0.0^{abc}	5.7 ± 0.5	31 ± 3

Superscript letters within a column represent significant differences (Fisher's LSD, $P \leq 0.05$).

Table 4-2. Screening of progeny seedlings germinated from seeds produced by hybridization experiments performed in the glasshouse and field. The male-sterile ('MS TN90') variety of tobacco was used as the pollen recipient plants and crossed with pollen from experimental transgenic lines (means ± SE).

Line	Bioconfinement efficiency (%)	Transgenic progeny	Seeds germinated	Seeds screened	Pollen germination (%)
Glasshouse 'MS TN90' cross - progeny screen					
Nontransgenic	N/A	0	36,860	38,800	42.33 ± 0.08^{a}
E1	100.00	0	38,000	40,000	18.33 ± 0.09^{c}
E2	100.00	0	28,310	29,800	21.25 ± 0.09^{bc}
E3	100.00	0	38,000	40,000	19.75 ± 0.04^{bc}
E4	99.12	248	38,000	40,000	30.58 ± 0.04^{b}
E7	99.99	1	38,000	40,000	17.58 ± 0.04^{c}
E8	99.77	36	22,420	23,600	17.33 ± 0.05^{c}
E9	99.85	17	15,200	16,000	12.50 ± 0.05^{c}
Field 'MS TN 90' cross - progeny screen					
Nontransgenic	N/A	0	8,124	9,249	38.51 ± 0.05
E1	100.00	0	1,690	1,764	28.34 ± 0.02
E2	99.95	1	2,037	2,167	27.94 ± 0.03
E3	100.00	0	861	909	34.26 ± 0.04
E4	100.00	0	1,021	1,253	30.73 ± 0.02

Superscript letters within a column represent significant differences (Fisher's LSD, $P \leq 0.05$).

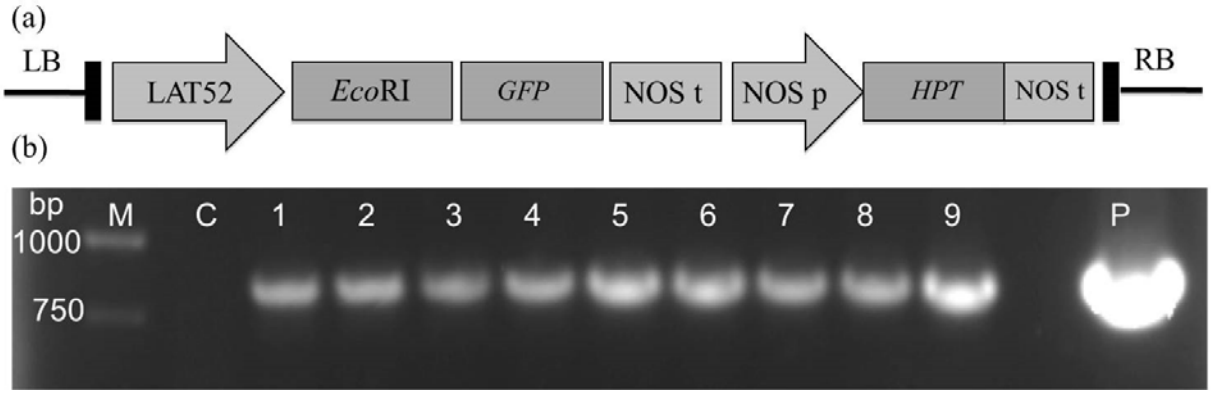


Figure 4-1. Transformation vector T-DNA used in plant transformation experiments and genomic PCR results. (a) T-DNA from plasmid pBC-LAT52-*Eco*RI used to transform tobacco. The *Eco*RI restriction endonuclease was translationally fused with the *green fluorescent protein* (*GFP*) gene, which was included for prospects of monitoring expression and estimating efficacy of the pollen ablation system; unfortunately, GFP did not enable monitoring in this system. The fused genes were under the control of the pollen-specific tomato LAT52 promoter. NOS t, nopaline synthase terminator; NOS p, nopaline synthase promoter; *hpt*, hygromycin phosphotransferase; LB, left border; RB, right border. (b) PCR analysis for the *Eco*RI gene was performed using genomic DNA extracted from leaves of putative transgenic T_1 *Eco*RI tobacco lines and nontransgenic controls (lane C). A DNA size marker was used to confirm band sizes (Lane M), and a plasmid containing the transgene (pBC-LAT52-*Eco*RI, lane P) was used as the positive control.

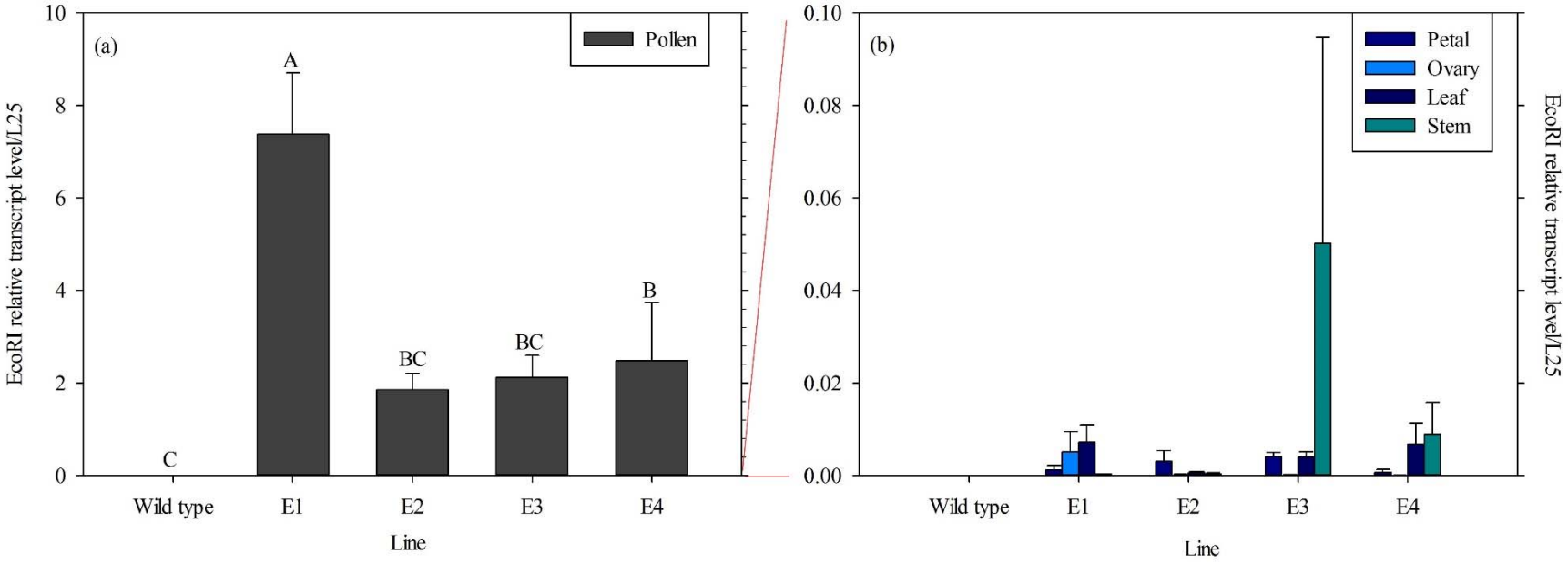


Figure 4-2. Quantitative RT-PCR of the *Eco*RI transcript in transgenic plants in various tissues. Relative quantification of *Eco*RI transcript was performed using the standard curve method, and each signal was normalized to transcript levels measured from the *L52* ribosomal gene from tobacco (GenBank L18908). (a) *Eco*RI transcript measured in mature pollen and (b) *Eco*RI transcript measured in other plant tissues. Bars represent means from three independent biological replicates. Letters represent significant differences between means (Fisher's LSD, $P < 0.05$), and error bars represent standard error of the means.

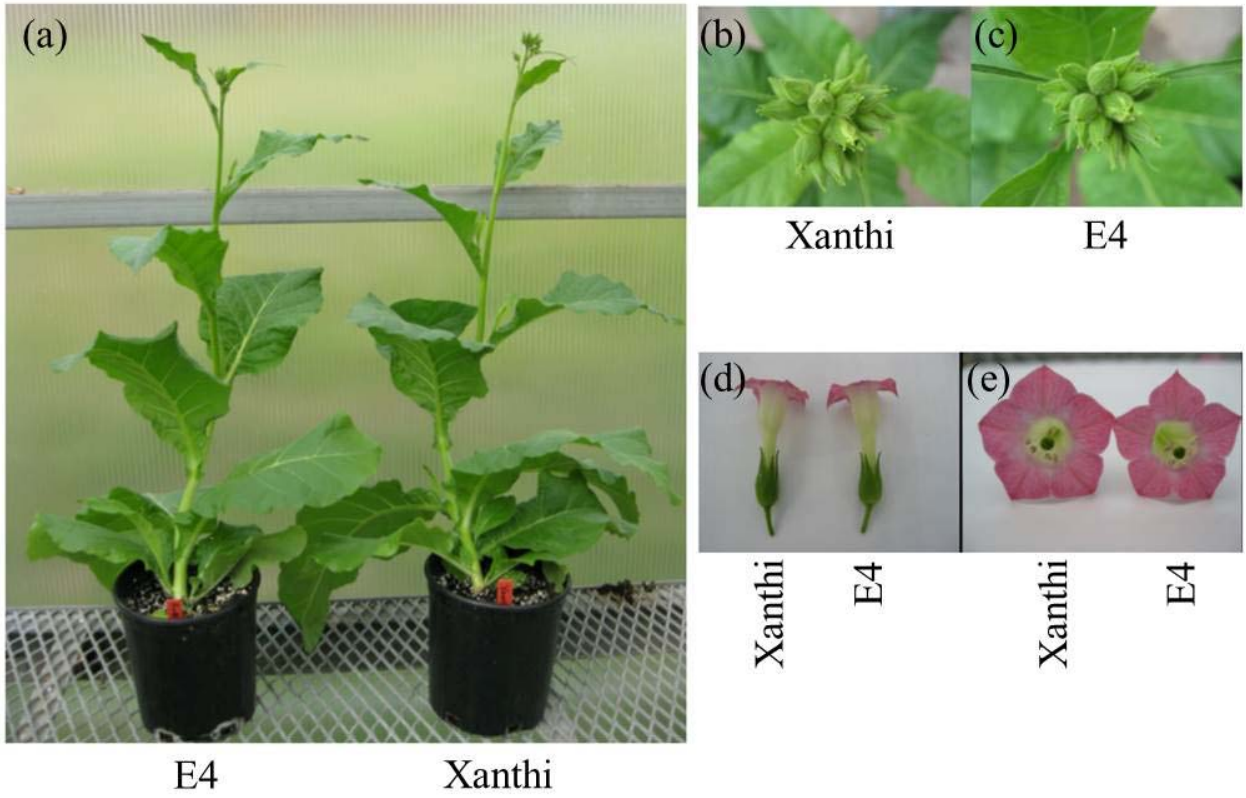


Figure 4-3. Phenotype of a representative T_1 transgenic event (E4) expressing *Eco*RI in pollen and a nontransgenic tobacco ('Xanthi'). (a) Three-month glasshouse-grown plants; (b, c) Flower buds; (d) Lateral view of flowers; (e) Front view of flowers.

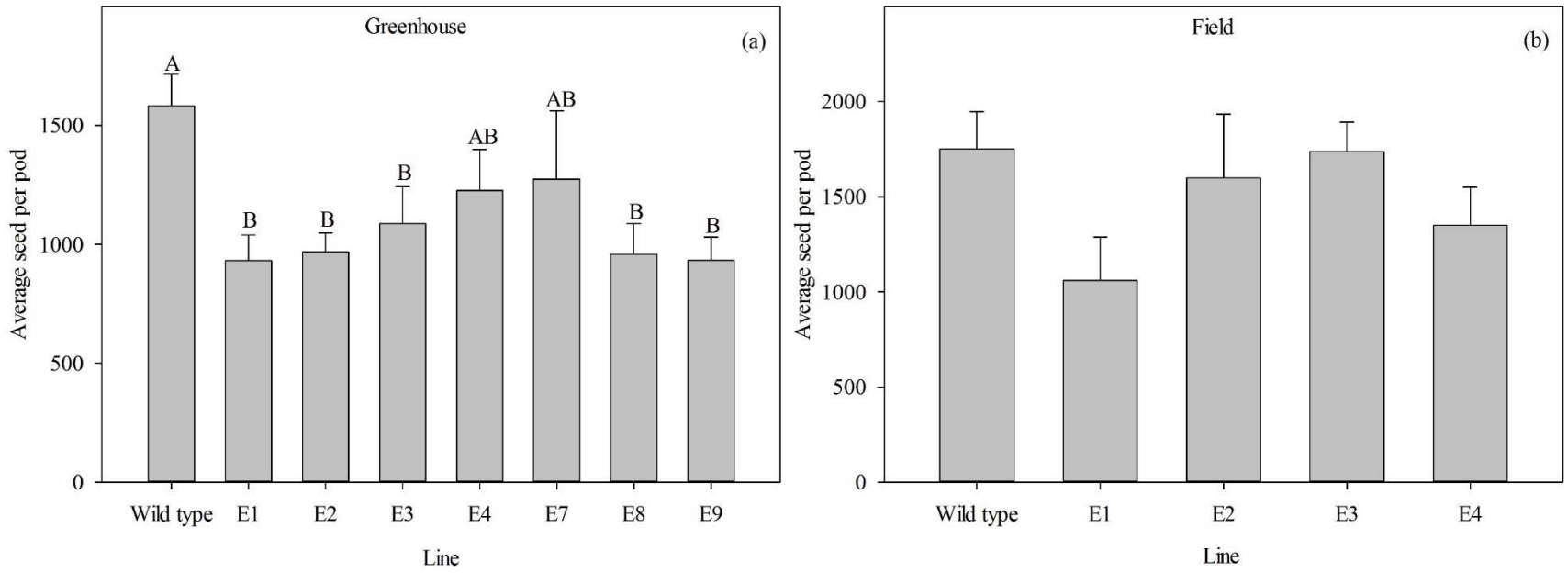


Figure 4-4. Average number of seeds produced per pod from transgenic lines expressing *Eco*RI in pollen under glasshouse and field conditions. Wild type: seed collected from the nontransgenic control plants. All other sample labels in both panels represent *Eco*RI transgenic lines. (a) Seed produced in plant productivity study grown under glasshouse conditions. (b) Seed produced under field conditions. Letters represent significant differences between means (Fisher's LSD, $P < 0.05$), and error bars represent standard error of the means. There were no significant differences observed in the field experiment.

Supplementary materials and methods

Transgene integration

Southern blot analysis was performed (Figure 4-S1) to estimate T-DNA insertion copy number for each transgenic line, and methods used are described in Balasubramaniam et al. (2013) with a few modifications. Genomic DNA from notransgenic 'Xanthi' tobacco and transgenic events (E1, E2, E3, E4, E7, E8, and E9) was isolated using a CTAB method (Stewart and Via, 1993) using 3 g of fresh leaf tissue. Genomic DNA (10 µg per sample) was then digested with *Hin*dIII and loaded into individual lane of a 0.8% (w/v) agarose gel (Figure 4-S1b). The gel also contained a size marker lane loaded with 4 µl of DIG labeled lambda DNA marker III (*Hin*dIII-*Eco*RI digested-Roche Applied Science) and a lane containing 120 pg/µl *Hin*dIII digested pBC-LAT52-*Eco*RI used a positive control. Subsequently, the gel was electrophoresed for 22 h, capillary blotting (Sambrook et al., 1989) was performed to transfer DNA to a nylon membrane (GE Healthcare), and lastly the membrane was cross-linked by UV irradiation. A pre-hybridization of the membrane was performed at 42 ºC in 40 ml DIG EasyHyb solution for 2 h. The membranes were hybridized in 35 ml of same solution that was freshly added with a DIG labeled 580-bp *hpt* hygromycin resistance (*hpt*) gene fragment for 15-18 h in a rocking hybridization oven. The *hpt* primer sequence used was: forward, 5′-GCGCTTCTGCGGGCGATTTG and reverse, 5′-GTCCGAATGGGCCGAACCCG (Balasubramaniam et al., 2013). Membrane hybridization, post-hybridization washes, and probe detection were performed as described in Balasubramaniam et al. (2013). Probe signals were visualized after a 6 h exposure on x-ray film.

Pollen production

Total pollen tallies per individual flower was taken on greenhouse-grown T_2 transgenic plants and a nontransgenic control (N=1, n>20,000 total; Figure 4-S3). Plants were grown under supplemental lighting at 16/8 hr day/night and 28/22 °C day/night. Pollen was collected from a single newly opened flower from one plant per type and pollen was collected for 4 days. The plant representative for line E9 died and was excluded from this analysis. After collection, pollen was counted using an automated Cellometer Vision® instrument (Nexcelom Biosciences, LLC, Lawrence, Mass,.USA). To prepare samples for counting, pollen was suspended in water and placed in a counting chamber. Pollen was imaged and counted using Cellometer Vision Software®.

Progeny analysis

Segregation ratios were recorded (Table 4-1) for each transgenic line and a nontrangenic control ('Xanthi'). Seeds were surfaced sterilized as previously described. Twenty-five seeds were placed on a single petri dish containing MSO medium supplemented with 50 $mg.L^{-1}$ hygromycin and replicated five times for each line (125 seeds total per line). After 14 days, the total number of germinated, hygromycin resistant (live/green), and hygromycin susceptible (dying/yellow) plants were counted. Data were analyzed using the Chi-squared goodness-of-fit test.

Supplementary tables and figures

Table 4-S1. Segregation analysis of T_2 progeny based on the hygromycin-resistant assay.

Line	Total germinated	Total transgenic (T)	Total nontransgenic (N)	Observed ratio (T:N)	Transgene copy number	χ^2 value under expected 1:1 ratio
nontransgenic	114	0	114	N/A	N/A	N/A
E1	119	13	76	0.6:1	1	40.92[a]
E2	110	56	54	1.0:1	≥2	0.03
E3	116	31	85	0.4:1	≥2	25.14[a]
E4	124	56	68	0.8:1	1	1.16
E7	125	103	22	4.7:1	≥1	52.49[a]
E8	90	51	39	1.3:1	≥1	1.60
E9	104	62	42	1.5:1	≥1	3.85

[a] $Pr > \chi^2 < 0.05$

Table 4-S2. Hygromycin selection results of F_1 and BC_1F_1 progeny seedlings collected from maternal emasculated 'Xanthi' plants hand-crossed with experimental pollen donors. Individual transgenic F_1 progeny were backcrossed to emasculated 'Xanthi' to produce BC_1F_1 progeny.

Emasculated wild-type 'Xanthi' cross - F_1 progeny screen				
Pollen donor lines	Bioconfinement Efficiency (%)	Seeds Plated	Seeds germinated	Transgenic progeny
nontransgenic	100.00	1108	550	0
E1	100.00	1147	347	0
E2	100.00	1284	402	0
E3	100.00	1054	465	0
E4	99.85	1303	428	65
E7	99.99	1122	487	3
E8	99.99	1356	487	5
E9	99.99	1129	587	3

Emasculated wild-type 'Xanthi' cross - BC_1F_1 progeny screen				
Line	Bioconfinement Efficiency (%)	Seeds screened	Seeds germinated	Transgenic progeny
Wild-type	N/A	698	558	0
E4 - Plant 1	100.00	744	592	0
E4 - Plant 2	99.99	738	594	3
E4 - Plant 3	100.00	711	606	0
E4 - Plant 4	99.91	672	583	55
E4 - Plant 5	99.99	723	407	4
E8 - Plant 1	99.98	656	500	9
E8 - Plant 2	99.99	681	552	3
E8 - Plant 3	99.99	808	551	8
E8 - Plant 4	99.98	777	564	9
E8 - Plant 5	99.99	1004	662	5
E9 - Plant 1	99.99	789	619	2
E9 - Plant 2	100.00	774	570	0
E9 - Plant 3	100.00	569	496	0

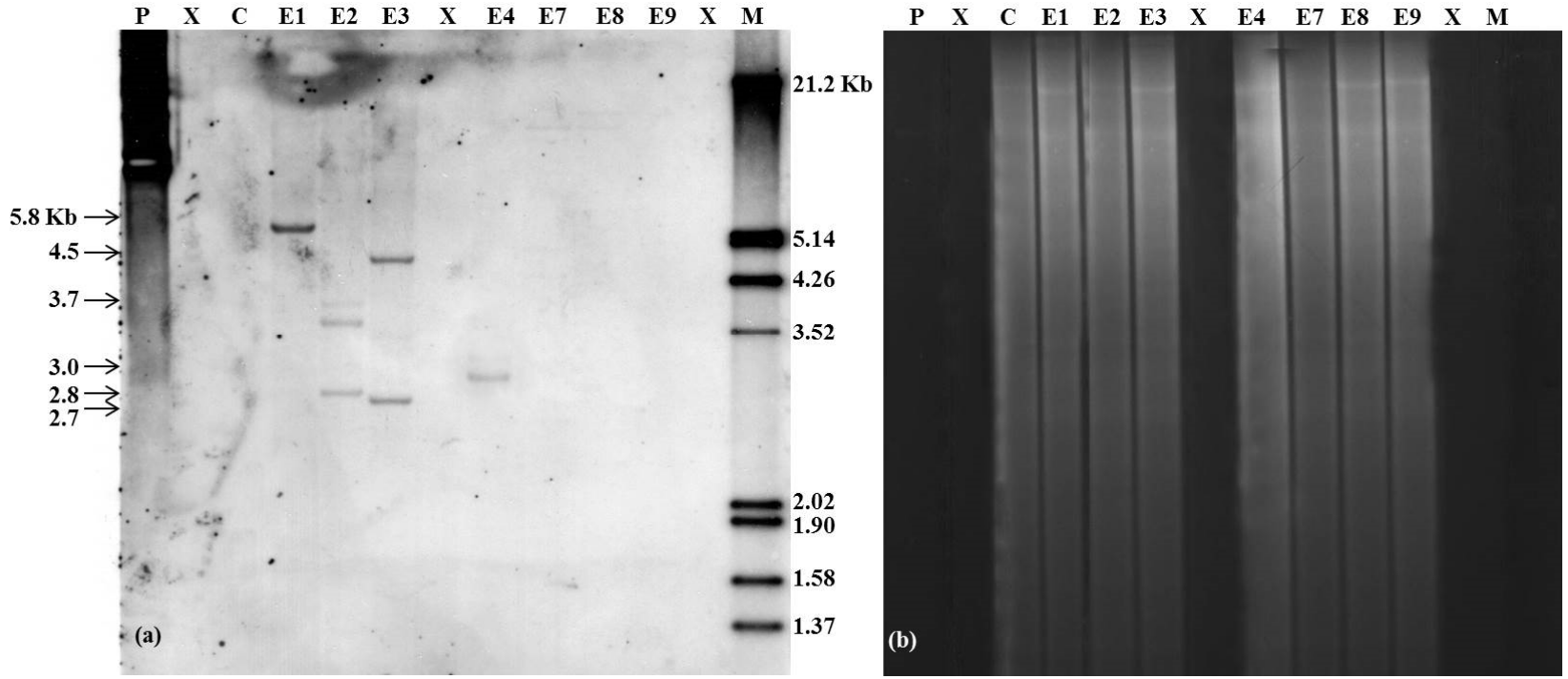


Figure 4-S1. Southern blot analysis of T_2 transgenic *Eco*RI tobacco plants. Panel 9a): Southern blot probed with *hygromycin phosphotransferase* (*hpt*) gene fragment. Lanes 'E1, E2, E3, E4, E7, E8, and E9' contain transgenic lines samples, lane 'C' is the nontransgenic control sample, lane 'P' contains *Hin*dIII digested pBC- Lat52-*Eco*RI plasmid DNA, lane 'M' contains DIG labeled Lambda DNA, and lanes labeled 'X' are deliberately empty lanes. Approximate band sizes are located on each side of the blot and image was adjusted for brightness and contrast to provide visual clarity. Panel (b): Agarose gel containing genomic DNA extracted from leaves of *Eco*RI lines and nontransgenic tobacco controls digested with *Hin*dIII.

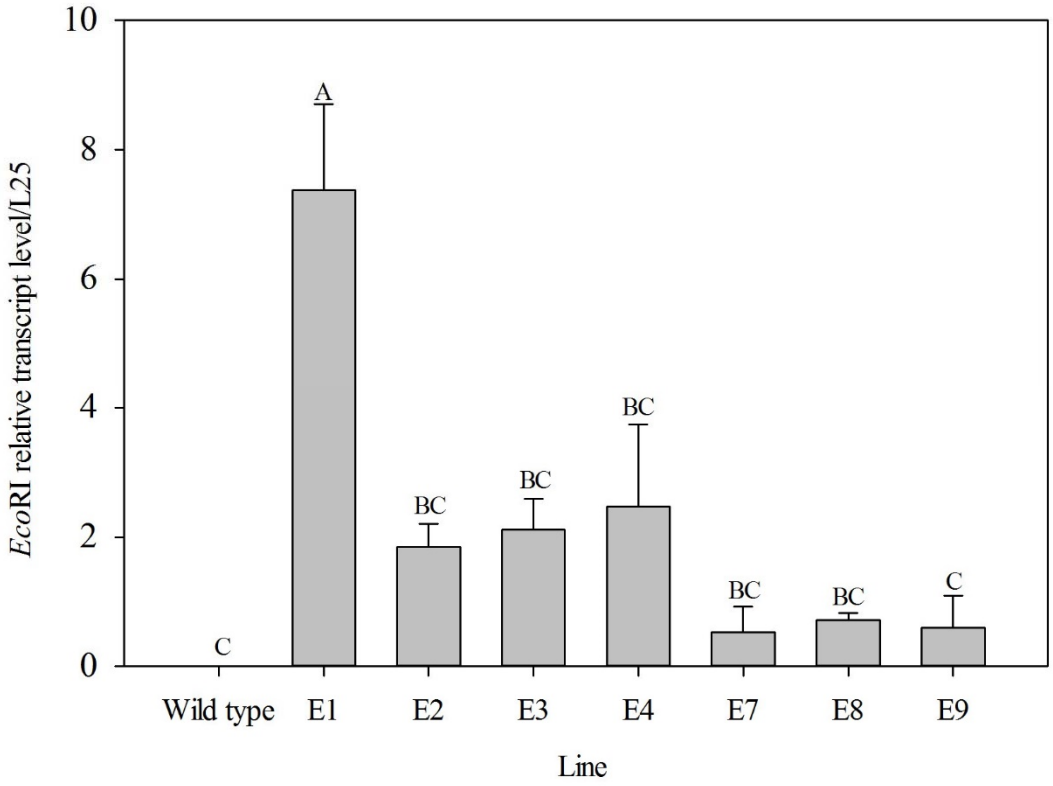


Figure 4-S2. Quantitative RT-PCR analysis of the *Eco*RI transcript of mature pollen in transgenic plants. Relative quantification of *Eco*RI transcript in plant tissues was performed using the standard curve method and each signal was normalized to transcript levels measured from the *L52* ribosomal gene from tobacco (GenBank L18908). Letters represent significant differences between means (Fisher's LSD, $P < 0.05$), and error bars represent ± standard error of the means.

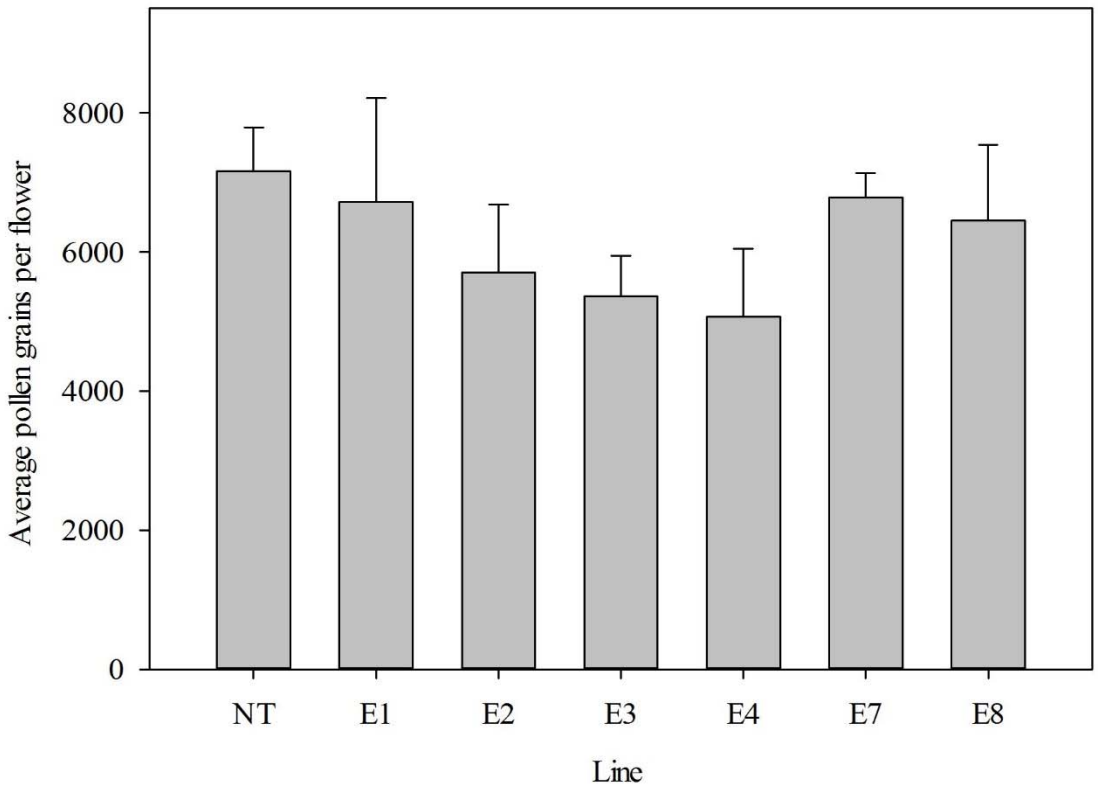


Figure 4-S3. Pollen grains produced per flower from *Eco*RI tobacco plants grown under greenhouse conditions. Pollen samples were collected from a newly-opened flower from one plant on four consecutive days. Pollen were suspended in water and counted using an automated Cellometer Vision® (Nexcelom Biosciences, LLC, Lawrence, Mass, USA).

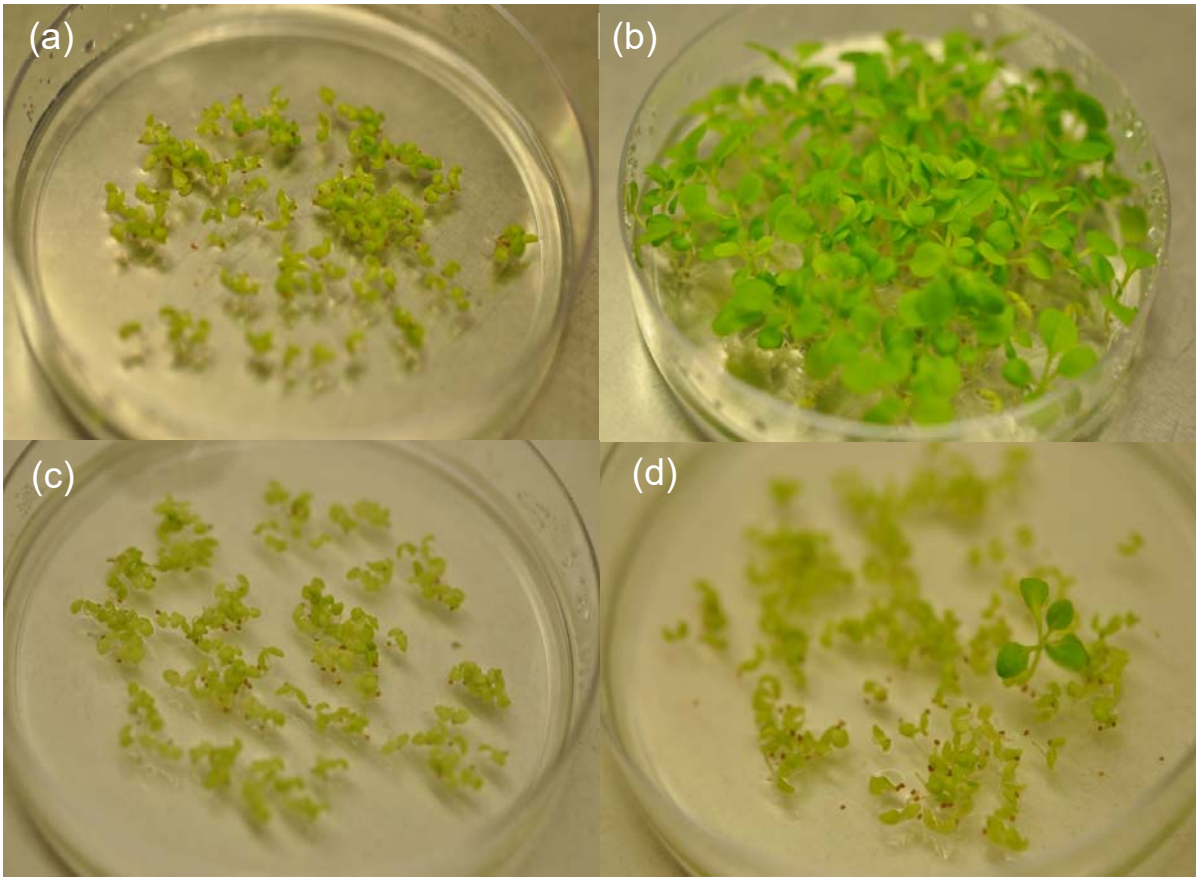


Figure 4-S4. Progeny screening method using MS medium supplemented with 50 $mg.L^{-1}$ hygromycin. F_1 seeds from crosses made between transgenic *Eco*RI *Nicotiana tabacum* tobacco lines and nontransgenic male sterile *N. tabacum* cv. 'MS TN90'. (a) Nontransgenic progeny control progeny. (b) Hygromycin-resistant transgenic tobacco without the *Eco*RI gene. (c) A subsample from screening progeny resulting from line E4 × 'MS TN90'. All seedlings shown were dead or dying. (d) A subsample from screening progeny resulting from line E9 × 'MS TN90' cross. A single survivor was present and shown, which was subsequently confirmed by PCR for the *Eco*RI gene.

Chapter 5: Conclusions

To meet US bioenergy goals, widespread plantings of switchgrass have been projected to occur within its native range within over next two decades. Indeed, numerous research institutions and companies are working to advance switchgrass cultivar development, via genetic engineering, to increase biomass yields and establish robust and cost-efficient biofuel production. GE switchgrass will undoubtedly be needed to achieve bioenergy yield and production targets. However, as reported in this dissertation, transgene flow was observed to occur frequently in relatively short distances (up to 100 m) from GE switchgrass field borders, and it is conceivable that pollen dispersal and pollination could occur at further distance on an agronomic scale. Up to this point, a limited amount of transgene flow has occurred with a few crops in the US, and has been accepted, to some degree, with little measurable negative impacts. However, this may not be the case with switchgrass. Currently, GE crops have mostly been engineered with transgenes that confer traits (e.g. herbicide, disease, and herbivory resistance) to protect against yield loss. Alternatively, GE switchgrass likely will contain transgenes that confer traits (as mentioned above) that do, indeed, boost biomass yield (as mentioned above) or provide tolerance to certain abiotic stress conditions (e.g. drought or salt tolerance) creating opportunities for switchgrass cultivation in new environments. Depending on selection, it is conceivable that these next-generation transgenes could lead to negative consequences if transgene flow occurs into wild switchgrass populations in unmanaged ecosystems. However, it should be noted that no evidence exists of such incidents of transgene flow in the scientific literature. Yet, it is likely that regulators, the public, and various stakeholders will have little tolerance for gene flow in switchgrass without additional risk assessment studies that garner long-distance (beyond 100 m) pollen dispersal and pollination data as well as careful evaluation of next-generation traits in unmanaged environments.

Although additional research is needed to evaluate environmental risks, genetically engineered cultivars with traits for "higher biomass and more fermentable sugars" have been classified as nonregulated via the BRS "Am I Regulated" process through the "Regulated Article Letters of Inquiry" (https://www.aphis.usda.gov/aphis/ourfocus/ biotechnology/am-i-regulated/ regulated_article_letters_of_inquiry/regulated_article_letters_of_ inquiry). The nonregulated classification of GE switchgrass occurred because current APHIS regulations, detailed in 7 CFR part 340 (https://www.aphis.usda.gov/ biotechnology/ downloads/7_cfr_340.pdf), are process-based, which includes any plant genetically engineered via a vector agent that is considered a plant pest or resulting GE plants containing plant pest DNA. Because the *Agrobacterium tumefaciens* (considered a plant pest by APHIS) transformation method is the mostly widely used to engineer GE plants, and many of the resulting plants contain regulatory sequences (e.g. promoters and terminators) from organisms considered plant pests, resulting plants are deemed regulated articles (with the exception of GE *Arabidopsis thaliana*) regardless of the biology of the plant, the trait conferred by the transgene, and the intended release environment. The GE switchgrass cultivars mentioned above do not fall under this regulatory definition, because these plants did not contain any plant pest DNA sequences and were engineered using a biolistic transformation method that did not employ a plant pest vector. Biolistic transformation was developed shortly after the implementation of APHIS regulations in 1986, and since all GE plants, to that point, were engineered via *Agrobacterium*-transformation, APHIS may not have envisioned regulating GE plants produced via other methods (see details in The National Academy of Sciences report *Genetically Engineered Crops: Experiences and Prospects* at https://www.nap.edu/catalog/23395/genetically-engineered-crops-experiences-and-prospects). Furthermore, APHIS currently has no authority to regulate novel traits alone, but generally

regulate GE plants based on their analysis of plant pest and noxious weed risks. Therefore, a determination was made that there was no evidence these GE switchgrass posed a risks as a plant pest, and APHIS was legally required to classify the plants as nonregulated.

APHIS has recently proposed a revision to regulations regarding GE plants. Among the proposed changes, is a shift in focus from the transformation method used and regulatory scrutiny of each transgenic event to one where plant pest risks are evaluated for unfamiliar transgenes and their conferred traits in combination with the biology of the plant to be engineered. These proposed changes, if implemented, would require GE plants, similar to the GE switchgrass mentioned above, to undergo regulatory review if transgenes/traits have not been reviewed previously. However, GE plants that have been previously deemed nonregulated through the "Am I regulated" process, will remain as such under the new regulations. The complete list of proposed rule changes can be found at https://www.aphis. usda.gov/brs/fedregister/BRS_20190606.pdf. Furthermore, it is the author's opinion that post-approval monitoring and management plans should be added to the proposed rule changes. These additions would aid in the discovery of any significant adverse environmental effects post-deregulation, and increase response time and remediation efficacy should that be necessary.

Lastly, switchgrass holds great potential as a lignocellulosic biofuel feedstock and as a next-generation GE crop. However, additional research is necessary to determine maximum effective pollination distances to aid regulatory environmental risk assessment and to determine minimum isolation distances for current and future regulated GE switchgrass studies. In addition, based on the results presented in this dissertation, it is clear the incorporation of bioconfinement strategies,

such as pollen-specific *Eco*RI selective male sterility, into GE crops where gene flow is of serious concern would provide an effective solution (> 99% bioconfinement efficiency) for limiting the exposure of novel transgenes in the environment. Going forward, large-scale field testing should be performed to evaluate the long-term efficacy of this bioconfinement strategy and its impact on regulatory, public, and various stakeholders' acceptance of GE switchgrass deployment as a biofuel crop in the US.

Vita

Reginald “Reggie” Jason Millwood was born in Shelby, NC on August 24, 1973. He grew up in the neighboring town of Forest City, NC where he graduated from East Rutherford High School in 1991. Reggie attended The University of North Carolina at Greensboro and graduated with a Bachelor of Arts degree in Biology in December 1998. In June 1998, Reggie began undergraduate research in Dr. Neal Stewart’s laboratory at the University of North Carolina at Greensboro and in January 1999 he began working there as a research technician. In May 2002, Reggie moved with Dr. Stewart’s laboratory to The University of Tennessee Knoxville where he continuing to work as a research associate in Dr. Stewart’s lab. In the fall of 2011, Reggie received a Masters in Science in plant molecular genetics while studying the consequences of gene flow from transgenic crops to their wild relatives. Soon thereafter, Reggie began his PhD work in the Department of Plant Sciences at The University of Tennessee Knoxville with a concentration in plant molecular genetics. His PhD project focused on pollen-mediated gene flow in the biofuel crop switchgrass and transgene bioconfinement strategies. Reggie and his wife, Meghan, and their three sons, Christian, William, and Reagan currently reside in Knoxville, TN.