Bioconfinement of a putatively sterile *Nicotiana* hybrid and development of tools for assessing gene flow

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Abstract

Production of transgenic crops in open field environments is an ongoing concern of due to the potential for gene flow. New transgenic crops, such as plant-made-pharmaceuticals may generate additional concerns about effects of adventitious transgenes. Use of a bioconfinement strategy may alleviate any consequences by preventing gene flow. The following chapters discuss previous and current research on gene flow, testing of a *Nicotiana* hybrid system for bioconfinement efficiency, and development of methods for transgene detection. The candidate 'platform plant' that was tested is a *Nicotiana* hybrid (*Nicotiana tabacum* 'TN 90' × *Nicotiana glauca*) previously identified to be sexually sterile. To quantify gene flow from hybrids, the *mGFP5ER* gene encoding for green fluorescent protein (GFP) was inserted into the paternal lines, which were crossed to form the hybrid. The DNA content and male fertility of these lines were used to characterize GFP-tagged hybrid lines. There were no differences in DNA content but significant differences in male fertility, in which pollen germination was observed at low rates. Two field gene flow studies revealed GFP-hybrids were not totally sterile since hybrids outcrossed and were pollinated by *N. tabacum* pollen, but they produced few viable seed. These results were confirmed with manual greenhouse crosses. Biomass studies revealed that the GFP-hybrids were comparable in productivity to an *N. tabacum* cultivar typically used in field production. An additional tagging strategy was created to produce the orange fluorescent protein (OFP), tdTomato-ER, in pollen by using pollen specific promoters in addition to the whole plant GFP cassettes. *N. tabacum* 'TN 90' and *N. glauca* were transformed, bred and hybridized to generate a hybrid that produced

pollen tagged with orange fluorescent protein. Manual crosses were performed in a greenhouse and partially similar results were obtained compared with previous GFP-tagged hybrid crosses. OFP-tagged hybrid outcrossing produced totally non-viable seed and when non-transgenic *N. tabacum* was supplied as a pollen donor to the OFP-tagged hybrids some non-tagged progeny were observed. The results suggest the *Nicotiana* hybrid could be a productive biomanufacturing platform and could provide total bioconfinement if grown in physical isolation from *N. tabacum* and *N. glauca.*

Table of Contents

1. Literature Review **1**
1.1 Introduction **1**
1.2 Gene Flow **1**
1.3 Fluorescent proteins to monitor gene flow **3**
1.4 Plant molecular farming **4**
1.5 Bioconfinement strategies **5**
1.6 Objectives **6**

2. Assessing the bioconfinement potential of a *Nicotiana* hybrid platform for use in plant molecular farming applications **8**
2.1 Abstract **8**
2.2 Background **10**
2.3 Methods **13**
2.3.1 Plants 13
2.3.2 Plant transformation 13
2.3.3 GFP *Nicotiana* hybrid production 15
2.3.4 Field outcrossing 16
2.3.5 Manual crosses 18
2.3.6 Aboveground biomass 19
2.3.7 Statistical analysis 20
2.4 Results **20**
2.4.1 Transformation and characterization of parental lines and hybrids 20
2.4.2 Field outcrossing experiments 21
2.4.3 Manual crosses 23
2.4.4 Above-ground biomass 24
2.5 Discussion **24**
2.6 Conclusions **28**

3. An orange fluorescent protein tagging system for real-time pollen tracking **29**
3.1 Abstract **29**
3.2 Background **31**
3.3 Methods **32**
3.3.1 Plant material 32
3.3.2 Transformation 33
3.3.3 Hybrid *Nicotiana* production 35
3.3.4 Fertility assessment in hybrids 37
3.3.5 Statistical analysis 37
3.4 Results and Discussion **37**
3.5 Conclusions **39**

References **40**

Appendices **47**

Vita **61**

List of Tables

Table 1. Plant genotypes used in the studies, including parentage, hybrid and transgene status. Not all genotypes were used in every experiment..... 48
Table 2. Summary of seed collection, germination, and analysis from hybrid GFP and MS TN 90 plant at the Kentucky and Tennessee field sites..... 49
Table 3. Results of crosses made in the greenhouse to examine the sexual compatibility of hybrid GFP plants and the fertility of their progeny 50
Table 4. Greenhouse crosses performed with hybrid OFP plants..... 52

List of Figures

Figure 1. Design of field gene flow experiment. A modified Nelder wheel design was used evaluate the gene flow of hybrid GFP plants. Three plant types were used in the experiment: male sterile *N. tabacum* 'MS TN 90' were pollen 'receptor' plants, hybrid GFP was fluorescently tagged to enable gene flow tracking, and *N. tabacum* type SN 2108 was used as a pollen donor to assure pollen flow was occurring in the field by seed set on MS TN 90 and to test for female sterility of the hybrid GFP plants. A center pollen source patch contained 50 alternating hybrid GFP and fertile SN 2108 plants, spaced approximately 1 m apart. Sixteen 1 m^2 blocks of male sterile MS TN 90 pollen receptor plants were placed at 9, 23, 38, and 54 m distances from the center and were used to detect pollen via seed formation. Each MS TN 90 plot was 22.5° relative to the adjacent plot as viewed from the center. A honeybee hive was placed at the center of the field site to vector pollen. 53

Figure 2. (A) DNA content and (B) pollen germination analysis. DNA content was estimated by flow cytometry and pollen viability was estimated by pollen germination. Hybrid GFP-plant lines: H1. H2, H3, H4, and H5 and non-transgenic parent lines NT-glauca (NG) and NT-TN 90 (NT) n=5. Mean separation is by Fishers LSD and bars marked by the same letter are not significantly different ($p < 0.05$). Error bars are the standard error of the means..... 54

Figure 3. Fresh aboveground biomass productivity. **(A)**Productivity was measured for nontransgenic hybrid plants and nontransgenic TN 90 plants in a field experiment over two years with three measurements per year. **(B)**A greenhouse study was conducted during one year with two measurements of the hybrid GFP line, the transgenic parental lines of the aforementioned hybrid, and a nontransgenic hybrid line. Mean separation is by Fishers LSD and bars marked by the same letter are not significantly different ($p < 0.05$). Error bars are the standard error of the means. .. 55

Figure 4. Cassettes used in transformation. Cassettes TD-GFP- H and TD-GFP-K were used in transformation of *N. glauca* and *N. tabacum* 'TN 90' with *A. tumefaciens* strain EHA105. Vectors are identical except for the *hpt* gene present in TD-GFP-H and the *npt II* gene present in TD-GFP-K**.** 56

Figure 5. PCR analysis of T0 TN 90 plants transformed with TD-GFP-H. Lane 1: DNA marker, Lane 2: purified mGFP5ER plasmid as a positive control. Lanes 3-11: genomic DNA from putative transgenic TN 90. The 743 bp band present in lanes 4 through 11 confirmed transgene presence in plants. All other transgenic events showed similar results..... 57

Figure 6. Average relative fluorescence intensity for TN 90 TD-GFP-H T_1 lines. Leaf tissue from a non-transgenic TN 90 line and ten transgenic T_1 lines were excited at 395 nm and measured at an emission of 509 nm with a spectrofluorometer. Fluorescence intensity values were normalized to an average measurement of TN 90 negative control plants outside the spectral range of GFP emission. For TN 90 n=2, TD-GFP-H lines1-8, and 10 n=34, line 9 n=33. Error bars represent the standard

error from the mean and different letters indicate significant differences at P < 0.05. 58

Figure 7. Average relative fluorescence intensity for *N. glauca* TD-GFP-K T_1 lines. Leaf tissue from a non-transgenic *N. glauca* line and eight transgenic T_1 lines were excited at 395 nm and measured at an emission of 509 nm with a spectrofluorometer. Fluoresence intensity values were normalized to an average measurement of *N. glauca* negative control plants outside the spectral range of GFP emission. For *N. glauca* n=2, TD-GFP-K lines 1 n=26, line 2 n=34, line 3 n=31, line 4 n=16, lines 5 and 6 n=33, line 7 n=28, and line 8 n=24. Error bars represent the standard error from the mean and different letters indicate significant differences at P < 0.05. 59

Figure 8. Fluorescent protein visualization in pollen. Orange fluorescent protein-tagged pollen as viewed under an epifluorescent microscope at 100×. *N. glauca* and TN 90 plants have been bred for homozygosity of tdTomato-ER, resulting in hybrid OFP plants. All white light images were captured at 80 ms exposure time. Panels (B) and (D) were captured at 50 ms exposure time. Panels (F) and (H) were captured at 80 ms exposure time. Panel (J) was captured at 180 ms exposure time. 60

1. Literature Review

1.1 Introduction

The global demand for food production as well as the necessity for pest control solutions has driven an unprecedented adoption of new technologies by the agricultural sector. Currently, transgenic crops cover 107.3 million hectares, up 6% from 2011; developing nations have surpassed industrial countries in total biotech crop acreage (52%), but the lack of scientific and cost-efficient regulatory systems are still a major barrier to acceptance [1]. As each alternative transgenic crop is planted into the agricultural ecosystem, dispersal of transgenes into new environments is an ongoing and increasing concern. Future transgenic traits and market demands may transform the agricultural landscape by the production of non-food or feed commodities in plants by transgene expression technologies, such as plant-made-pharmaceuticals (PMPs) or improved cellulosic biomass. Each of these traits creates a new dynamic for the possible effects of gene flow, and raises questions of how transgene dispersal will be monitored, what possible consequences may arise, and how transgene flow will be mitigated.

1.2 Gene Flow

An issue that has remained at the forefront of controversy over transgenic crops is crop-to-crop and crop-to-weed transfer of transgenes. Open movement of deregulated transgenes (i.e. encoding herbicide resistance or insect toxin production) has generated

widespread criticism for the unrestricted introduction of non-native genes to wild relatives and open pollinated crops. This has been furthered by use of the term 'contamination' to describe gene flow from transgenic to non-transgenic fields [2] and 'superweed' to describe a scenario where viral or herbicide resistance genes could be transferred from a plant to wild relatives [3]. The latter term 'superweeds' has confusingly also been used to describe naturally occurring herbicide resistant weeds arising from herbicide overuse. Future development of transgenics destined for field production are likely to face opposition due to their potential for unwanted gene flow.

Gene flow can be described as movement of genes, through pollen, seed, and vegetative structures from one plant or population of plants to another [4]. This is in contrast to introgression, where genes are stably incorporated into a population from another [5]. Both gene flow and introgression play a major role in the evolution of all plant species. Two types of gene flow exist; horizontal gene transfer is where genes are transferred between unrelated species, and vertical gene flow where genes are transferred between more closely related species [6]. In terms of gene flow in plants, vertical gene flow is much more prevalent, where horizontal gene transfer is rare [5].

Pollen-mediated transgene flow is the most notable type of gene flow in plants. Pollen is produced in high numbers by plants and is spread by wind and many pollinators, making this method of gene flow very prolific. Transgene flow via pollen has been documented in many species in agronomic systems, including rice, wheat, oilseed rape, and sugarbeet,

[7-10]. Crop to wild relative gene flow has also been documented to occur in many species (reviewed in [11]). Future transgenic cultivation of currently unmodified species may warrant use of bioconfinement strategies [12] and new transgenic technologies, such as PMPs, may require additional biosafety measures [13].

1.3 Fluorescent proteins to monitor gene flow

Fluorescent proteins have proven to be extremely useful tools in biotechnology. Multiple fluorescent proteins have been identified that can be adapted for a wide range of experimental functions [14, 15]. The green fluorescent protein is one such protein that has a proven track record in biotechnological research. The GFP protein was first isolated from a jellyfish, *Aequoria victoria*, to determine the properties of its bioluminescence in 1962 [16]. It was subsequently cloned [17] and adapted for use in biotechnology. GFP has become widely used in research, as an *in vivo* marker for multiple applications including gene flow monitoring [18-20]. Whole plant tagging by GFP is an effective method to monitor gene flow because GFP fluoresces green (507 nm) in the presence of UV or blue light, while non-tagged plants autofluoresce red under UV light [21]. GFP-tagged plants also do not show any measurable reduction in fitness [22]. Spectrofluorometry can also be used to quantify fluorescence that can distinguish between hemi- and homozygous GFP-tagged plants [23, 24]. In addition to whole plant tagging, pollen tagging with GFP is also possible [25, 26] and has been shown to not affect pollen fitness [27]. This tagging system also serves as a useful method in assessing

zygosity in plants. New fluorescent proteins may serve as additions to these systems [20] and enable dual FP tagging of one plant.

1.4 Plant molecular farming

The first transgenic plant, created by expressing a bacterial gene in a tobacco, was reported in 1983 [28]. By 1986 the first plant-made-pharmaceutical, tobacco and sunflower cells producing human growth hormone, had been accomplished and reported [29]. Progress toward plant molecular product development has been made in plant-derived antibodies, human and animal vaccines, pharmaceutical proteins, and industrial enzymes [30]. Multiple expression systems with applicability to PMPs exist, including cell culture, whole plant production, secretion from roots or leaves, chloroplast expression, viral infection, transient *Agrobacterium*-mediated expression, and seed expression [31, 32]. The choice of production platform is dependent upon expression system and downstream processing requirements and can be a leafy, cereal, or fruit crop [33]. These advancements in expression and with refining technology have allowed for the further development of plant-made-pharmaceuticals from concept to product.

The first human trials for a plant produced vaccine occurred in 1997 [34]. In 2006, the first approval for a PMP was given to a poultry vaccine produced in tobacco cells [35]. Despite many attempts of PMP development intended for human use, only one currently has approval [36]. An HIV prophylactic has already entered human clinical trials and rice has recently been shown to be an effective production platform for human serum

albumin [37, 38], and more commercialization of PMPs as human therapeutics could happen soon. The potential advantages of PMP production are somewhat overshadowed by a lack of regulatory framework and establishment of best practices for full scale production, as well as reservation for public adoption in some developed nations [39, 40]. Concerns have been raised about using food crops for plant pharmaceutical production due to potential effects of gene flow and admixtures into the food supply [14].

1.5 Bioconfinement strategies

There are a plethora of strategies that may serve to prevent transgene flow and ameliorate concerns over the introduction of transgenic crops. Currently, many PMP plants in development are grown in greenhouses or laboratories, a form of physical confinement. To produce plants on a large and economical scale, field production is desired. Manual removal of flowers or harvesting prior to flowering can be used effectively but this is laborious and subject to crop uniformity. Spatial confinement is currently used for many transgenic field trials by eliminating any sexually compatible species within a range of the field site [41]. Transient expression systems also provide bioconfinement by temporary transgene expression. Many biological confinement systems have been developed that give the plant a built-in bioconfinement mechanism. Chloroplast based expression systems eliminate the transgenes from pollen, thus limiting gene flow to seed movement or vegetative structures only [42]. Cytoplasmic male sterile lines often used for hybrid seed production can be used to eliminate pollen all together. Male sterility has also been engineered via expression of chimeric ribonuclease genes (Barnase) to

eliminate pollen formation and the use of a Barnase inhibitor (Barstar) can re-induce fertility [43]. Gene use restriction technologies eliminate transgene flow by generating non-viable progeny or using an activator to control the transgene expression [44]. Transgene excision through site specific recombination recognizes and removes the transgene in the pollen, resulting in transgene free pollen [45]. A strategy using the sterility of an interspecific hybrid for bioconfinement is described in the following text.

1.6 Objectives

The first objective was to produce a Nicotiana hybrid (*Nicotiana tabacum* × *Nicotiana glauca*) that was tagged with fluorescent proteins for the purpose of gene flow monitoring. The second objective is built upon a previous body of research determining zygosity and gene flow using fluorescent proteins [23, 26, 46-49]: to determine bioconfinement by measuring gene flow in two field experiments and with greenhouse crosses. The questions addressed are: 1) Do hybrids express fluorescent proteins needed to quantify gene flow? 2) Are transgenes effectively confined by the hybrid? In addition to the following main objectives, studies on DNA content and male fertility of the hybrids, hybrid pollen tube growth, and above-ground biomass were also performed.

Objective 1: Transformation of *N. tabacum* cv. TN90 and *N. glauca* to express fluorescent marker genes and production of an interspecifc hybrid with fluorescent marker genes.

Hypothesis. *Transformation and breeding will produce a hybrid that functionally expresses fluorescent marker genes.*

Objective 2: Gene flow experiments to evaluate outcrossing and selfing capabilities of hybrid *Nicotiana* plants.

Hypotheses, *1. In field gene flow experiments, pollen flow from the center plot to the peripheral plots will be verified by seed set on MS TN90 plants.*

2. Transgenic hybrids will not produce seeds or cross-pollinate with male sterile TN 90 plants to produce transgenic seeds in greenhouse of field gene flow studies.

2. Assessing the bioconfinement potential of a *Nicotiana* hybrid platform for use in plant molecular farming applications[1]

2.1 Abstract

Background

The introduction of pharmaceutical traits in tobacco for commercial production could benefit from the utilization of a transgene bioconfinement system. It has been observed that interspecific F_1 *Nicotiana* hybrids (*Nicotiana tabacum* × *Nicotiana glauca*) are sterile and thus proposed that hybrids could be a morphologically unique and suitable bioconfined hosts for biomanufacturing. We developed genetically tagged hybrids with green fluorescent protein (GFP), which was used as a visual marker to enable gene flow tracking and quantification for field and greenhouse studies. GFP was used as a useful proxy for pharmaceutical transgenes. The hybrids were additionally characterized by measuring DNA content, pollen germination, and above-ground biomass.

[1] A paper with this title was submitted to BMC Biotechnology. Contributions were made as follows: **J. Hollis Rice**: Performed all plant transformation experiments, carried out the field experiment in Tennessee, performed pollen germination experiments, DNA content analysis, greenhouse biomass studies, and drafted the document: **Richard E. Mundell**: Conceived portions of the study, assisted in the field design, bred the original interspecific hybrid, produced the experimental hybrids, performed manual greenhouse crosses, and carried out biomass and gene-flow field experiments in Kentucky. **Reginald J. Millwood**: Coordinated the study and assisted with analysis. **Orlando D. Chambers**: Conceived portions of the study and assisted with coordination and execution of the study. **C. Neal Stewart Jr.**: Conceived portions of the study, coordinated the study and assisted with revisions **H. Maelor Davies**: Conceived portions of the study, coordinated the study and provided critical review. All authors contributed to and approved the final text.

Results

Analysis of DNA content revealed significant differences in the hybrid relative to the cultivated *N. tabacum*. Hybrid pollen was capable of germination *in vitro*, albeit with a very low frequency and with significant differences between plants. In two field experiments, one each in Tennessee and Kentucky, we detected outcrossing at only one location (Tennessee) at 1.4 % from all progeny screened. Additionally, from 50 hybrid plants at each field site, formation of 16 and 84 seed was observed, respectively. Similar conclusions about hybrid fertility were drawn from greenhouse crosses. In terms of above-ground biomass, the hybrid yield was not significantly different to that of *N. tabacum* in the field.

Conclusion

N. tabacum × *N. glauca* hybrids show potential to contribute to a bioconfinement and biomanufacturing host system. Hybrids exhibit exceptionally low fertility without sacrifice of green biomass yields relative to *N. tabacum.* In addition, hybrids are morphologically distinguishable from tobacco allowing for identity preservation. This hybrid system for biomanufacturing would optimally be used where *N. glauca* is not present and in physical isolation of *N. tabacum* production to provide total bioconfinement.

2.2 Background

Development of plants as biofactories has progressed since the advent of biotechnology and has rendered the concept of plant molecular farming into existence. Transgene-expression technologies that enable plants to produce large quantities of non-native proteins have useful properties in industrial or pharmaceutical applications, such as production of antibodies, vaccines, and enzymes [38, 50]. These technologies have formed the basis of several prospective commercial strategies for biomanufacturing these materials, with advantages of superior economics and ease of scale-up relative to the commonly used microbial and mammalian cell-based fermentation systems [39, 51]. Recently the first official clinical-use approval was made for transgenic carrot (*Daucus carota* subsp. *sativus*) cells expressing a human gene for treatment of Gaucher's disease [36]. This development could be a stepping-stone to using field-grown plants for protein biomanufacturing. Open-field production renders low facilities costs and high scalability, but raises questions of field-based risks. The choice of production platforms (e.g. plant species) is a crucial decision; use of major commodities for plant-made pharmaceuticals (PMP) could warrant extra precaution [14]. Fortunately, there are alternative, non-food species in which the gene-expression technologies are effective. Tobacco (*Nicotiana tabacum*) has been extensively explored as a PMP host [36, 52]. However, the possibility of transgene flow to commercial tobacco is a concern. Tobacco is primarily self pollinated, but can outcross as well (typically less than 5%; [53]). As transgenic varieties of tobacco are not used in any traditional tobacco production, accidental co-mingling of seed or genetic outcrossing, could cause regulatory, legal, or possibly health issues.

There have been several documented examples of unintended gene flow from transgenic plants. In creeping bentgrass (*Agrostis stolonifera* L.), for example, transgene escape to conspecific hosts via pollen and seed can occur over tens of kilometers [54]. ProdiGene, Inc., a former PMP company, was fined by regulatory authorities and was compelled to conduct an expensive clean-up effort for maize PMP volunteers that were detected in a former field site intermingled with soybeans [55]. Such incidents must be avoided if PMPs are to become a commercial reality. There are many possible solutions to the challenge of providing adequate transgene confinement. [41]. Harvesting prior to flowering, or manual flower removal might seem obvious and attractive solutions, as this would simultaneously obviate gene flow to other plants via outcrossing and seed formation from the transgenic crop itself. However, the challenge of preventing any flowers from forming in large production acreage necessitates a more reliable system, e.g., bioconfinement.

In this regard, cultivated tobacco and other species in the *Nicotiana* genus provide some potentially useful attributes. Many uncultivated *Nicotiana* species, such as *N. glauca* used in this study, produce biomass yields required for economical leaf-based biomanufacturing. Morphologically, F_1 hybrids between *N. tabacum* and *N. glauca* are readily distinguishable from cultivated tobacco, have high biomass, and have been reported to be sexually sterile [56-59]. These properties lead us to consider interspecific *Nicotiana* hybrids as a potential PMP production platform. PMP constructs could be introduced into the *N. tabacum* and *N. glauca* parents using existing methods. Hybrid seed for field production would then be generated by hybridizing *N. tabacum* with *N.*

glauca, an efficient process, as seed yields per plant are very high in these species. Use of these hybrids would provide bioconfinement attributes of (1) production of little or no viable pollen that could transfer transgenes to tobacco fields, and (2) production of little or no viable seed from the PMP 'production vehicle' owing to the lack of viable pollen.

However, the constraints and limitations of F_1 sterility and production of this system need further research using transgenes to place bioconfinement in the context of biosafety regulations in relevant field settings. A complicating factor is that no regulatory agency has declared specifications for any commercial crop/transgene with regards to field-level gene flow, although thresholds for presence of transgenic material in conventional food or feed exist. That said, we assume that bioconfinement should be very high to be effective. Therefore, we set out to assay interspecific hybrid plant pollination of hybrid to tobacco as well as the reciprocal cross. To create a suitable *N. tabacum* ♀ × *N. glauca* ♂ hybrid line for these experiments, we transformed both parents with a green fluorescent protein (GFP) gene as a proxy for a PMP, providing a convenient way of monitoring transgene flow. DNA content and pollen germination were measured to assess possible differences among multiple hybrid lines and parents. The fertility of the hybrid was characterized by a field gene flow study and by manual crosses in a greenhouse. The productivity of the hybrid, in terms of aboveground green biomass was also determined.

2.3 Methods

2.3.1 Plants

A summary of plant genotypes used in our studies is listed in Table 1. *N. glauca* was obtained from the US National Plant Germplasm System (NPGS) (plant introduction 307908, accession TW55 from Peru). The following *N. tabacum* lines were obtained from the Kentucky Tobacco Seed Improvement Association, Inc. in Lexington, KY, USA (38°8'N, 84°29'W): *N. tabacum* 'TN 90' was from foundation seed lot # 86-02-K-4A. *N. tabacum* 'MS TN 90' is a male sterile variety of TN 90 from seed lot # 86-03-KLC-15. *N. tabacum* 'SN 2108' is a "dark type" tobacco that is morphologically distinct from the TN 90 cultivar was from seed lot -KT D4LC. Several F_1 hybrids were used in our studies. The term 'hybrid GFP' is used to denote those F_1s containing a green fluorescent protein (GFP) marker gene. The F_1 amphihaploid hybrids we produced were the product of unidirectional pollination of *N. tabacum* with *N. glauca* pollen.

2.3.2 Plant transformation

N. tabacum 'TN 90' and *N. glauca* were transformed with the vector, pBIN mGFP5-ER, which contains the *mGFP5-ER* gene under the control of the constitutive *CaMV 35S* promoter and an *nptII* kanamycin resistance gene [60]. *mGFP5-ER*, a GFP variant, emits green light (λ_{max} = 509 nm) when excited by wavelengths of either ultraviolet (UV) (395 nm) or blue (465 nm) light, which is bright enough to be readily observed under UV excitation and quantifiable with appropriate instrumentation [24, 61].

Plants were transformed via *Agrobacterium tumefaciens* mediated transformation [62]. Leaf explants were prepared by sterilizing young leaves with a mixture of 10% commercial bleach and 0.01% Tween 20, washed with sterile water, then excised into 6 mm^2 segments. Explants were allowed to soak in a suspension of liquid MS salts containing B_5 vitamin (DBI medium) and *A. tumefaciens* strain GV3850 harboring the constructs of interest for 30 minutes. Transformed explants were allowed to co-cultivate on solid DBI medium for 48 h prior to transfer to DBI containing Timentin® (400 mg/L) and kanamycin (200 mg/L). Shoots arising from callus were transferred to MS medium containing kanamycin (200 mg/L) for root development [63]. Shoot cultures were grown at 24° C under 16/8 h light/dark periods until rooting occurred. Shoots were then transferred to potting media and acclimated for two weeks. GFP-expressing plants were selected visually with a hand-held longwave UV light (UVP model B-100AP 100 W:365 nm) as previously described by Millwood et al. [24]. For further confirmation of the presence of *mGFP5-ER*, genomic DNA was extracted [64] from leaf tissue, and PCR was performed according to Hudson et al. [25]. Confirmed T_0 plants were then transferred into 4 L pots and grown to maturity in a greenhouse under 16/8 h light/dark periods and corresponding 27°/20° C thermoperiods. Upon flowering, plants were individually bagged with breathable mesh pollination bags (DelStar Technologies, Inc., Middleton, DE, USA) and manually shaken to promote pollination. T_1 seed were collected at maturity and this process was repeated to obtain T_2 generation seeds.

2.3.3 GFP *Nicotiana* hybrid production

Our goal was to produce a *Nicotiana* hybrid with sufficient copies of *mGFP5-ER* for tracking purposes, whereby a copy of the *mGFP5-ER* transgene should be present in the genome of each pollen grain. Transformed parent lines were bred to homozygosity for *mGFP5-ER* to the T_2 generation. Plants were screened for GFP using a handheld UV light to select the brightest GFP-expressing seedlings. GFP expression was then measured by a spectrofluorometer (Fluorolog®-3 HORIBA Jobin Yvon, Edison, NJ, USA)[23, 24] and analyzed with software (FluorEssence™ Version 2.5.2.0.HORIBA Jobin Yvon, Edison, NJ, USA) to measure GFP fluorescence. Two strategies were employed to assure homozygosity of each T_2 line. First, lines were germinated in two flats each and screened with the handheld UV light to determine zygosity of each T_2 line (using ratios of GFP to non-GFP plants) and inheritance of antibiotic resistance traits among T_2 lines germination on MS medium containing kanamycin (200 mg/L). Selected T_2 homozygous lines were designated as "TN 90 GFP" or "glauca GFP" and grown to maturity. These plants were then crossed (TN 90 GFP ♀ × glauca GFP ♂), to produce the putatively sterile hybrid designated as "hybrid GFP" (see Table 1),

2.3.3.1 Estimation of nuclear DNA content

N. tabacum has twice as many chromosomes as *N. glauca* (2n=48 vs 2n=24), resulting in low likelihood of meiotic chromosome pairing in the F_1 interspecific hybrid. The absolute DNA content of five hybrid GFP lines and each non-transgenic parental line was estimated by flow cytometry with five replicates each. Plant tissue samples were processed as previously described by Galbraith [65] and analyzed using an Accuri C6

flow cytometer (Accuri Cytometers, Ann Arbor, MI, USA). The known genome size of *Solanum lycopersicum* 'Roma,' 2C= 1.96 pg [66] was used as an internal standard for estimating DNA content in *N. tabacum* (2C = 9.67), *N. glauca* (2C = 6.91), and the *Nicotiana* hybrid [67].

2.3.3.2 Pollen germination

To determine male fertility pollen germination rates of five hybrid GFP lines, wild-type *N. tabacum* (NT-TN 90), and wild-type *N. glauca* (NT-glauca) were compared with five replicates of each. Pollen grains were collected and germinated as previously described [27, 68]. Pollen was placed on a microscope slide for observation with an Olympus BX 51 microscope (Olympus Corporation, Shinjuku, Tokyo, Japan) at 100x magnification. A randomly sampled field of view was captured by a digital camera (Olympus Q Color 3) and imaging system. Between 722 and 993 pollen grains were counted for each hybrid line, 1678 grains were counted for non-transgenic (NT)-TN 90 and 1262 grains were counted for NT-glauca. Germination percentage was calculated by dividing the number of germinated pollen grains by the total number of observed pollen grains.

2.3.4 Field outcrossing

Natural outcrossing rates of fluorescently tagged hybrids were estimated in two field experiments conducted at Versailles, Kentucky, USA (38.075784, -84.740575) and Knoxville, Tennessee, USA (35.891769, -83.959786). A modified Nelder wheel design (Figure 1) [69] covered approximately 0.931 hectares and contained three plant types. GFP-tagged hybrids and non-transgenic SN 2108 plants were used as pollen donors in the

center of the plot, and located along the spokes of the Nelder wheel MS TN 90 plants were used as pollen recipients (Figure 1). The pollen source plot measured approximately 15 m in diameter and contained 3 concentric circles consisting of 50 plants each of alternating hybrid GFP and SN 2108 spaced approximately 1 m apart; a honeybee hive was located at the center of the experiment. Surrounding this central plot were sixteen blocks of five MS TN 90 plants, each used to detect outcrossing at 9, 23, 38, and 54 m from the center plot in each cardinal direction. Each block of MS TN 90 plants were 22.5° relative to the adjacent block with respect to the center plot in contrast to Nelder's design where the outer plots are arranged in a linear fashion. This modification was made to take advantage of honeybee flight patterns [70], for flight to and from the center patch. Blocks of MS TN 90 plants and hybrid GFP flowers were monitored throughout the season for formation of pods, which were promptly collected at maturity. SN 2108 plants were used solely as a pollen source and seed set was not of interest because of high self-fertilization rates.

Seed pods were collected at maturity continuously throughout the growing season from both MS TN 90 and hybrid GFP plants and subsequently dried. In Kentucky seed pods were dried after being placed into coin envelopes which were placed into a wooden container with a perforated bottom and shelves. Room temperature air was continuously blown into the bottom of the container until pods were dry. In Tennessee, seeds pods were placed into envelopes and stored in a drying oven at 28 °C for 48 hr. After drying, were germinated on filter paper moistened with 0.2% KNO_3 at alternating 25°C 16 h light: 20°C 8 h dark in accordance with the International Seed Testing Association

standards [71]. After three weeks 100 μM GA 4+7 was applied to germinate any remaining seed. Seedlings were screened with a handheld UV light to detect GFP-expressing progeny, which were transplanted into potting media and analyzed with a spectrofluorometer as previously described.

2.3.4.1 Pollen tube growth

There could be competition between hybrid GFP and SN 2108 pollen for pollination of MS TN 90 plants in the field. Therefore, pollen tube growth rates were compared. Two plants per line were used, with three replicates per plant measured for 3 d. Pollen was germinated as described above, except 15 μl of BK medium and pollen was taken every 15 min for 5 h to perform a time-series analysis. Micrographs of pollen tubes were captured and tube lengths were measured against a gridline on a Hausser Scientific brightline hemacytometer (Horsham, PA, USA). A total of 8750 pollen grains were observed from SN 2108 plants and 7,524 grains were observed from hybrid GFP.

2.3.5 Manual crosses

Manual crosses were conducted in a greenhouse in Lexington, KY, and were performed to mirror the possible crosses expected in the field experiment. To determine the outcrossing capability of the hybrid, hybrid GFP plants were crossed with MS TN 90. To evaluate the seed setting capacity of the hybrid, SN 2108, the pollen source plant type used in the field experiment were crossed to hybrid GFP plants. Hybrid GFP plants were also intercrossed to determine any transgene event reproduction variability. In addition, both fertile plants, SN 2108 and MS TN 90, were crossed as controls. Pollen-recipient

flowers were emasculated prior to crossing. A total of 96 crosses were performed for hybrid GFP ♀ × SN 2108 ♂ crosses and 95 crosses were performed for MS TN 90 ♀ × hybrid GFP ♂ crosses. Ten crosses were performed between a pair of MS TN 90 ♀ × SN 2108 ♂ plants and also for the pair of crossed hybrid GFP plants. Seeds derived from crosses containing GFP-tagged plants were germinated, transplanted to potting mix, and analyzed with a spectrofluorometer as previously described.

2.3.6 Aboveground biomass

2.3.6.1 Field study

Vegetative biomass of NT-TN 90 and NT-hybrid plants were measured. The study employed a complete randomized block design with three replications. Seeds were germinated in float trays and transplanted at a density of approximately 12,000 plants/hectare. Plots consisted of four 6 m rows containing 80 plants. Drip irrigation was employed for supplemental watering. Harvesting occurred during the budding stage by trimming rows to 4.6 m for standardization and measuring all green biomass above the 30 cm mark above the soil line. Subsequent harvests occurred every 28 to 35 days for a total of three harvests and the study was repeated in two different years.

2.3.6.2 Greenhouse study

To determine the productivity of hybrid GFP relative to the parent lines NT-hybrids, five replicate plants from hybrid GFP, glauca GFP, TN 90 GFP, and NT-hybrid lines were grown. A completely randomized design was used to determine fresh biomass productivity. All plants were germinated in 4 L pots in a greenhouse and transplanted to

12 L pots. Plants were grown under a 16/8 h light/dark periods and corresponding 27°/20° C thermoperiods and spaced at 1 m centers. Plant productivity was analyzed by measuring fresh weight harvested at the budding stage 30 cm above the soil line. Plants were allowed to re-grow to the budding stage for two measurements.

2.3.7 Statistical analysis

All analysis of variance (ANOVA), regression, and chi-squared tests were performed using SAS (Version 9.3 SAS Institute Inc, Cary, NC, USA) with a significance level of $p < 0.05$. The MIXED procedure was used for all ANOVA calculations. The least significant difference was used for mean separations if ANOVA results were found to be statistically significant. Log and rank transformations and were used when data did not meet the assumptions of a normal distribution by the Shapiro-Wilk test [72] or equal variance by the Levene test [73].

2.4 Results

2.4.1 Transformation and characterization of parental lines and hybrids

Multiple transgenic events from *N. tabacum* 'TN 90' and *N. glauca* were obtained. T_1 plants that highly expressed GFP were selected and self-pollinated. Homozygosity of T_2 lines was confirmed by progeny analysis by antibiotic screening on media and GFP expression. The homozygous T_2 *N. tabacum* and *N. glauca* lines were crossed to produce hybrid GFP lines, where GFP was visible in the stems and leaves of the plants.

To assess the effectiveness of the sterile hybrid system as a bioconfinement platform, it was important to characterize hybrid-line variation in DNA content and male fertility. The absolute DNA content of the five hybrid GFP lines did not differ significantly from each other or the paternal line *N. glauca*, but differed from the maternal line *N. tabacum* ($p = 0.010$)(Figure 2A). Although pollen germination differed across hybrid and parent lines ($p < 0.001$), only one hybrid line differed from the other four with a higher percentage of germination (Figure 2B). Regression analysis revealed no association between pollen germination and DNA content.

2.4.2 Field outcrossing experiments

Preliminary hand-crosses resulted in very low fertility of the interspecific hybrid plants, which prompted us to assay natural outcrossing in the field. We observed no aberrations of pod formation and seed-set on the MS TN 90 plants in each field experiment. No GFP-positive seedlings were found after germination of 7,340 MS TN 90 seeds from the KY field site, however, one single GFP positive seedling (collected from a distance of 9 m from the field center) was found from 74 germinated seeds from the TN field site (a 1.4% outcrossing rate) (Table 2). The sole survivor died several weeks after germination. In the testing of our initial hypothesis that there would be no hybrid GFP outcrossing, a Pearson's chi squared test indicated a nonsignificant deviation from the null hypothesis ($p < 0.05$) with $p=1.0$ and $p=0.9$ for KY and TN field sites, respectively. Whereas pollen movement occurred in these fields there was nearly no transgene 'outflow' from the hybrids.

All pods formed on the hybrid GFP plants were examined; only 16 seeds were derived from 5 plants at the KY site and 84 seeds formed from 11 plants at the TN field site. A total of 47 of these seeds from both sites germinated, yielding 37 seedlings and hence mature plants. Thirty-five seedlings were GFP-positive, both by observation under UV light and by spectrofluorometric analysis (Table 3). These observations suggest that while seeds containing the transgene can be formed on the hybrids in an open-pollination environment, the total seed-set on these plants is very small in comparison with that of conventional *N. tabacum*. Moreover, none of the progeny grown from the very limited hybrid GFP seed production in the field experiment produced any seed when self-pollination was attempted.

2.4.2.1 Pollen tube growth: pollen competition experiment

To estimate the degree of competition from hybrid GFP and SN 2108 to pollinate MS TN 90 plants in the field experiments, pollen tube growth was measured. Simple linear regression of the SN 2108 pollen samples that germinated revealed that pollen tube length and time was positively correlated ($R^2 = 0.203$) with an average growth rate of 0.043 mm per 15 minutes ± 0.005 mm. For hybrid GFP plants, linear- or polynomial regression models did not reveal any association of pollen tube length and time ($p = 0.206$), thus pollen competition might have occurred between plant types.

2.4.3 Manual crosses

Crosses were performed under greenhouse conditions among the transgenic hybrid GFP lines, MS TN 90, and SN 2108 (Table 3). We observed robust seed production from the MS TN 90 by SN 2108 cross (data not shown). However, the pollination of hybrid GFP plants by SN2108 resulted in few seeds from 7 of 96 crosses (Table 3). Five of these seeds germinated (71% viability) but only 3 plants survived to maturity. Testing the outcrossing potential of the hybrid GFP to MS TN 90 resulted in seed from 9 out of 96 crosses. Viability of this seed was extremely low (< 0.7%); just 3 out of 445 seeds germinated (Table 3), and one plant died shortly after germination. Attempted crosses between two hybrid GFP plants resulted in no seed.

Subsequently, crosses were performed to determine fertility of the progeny derived from the hybrid GFP ♀ × SN 2108 ♂ cross, designated as HYB BC_1F_1 and the MS TN 90 ♀ × hybrid GFP ♂ cross, designated as MS BC_1F_1 (Table 3). This germplasm, in effect, simulates the possible fates of volunteer plant populations as a result of comingling of genetic material from a hybrid GFP field and nearby *Nicotiana* individuals. HYB BC_1F_1 pollen was crossed to MS TN 90 plants to test if a volunteer produced from seed set on hybrid F_1 plants could pollinate a neighboring *N. tabacum* field; none of the 60 crosses set any seed. MS BC_1F_1 plants, the result of the few successful hybrid GFP outcrossing to MS TN 90, were pollinated with non-transgenic *N. glauca* and TN 90 pollen, and hybrid GFP pollen. Only the MS BC_1F_1 ♀ × NT-glauca crosses produced any seed, confirming earlier findings that *N. glauca* can successfully pollinate hybrids. No other crosses produced seed.

These results suggest that the small amount of viable seed formation that could occur in field production of the hybrids via pollen introduced from within (hybrid plants) or outside that field (conventional tobacco) would not result in the persistence of transgenic plants in that field beyond one generation. The only exception to this limited persistence would be if *N. glauca* plants grew inside or adjacent to a hybrid field site.

2.4.4 Above-ground biomass

For interspecific hybrid *Nicotiana* plants to have potential as production-host plants in biomanufacturing, they should produce high biomass (e.g., comparable to commercial tobacco). There were not significant differences of biomass between hybrids and TN 90 in the field experiment (p=0.738; Fig 3A). There were biomass differences among plant types in the greenhouse study (Fig 3B); the TN 90 and hybrid GFP plants were not significantly different and produced the highest amount of biomass while the non-transgenic hybrid and *N. glauca* GFP produced significantly lower biomass. We conclude that interspecific hybrid tobacco performed comparably to *N. tabacum* in biomass

2.5 Discussion

Tobacco has several qualities that make it suitable for use as a production platform for biomanufacturing. It is easily transformable to achieve a high content of soluble heterologous protein, and accommodates a range of alternative gene-expression systems including viral transfection, transient expression via *Agrobacterium* vectors, and stable

nuclear and chloroplast transformation methods [74]. The tobacco system is very productive; the above-ground portion of the plants can be harvested several times per growing season, producing up to 25 tonnes ha^{-1} of biomass [75]. In addition, tobacco has a track record of experimental use as a bioreactor for producing vaccines, antibodies, and cytokines [76-78], and its high biomass yields and prolific production of seed suggest efficiency and flexibility of scale-up. Tobacco is being used in biomanufacturing in the private sector currently. Planet Biotechnology has a dental caries product in Phase II clinical trials, and Medicago has an influenza vaccine in Phase I clinical trials. The interspecific hybrid (*N. tabacum* ♀ × *N. glauca* ♂) has been described as an effective production platform for an animal vaccine [76]. The 'sterility' of this hybrid has been cited as a distinct benefit for production in the environment over tobacco [76] contributing to the desired goal of complete bioconfinement of heterologous pharmaceutical genes and proteins in biomanufacturing applications, but until now this attribute has not been evaluated in detail.

Previous studies on the fertility of the *Nicotiana* interspecific F_1 hybrids concluded that they were infertile [36]; Trojak-Goluch and Berbec described the F_1 amphihaploids resulting from pollinating *N. tabacum* with *N. glauca* as "completely self- and cross-sterile" [56, 58, 76]. Al-Ahmad et al. observed their *N. sylvestris* × *N. tabacum* hybrids were incapable of self-pollination or of successfully generating any progeny when backcrossed to the female parent *N. sylvestris* [59]. The principal difference between our findings and those earlier studies is our observation of occasional, albeit very minimal, fertility of *N. tabacum* ♀ × *N. glauca* ♂ hybrid plants, both in the greenhouse and in the

field. Similarly the germination rate of pollen formed on different (transgenic) hybrid individuals was very low but measurable and variable (Figure 2B), contrasting with the observation from Ling et al. that the pollen from transgenic *N. tabacum* ♀ × *N. glauca* ♂ hybrids was non-viable [76]. This disparity is important in relation to the goal of obtaining optimum bioconfinement, as it cautions that the 'sterility' trait may not be sufficiently predictable for this hybrid host to be the sole strategy employed.

Differences in residual fertility among hybrid plants may have resulted from variation among transgenic events following fusion of the gametes owing to amphiploidy effects. Trojak-Goluch and Berbec concluded that meiosis in pollen mother cells was mostly asynaptic, based on the number of univalent chromosomes, which varied from cell to cell during metaphase I; they also noted that chromatid bridges, lagging chromosomes and a lack of one of the meiotic divisions were common observations during anaphase and telophase I [56]. Hence we were interested in variation of total DNA content among types, especially among hybrids. The absolute DNA content of NT-TN 90 was similar to previously reported values for *N. tabacum* [67, 79] (Fig. 2A). Also, our estimations for *N. glauca* reflected a wide range of previously reported 2C values [67, 80]; Fig. 1A). The average 2C value of hybrid GFP ranged from 8.92 pg to 9.16 pg among five lines, (Fig. 1A). The DNA content of these hybrid GFP plants, which are amphiploid/tetraploid (3n = 2x= 36) [58], were not significantly different from that of NT-glauca (2n = 2x = 24). One might be concerned that changes in amount of DNA during the hybridization process could compromise subsequent transgene expression levels. However, we consistently observed that F_1 hybrid GFP, which was produced from parent lines homozygous for

mGFP5-ER, produced GFP-expressing progeny in subsequent crosses, and therefore apparently retained multiple copies of the *mGFP5-ER* transgene.

Even with the very limited fertility that we observed, the interspecific hybrid strategy does represent a bioconfinement improvement over expressing genes in *N. tabacum* in the context of bioconfinement. The manual plant crosses showed low production of viable seeds: from *N. tabacum* type SN 2108 to the hybrid, and from hybrid to *N. tabacum* MS TN 90 (Table 3). This finding was confirmed in the field setting, as evidence of GFP presence in the *N. tabacum* MS TN 90 progeny was found among thousands of seeds (Table 2). In contrast, a very small number of viable seeds were set on the hybrids in the field (Table 2). However, we note that the Tennessee field study data are very limited owing to very low seed germination; we cannot explain this finding.

The use of the interspecific hybrid host for biomanufacturing should not be considered to be a failsafe bioconfinement solution. A male-sterility trait could be employed in *N. tabacum* to improve bioconfinement. Although, cytoplasmic male sterility (CMS) sometimes suffers from reversion of the phenotype [81, 82], CMS might also be useful. However, if the hybrid is used in isolation from tobacco production fields (say, 10 km), AOSCA seed production regulations state only 0.40 km of isolation of different fertile tobacco cultivars the combination of partial bioconfinement in the hybrid system and physical isolation could be sufficient to mitigate risks.

Of the crosses that were attempted, the most productive was the pollination of the hybrid by *N. glauca* (data not shown). *N. glauca* is currently present in twelve U.S. states, [83] including regions in which tobacco agriculture occurs (e.g. Ohio). A regulatory process examining the hybrid for use in outdoor production of recombinant proteins would need to evaluate wild *N. glauca* distribution and proximity to PMP-production fields. However, the pollination of *N. glauca* by the hybrid was consistently unsuccessful in our hands (data not shown), which is encouraging in relation to concerns about transgene transfer to the wild species in the environment.

2.6 Conclusions

The extremely low fertility of interspecific *Nicotiana* hybrid plants could contribute to an effectively bioconfined biomanufacturing platform, which would also likely require physical isolation from commercial tobacco production as well as from wild *N. glauca*. The hybrid progeny obtained from pollinating *N. tabacum* using the uncultivated species *N. glauca* represent a good candidate for a bioproduction host since it has very low fertility and sufficient above-ground biomass in the field.

3. An orange fluorescent protein tagging system for real-time pollen tracking[2]

3.1 Abstract

Background

Monitoring gene flow could be important for future transgenic crops, such as those producing plant-made-pharmaceuticals (PMPs) in open field production. A *Nicotiana* hybrid (*Nicotiana. tabacum* × *Nicotiana glauca)* shows limited male fertility and could be used as a bioconfined PMP platform. Effective assessment of gene flow from these plants is augmented with methods that utilize fluorescent proteins for transgenic pollen identification.

Results

We report the generation of a pollen tagging system utilizing an orange fluorescent protein to monitor pollen flow and determine transgene zygosity. This system was created to generate a tagged *Nicotiana* hybrid that could be used for the incidence of gene flow. *Nicotiana tabacum* 'TN 90' and *Nicotiana glauca* were successfully transformed

[2] This paper is intended for future submission. Contributions were made as follows: **J. Hollis Rice**: Performed all plant transformation experiments and transgenic line analysis, and drafted the document: **Richard E. Mundell**: Conceived portions of the study, bred the original interspecific hybrid, produced the experimental hybrids, and performed manual greenhouse crosses. **Reginald J. Millwood**: Coordinated the study and assisted with analysis. **Laura L. Abercrombie** created vectors for transformation. **Orlando D. Chambers**: Conceived portions of the study and assisted with coordination and execution of the study. **C. Neal Stewart Jr.**: Conceived portions of the study, coordinated the study and assisted with revisions **H. Maelor Davies**: Conceived portions of the study, coordinated the study and provided critical review.

via *Agrobacterium tumefaciens* to express the orange fluorescent protein gene, *tdTomato-ER*, in pollen. A green fluorescent protein, mGFP5-ER, was synthesized in vegetative structures of the plant and used as a control. Hybrids were created that combined both fluorescent proteins as a research tool for monitoring pollen movement and gene flow. Manual greenhouse crosses were used to assess hybrid sexual compatibility with *N. tabacum*, resulting in seed formation from hybrid pollination in 2% of crosses, which yielded non-viable seed. Pollen transfer to the hybrid formed seed in 19% of crosses and 10 out of 12 viable progeny showed GFP expression.

Conclusion

The orange fluorescent protein is visible when expressed in the pollen of *N. glauca, N. tabacum*, and the *Nicotiana* hybrid, although hybrid pollen did not appear as bright as the parent lines. The hybrid plants, which show limited ability to outcross, could provide bioconfinement with the benefit of detectable pollen. Fluorescent protein-tagging could be a valuable tool for breeding and *in vivo* ecological monitoring.

3.2 Background

Increased use of transgenic crops has prompted the necessity of monitoring transgene flow in agroecological systems. Previous investigations have ascertained the utility of gene flow tracking with fluorescent proteins (FPs) [25, 26, 46, 49]. These studies have shown that green fluorescent protein (GFP) is an effective tool for the purpose of gene flow tracking and can be targeted to various organs and tissues within plants, including pollen. This technology, in effect, could be used in an environmental monitoring system, one of the many uses of FPs in plants [15]. One drawback of using native GFP as a marker in plants is the signal-to-noise ratio at GFP's maximum excitation wavelength of 395 nm, often resulting in autofluorescence of plant tissue components [20]. Fluorescent proteins emitting in the red/orange spectrum that require longer wavelengths for excitation have lower levels of autoflorescence in plant tissues compared to blue or UV light [20]. One such widely used orange fluorescent protein (OFP), DsRed, is derived from *Discosoma* sp. its mutant variants have higher extinction coefficients and quantum yields [84]. Coral-derived FPs should be useful for monitoring gene flow.

Nicotiana tabacum (tobacco) and *Brassica napus* (canola) plants have been transformed to synthesize GFP in pollen, using pollen-specific promoters [25, 26]. Long-range pollen tracking was conducted in canola species to assay pollen movement in real time (e.g. immediate detection of tagged pollen) using traps at various distances within field and greenhouse experiments. This method is quicker and less laborious for determining pollen flow than analyzing progeny from recipient plants (e.g. antibiotic screening, PCR,

FP screening) [21]. Drawing upon this previous body of work, it is logical to conceptualize a method to determine bioconfinement efficacy using FP tagging.

The *Nicotiana* hybrid (*Nicotiana tabacum* × *Nicotiana glauca*), is highly sterile and prompted a further examination of bioconfinement through gene flow monitoring. Previously, we have shown that GFP tagging in vegetative plant tissues of this hybrid allows for gene tracking and assists with sterility assessments (unpublished data). Here we describe a modified system applicable to a real-time assay of pollen flow from FP-tagged plants. Our goal was to engineer pollen to synthesize an orange fluorescent protein, using a pollen-specific promoter, and breed this into the hybrid *Nicotiana* without losing functionality of the FP. To achieve this goal, parent plants *N. tabacum* 'TN 90' and *N. glauca* were transformed to synthesize tdTomato-ER in the pollen via *Agrobacterium*-mediated transformation, breed to homozygosity, and then create the transgenic hybrid. Manual greenhouse crosses were performed to assess sexual compatibility and functionality of the system.

3.3 Methods

3.3.1 Plant material

N. tabacum 'TN 90' used for transformation was from foundation seed lot # 86-02-K-4A, *N. tabacum* 'MS TN 90' from foundation seed lot # 86-03-KLC-15 is a male sterile variety of TN 90 that was used as a pollen recipient plant in crosses. *N. tabacum* 'SN 2108,' a morphologically distinct variety from the TN 90 cultivar used as a pollen donor

in greenhouse crosses, is an experimental line developed into 'KT D4'; all *N. tabacum* were obtained from the Kentucky Tobacco Seed Improvement Association, Inc. in Lexington, KY, USA. (38°8'N, 84°29'W). *N. glauca* used for transformation was from the US National Plant Germplasm System (NPGS) (plant introduction 307908, accession TW55 from Peru).

3.3.2 Transformation

3.3.2.1 Vector contstruction

Two fluorescent proteins were used to mark plants. The *mGFP5-ER* gene encodes a green fluorescent protein that emits green light (λ_{max} = 509 nm) when excited by wavelengths of blue (465 nm) or ultraviolet (UV; 395 nm) light that is targeted and retained to the endoplasmic reticulum (ER) within cells. GFP in transgenic plants is observable by UV light illumination in the dark or epifluorescence microscopy, and is quantifiable using fluorescence spectrometry [24, 61]. *tdTomato-ER*, is a *DsRed* variant that is a tandem dimer FP that is retained in the ER. It is an orange fluorescent protein (λ_{max} = 581 nm) excited by green light (554 nm) [84, 85]. To create dual fluorescent protein marker vectors, the Gateway compatible vector backbone pMDC99, containing a hygromycin resistance cassette, and pMDC100, containing a kanamycin resistance cassette, were utilized as Gateway destination vectors [86]. An entry vector containing a pollen-specific promoter, *LAT52* [87], driving expression of the fluorescent protein *tdTomato-ER* and a nos terminator was recombined with the destination vectors, creating the intermediate vectors pMDC99-tdTomato-ER and pMDC100-tdTomato-ER. Subsequently a GFP expression cassette (containing CaMV35S-mGFP5-ER-nosT) was

amplified from pBIN19-mGFP5-ER and cloned into the intermediate vectors, creating the binary vectors TD-GFP-H (containing hygromycin selection) and TD-GFP-K (containing kanamycin selection), respectively (Figure 1). These vectors were identical except for the antibiotic resistance genes to facilitate screening by using dual antibiotic selection after hybridization of *Nicotiana* species to incorporate both constructs into the F_1 hybrid.

3.3.2.2 Generation of transgenic plants

Plant transformation experiments were performed using *Agrobacterium tumefaciens* strain EHA 105 using the previously-described leaf disc method [62]. Sterilized leaf explants were soaked for 30 min in a mixture of *Agrobacterium* and liquid MS salts containing B_5 vitamin (DBI). Transformed explants were then co-cultivated on solid DBI media for 2 days before being transferred to solid DBI containing Timentin® (400 mg/L) and either kanamycin (200 mg/L) or hygromycin (50 mg/L) for selection. Shoots generated from transformed callus were transferred to MS media containing respective selective antibiotics [63]. Shoots were maintained at 24° C under 16/8 h light/dark periods until rooting, then transferred to soil in 18 cell flats with humidity domes to allow for acclimation for approximately two weeks. Plants were then screened with a handheld UV light (UVP model B-100AP 100 W:365 nm) to detect any non-fluorescent plants as previously described [24]. The presence of *mGFP5-ER* was confirmed in each T_0 plant by DNA extraction and PCR (Figure 2) as previously described [25, 64]. Plants confirmed visually and with PCR were transferred into 4 L pots in a greenhouse under 16/8 h light/dark periods at 27°/20° C, respectively. Seeds were harvested from each

plant by covering flowers with breathable mesh pollination bags (DelStar Technologies, Inc., Middleton, DE, USA) and manually shaken until seed pods developed and were harvested. In all, 20 events were generated per construct per plant.

3.3.3 Hybrid *Nicotiana* production

Plants were bred to obtain lines homozygous for the transgenes *mGFP5-ER* and *tdTomato-ER* to allow for a complete tracking of pollen. To ensure multiple transgene copies were stacked into the hybrid, our goal was to produce hybrids containing one TD-GFP-K and one TD-GFP-H construct, using dual antibiotic screening to ensure integration into the hybrid genome.

3.3.3.1 Fluoresence measurements and observations

Brightly fluorescent T_0 plants, as determined by visual observation, were selected for analysis and further breeding; ten T_1 TN 90 GFP-H lines and eight *N. glauca* T_1 TD-GFP-K lines were selected. T_1 seeds were germinated and handheld UV light was used to select the brightest GFP-expressing seedlings. GFP fluorescence was measured by a spectrofluorometer (Fluorolog®-3 HORIBA Jobin Yvon, Edison, NJ, USA)[23, 24] and analyzed with its software (FluorEssence™ Version 2.5.2.0.HORIBA Jobin Yvon, Edison, NJ, USA) to quantify average fluorescence (photon counts per second) from each line (Figures 3 and 4). Individual plants were selected that had the highest measured fluorescence, and thus, were most likely to be homozygous for the *mGFP5-ER* transgene. When plants flowered, pollen was taken from each plant, suspended in 200 µl of water, and 15 µl of the suspension transferred to a microscope slide and observed under an

epifluorescent BX 51 microscope (Olympus Corporation, Shinjuku, Tokyo, Japan). A Texas Red®/Cy3.5 (TxRed) filter set (Chroma Technology Corporation, Bellows Falls, VT, USA) was used to view fluorescent pollen grains. The field of view was captured by a digital camera (Olympus Q Color 3) and Qcapture imaging system (Q Imaging Corp., Burnaby, Canada) (Figure 5).

3.3.3.2 Transgenic line selection

Using epifluorescence microscopy, transgene zygosity was determined by dividing the number of fluorescent pollen grains by the number of non-fluorescent grains within the field of view. Plants with 100% fluorescent pollen were bagged and self-fertilized as previously described. Multiple strategies were employed to assure homozygosity of each T_2 line. Germinated seed was screened with a handheld UV light to determine zygosity of each T_2 line (using ratios of GFP to non-GFP plants). T_2 lines of seed were also screened for inheritance of antibiotic resistance genes by germination on MS media [63] containing kanamycin (200 mg/L) or hygromycin (50 mg/L). Observation of plant health allowed for determination of segregation; 100% survival indicated a homozygous line for selection. The selected transgenic T_2 lines, TN 90 TD-GFP-H and *N. glauca* TD-GFP-K, were crossed (TN 90 TD-GFP-H × *N. glauca* TD-GFP-K). Hybrid lines derived from parents lines transformed with the TD-GFP-K/TD-GFP-H constructs were named 'Hybrid OFP' plants. These hybrid seeds were germinated on MSO media containing both kanamycin (200 mg/L) and hygromycin (50 mg/L) to ensure both constructs were integrated into the hybrid genome.

3.3.4 Fertility assessment in hybrids

Manual crosses were conducted in a greenhouse in Lexington, Kentucky (Table 1). Hybrid OFP plants were crossed with the male sterile *N. tabacum* 'MS TN 90' to determine hybrid outcrossing potential and transgene transmission rates. To evaluate female fertility, SN 2108, a pollen donor was crossed to hybrid OFP plants, which were emasculated prior to crossing. For both types of crosses, 8 pairs of plants were crossed, with 12 crosses per pair except of one pair (hybrid OFP × SN 2108) of plants where 11 crosses were made. Seeds from crosses were germinated and screened visually as previously described for GFP presence. Fertility rates were determined by the number of successful crosses divided by the total number of crosses attempted. Detectable gene flow was determined by dividing the total number of germinated GFP expressing seed by the total number of surviving seedlings.

3.3.5 Statistical analysis

All analysis of variance (ANOVA) routines were performed using SAS (Version 9.3 SAS Institute Inc, Cary, NC, USA) using the MIXED procedure with a significance level of $p < 0.05$. When ANOVA results were found to be statistically significant, the least significant differences were used for mean separations.

3.4 Results and Discussion

Transformation of *N. tabacum* 'TN 90' and *N. glauca* were successful except for *N. glauca* TD-GFP-H where multiple attempts failed to produce hygromycin-resistant plants. GFP was visible in leaves, stems, and roots (data not shown) and OFP was visible

in pollen under a microscope (Figure 5) with the aforementioned filter set. GFP, regulated by the CaMV 35S promoter, was not visible in pollen in accordance with previous findings [22, 88]. Highly fluorescent individual plants from the most fluorescent *N. glauca* TD-GFP-K lines were crossed with highly fluorescent TN 90 TD-GFP-H lines to ensure hybrids had both antibiotic resistance genes and would fluoresce brightly, thereby facilitating detection. Hybrid OFP lines were 100% resistant to kanamycin and hygromycin when screened on MSO media containing both antibiotics (data not shown) and fluorescence was visually observed in all plants, indicating inclusion of both cassettes into the F_1 hybrids.

Manual plant crosses revealed that the hybrids were able to backcross to a non-transgenic male sterile *N. tabacum* 'MS TN 90' (Table 1), forming entirely non-viable seed in 2% of the crosses (98% of the crosses produced no seed), thus restricting detectable transgene transmission rates in progeny to 0%. This result was in contrast to our previous findings where few viable seeds were generated from a similar (MS TN 90 × hybrid) cross (unpublished data). However, we have shown that male fertility varies among hybrid lines (unpublished data). When the fertile line SN 2108 was used to pollinate hybrid OFP plants, limited seed set (19% of crosses) was observed. Only 10 germinated seedlings out of 12 expressed GFP, (83% detectable transgene transmission), indicating that transgenes might be segregating out of some hybrid OFP × SN 2108 progeny.

It was unknown if tdTomato-ER would be visible in the pollen of the *Nicotiana* hybrid as the plant largely produces immature pollen where many pollen mother cells cease to

develop past the tetrad stage [56]. Many of the immature pollen grains apparently did not synthesize sufficient tdTomato-ER for visual detection. The FP was only obvious in larger, more mature hybrid OFP pollen and did not appear to fluoresce as brightly as TN 90 TD-GFP-K and *N. glauca* TD-GFP-H. The pollen-specific promoter *LAT52*, regulates gene expression during microspore mitosis, allowing transcription until anthesis [87, 89]. Our observation of few mature fluorescent pollen grains produced in the hybrids demonstrates that the hybrid system could be a viable candidate for transgene bioconfinement.

3.5 Conclusions

A bright orange fluorescent protein, tdTomato-ER, can be synthesized in pollen when its gene is under the control of the LAT52 pollen promoter. Fluorescently-tagged pollen is very distinguishable from non-tagged pollen, and shows low autofluorescence. The plants produced in this study further increase tools for gene flow studies. Crossing studies demonstrated that hybrid OFP plants had low fertility and provided bioconfinement by limiting successful crosses made to the maternal line, *N. tabacum*. As pollen tracking is possible with this fluorescently tagged hybrid, more research is needed to determine the efficacy of pollen detection with this system and how it relates to bioconfinement in a field setting.

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Appendices

Table 1. Plant genotypes used in the studies, including parentage, hybrid and transgene status. Not all genotypes were used in every experiment

Genotype designation	Species or F_1 hybrid	Transgenic status	Marker gene	Maternal parent	Paternal parent
TN 90 GFP	*Nicotiana tabacum*	Transgenic	GFP	*Nicotiana tabacum*	*Nicotiana tabacum*
glauca GFP	*Nicotiana glauca*	Transgenic	GFP	*Nicotiana glauca*	*Nicotiana glauca*
hybrid GFP	hybrid	Transgenic	GFP	TN 90 GFP	glauca GFP
MS TN 90	*Nicotiana tabacum*	Non-transgenic	N/A	*Nicotiana tabacum*	*Nicotiana tabacum*
SN 2108	*Nicotiana tabacum*	Non-transgenic	N/A	*Nicotiana tabacum*	*Nicotiana tabacum*
HYB BC_1F_1	hybrid	Transgenic	GFP	hybrid GFP	SN 2108
MS BC_1F_1	hybrid	Transgenic	GFP	MS TN 90	hybrid GFP
NT-TN 90	*Nicotiana tabacum*	Non-transgenic	N/A	*Nicotiana tabacum*	*Nicotiana tabacum*
NT- glauca	*Nicotiana glauca*	Non-transgenic	N/A	*Nicotiana glauca*	*Nicotiana glauca*
NT- hybrid	hybrid	Non-transgenic	N/A	*Nicotiana tabacum*	*Nicotiana glauca*

Table 2. Summary of seed collection, germination, and analysis from hybrid GFP and MS TN 90 plant at the Kentucky and Tennessee field sites

site	Kentucky		Tennessee	
plant	Hybrid GFP	MS TN 90	Hybrid GFP	MS TN 90
total pods collected	73	155	263	118
blocks setting seed[a]	N/A	15	N/A	9
plants setting seed	5	N/A	11	N/A
seeds collected	16	11170	84	5968
seeds germinated	9	7340	38	74
total surviving seedlings	5	7340	32	73
GFP positive seedlings[b]	4	0	31	1
GFP negative seedlings[b]	1	7340	1	83
surviving GFP positive seedlings confirmed with spectrofluorometer	4	0	31	0

[a] *N. tabacum* 'MS TN 90' plants were planted and harvested in blocks of five plants.
[b] Presence of green fluorescent protein was confirmed visually with a handheld UV light.

Table 3. Results of crosses made in the greenhouse to examine the sexual compatibility of hybrid GFP plants and the fertility of their progeny

genotypes[a]	plants crossed[b]	total crosses[c]	crosses resulting in seed production	total seed count	seeds that germinated	surviving plants
(A) F1 crosses						
(hybrid GFP × SN 2108)[d]	12:12	95	7	12	5	3
(MS TN 90 × hybrid GFP)[e]	12:12	96	9	445	2	2
(hybrid GFP × hybrid GFP)	1:1	10	0	0	0	0
(B) backcrosses						
(MS TN 90 × HYB BC_1F_1)	2:1	60	0	0	0	0
(MS BC_1F_1 × NT-glauca)	1:1	20	2	128	74	74
(MS BC_1F_1 × NT-TN 90)	1:1	20	0	0	0	0
(MS BC_1F_1 × hybrid GFP)	1:1	20	0	0	0	0

[a] Crosses listed by (female ♀ × male ♂)
[b] Refers to numbers of each plant used in crosses with respect to the plant order in the cross column.
[c] A 'cross' is constituted by a pollen transfer from one plant to the flower of another plant.
[d] Progeny of this cross formed the individuals named HYB BC_1F_1

[e] Progeny of this cross formed the individuals named MS BC_1F_1

Progeny generated from the initial hybrid GFP crosses were used in a subsequent set of crosses to test sexual compatibility of potential volunteers with other *Nicotiana*s. The light-shaded rows indicate which progeny were used for additional crosses. See Table 1 for plant nomenclature.

Table 4. Greenhouse crosses performed with hybrid OFP plants

genotypes[a]	plants crossed[b]	total crosses[c]	crosses forming seed	total seed count	germinated	survived	GFP positive	fertilization rate	detectable gene flow
(MS TN 90 × hybrid OFP)	8:8	96	2	51	0	N/A	N/A	2%	0%
(hybrid OFP × SN 2108)	8:8	95	18	34	14	12	10	18%	83%

[a] Crosses listed by (female ♀ × male ♂).
[b] Refers to numbers of each plant used in crosses with respect to the plant order in the genotypes column. 12 crosses were attempted between each pair of plants, except one pair of hybrid OFP and SN 2108 where 11 crosses were made.
[c] A 'cross' is constituted by a pollen transfer from one plant to the flower of another plant.

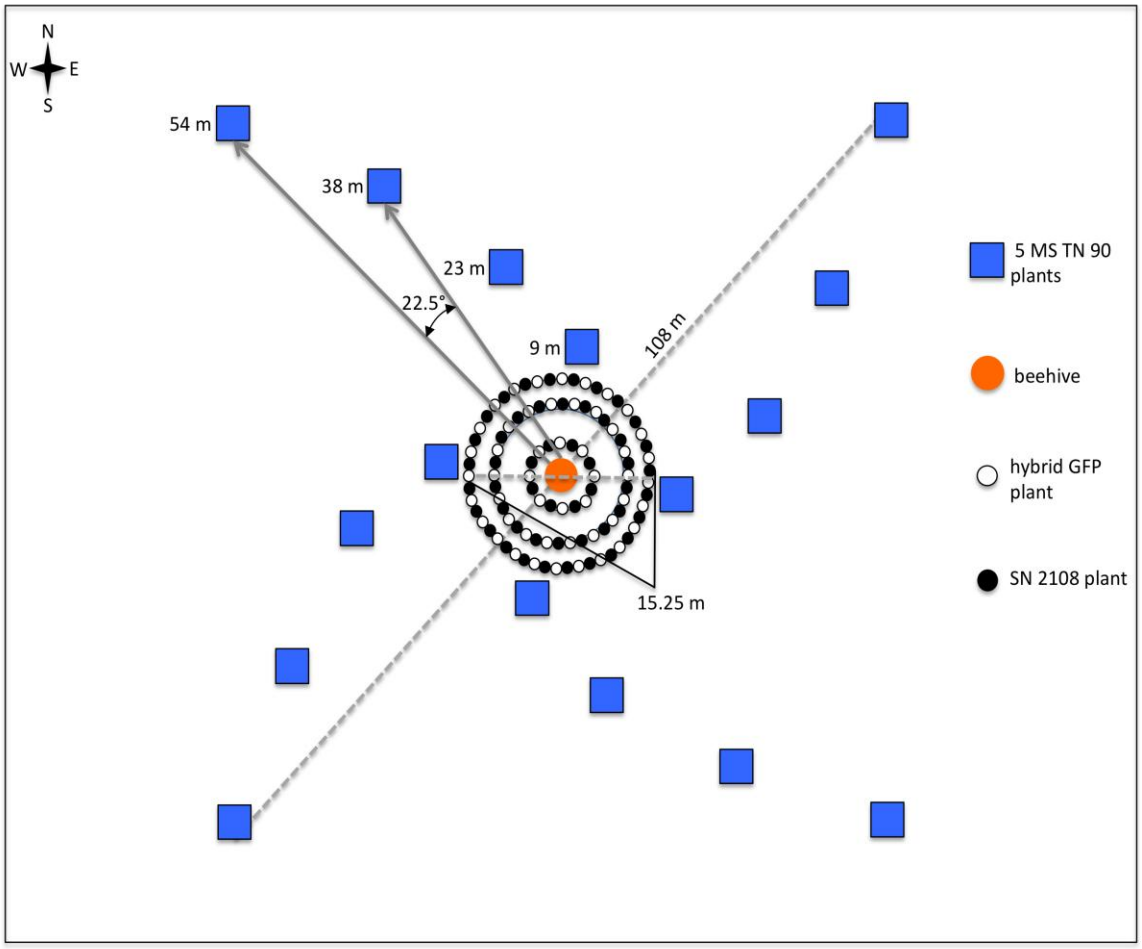

Figure 1. Design of field gene flow experiment. A modified Nelder wheel design was used evaluate the gene flow of hybrid GFP plants. Three plant types were used in the experiment: male sterile *N. tabacum* 'MS TN 90' were pollen 'receptor' plants, hybrid GFP was fluorescently tagged to enable gene flow tracking, and *N. tabacum* type SN 2108 was used as a pollen donor to assure pollen flow was occurring in the field by seed set on MS TN 90 and to test for female sterility of the hybrid GFP plants. A center pollen source patch contained 50 alternating hybrid GFP and fertile SN 2108 plants, spaced approximately 1 m apart. Sixteen 1 m^2 blocks of male sterile MS TN 90 pollen receptor plants were placed at 9, 23, 38, and 54 m distances from the center and were used to detect pollen via seed formation. Each MS TN 90 plot was 22.5° relative to the adjacent plot as viewed from the center. A honeybee hive was placed at the center of the field site to vector pollen.

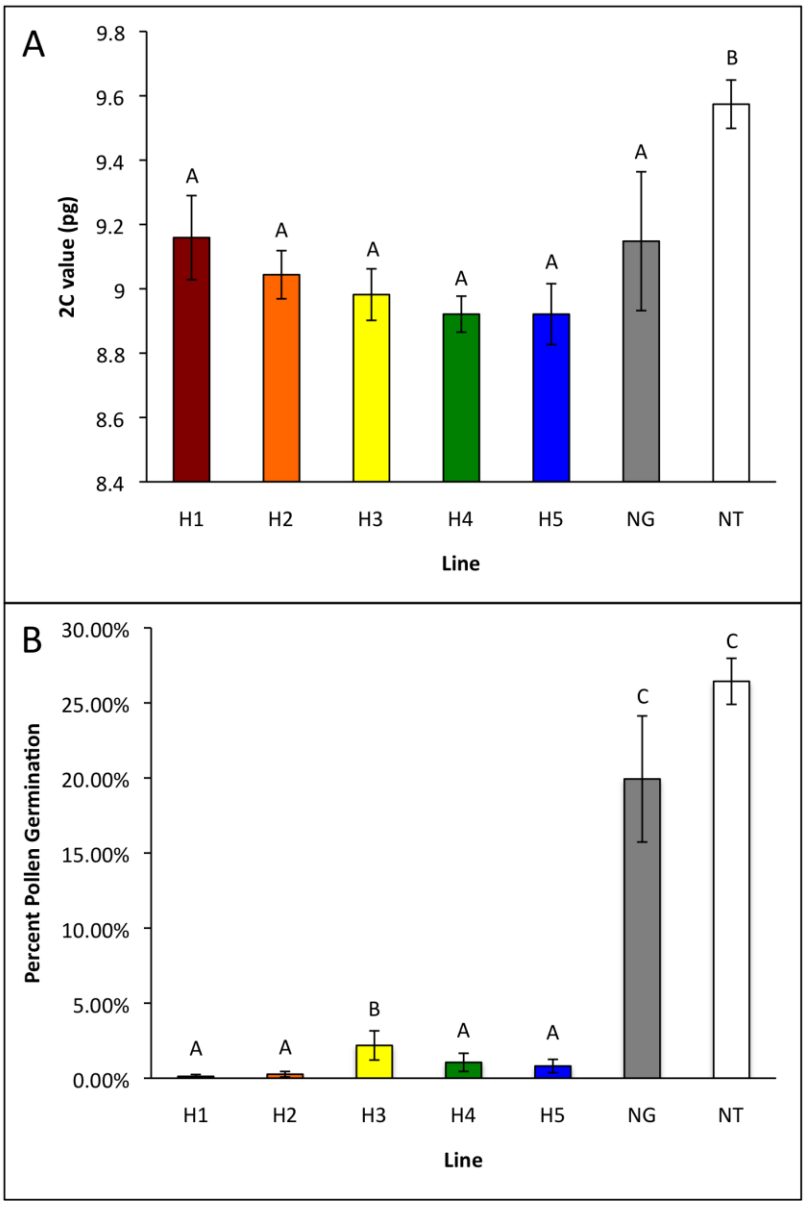

Figure 2. (A) DNA content and (B) pollen germination analysis. DNA content was estimated by flow cytometry and pollen viability was estimated by pollen germination. Hybrid GFP-plant lines: H1. H2, H3, H4, and H5 and non-transgenic parent lines NT-glauca (NG) and NT-TN 90 (NT) n=5. Mean separation is by Fishers LSD and bars marked by the same letter are not significantly different ($p < 0.05$). Error bars are the standard error of the means.

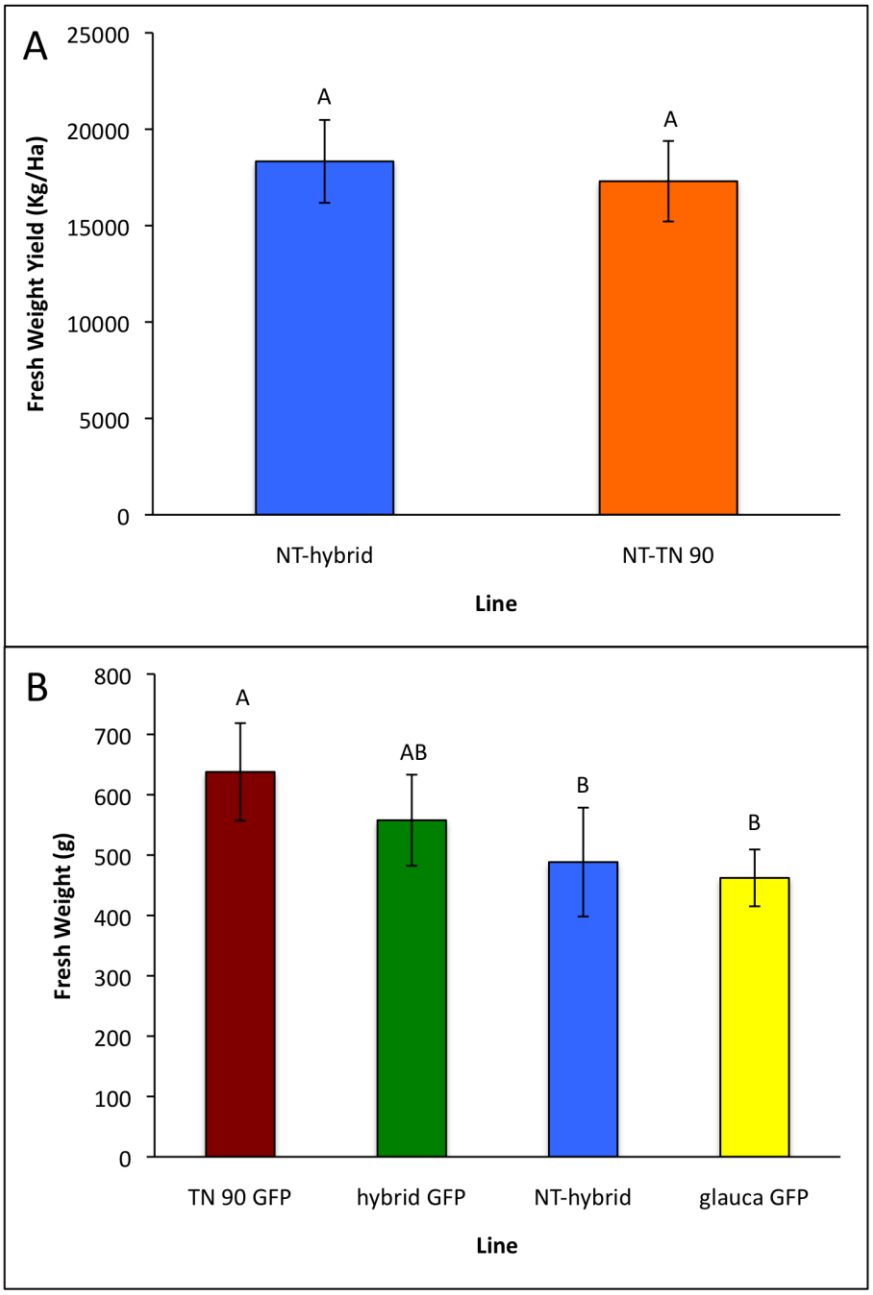

Figure 3. Fresh aboveground biomass productivity. **(A)**Productivity was measured for nontransgenic hybrid plants and nontransgenic TN 90 plants in a field experiment over two years with three measurements per year. **(B)**A greenhouse study was conducted during one year with two measurements of the hybrid GFP line, the transgenic parental lines of the aforementioned hybrid, and a nontransgenic hybrid line. Mean separation is by Fishers LSD and bars marked by the same letter are not significantly different ($p < 0.05$). Error bars are the standard error of the means.

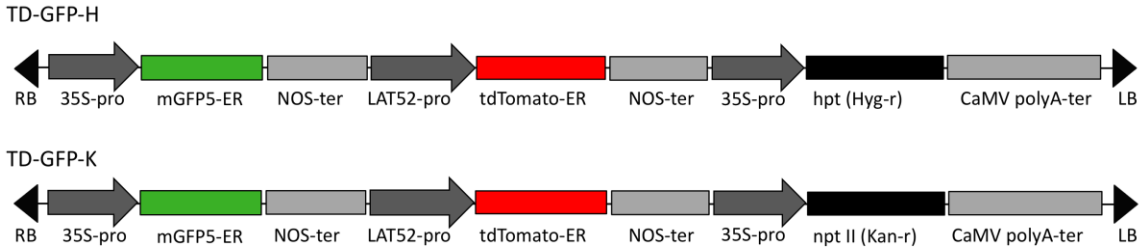

Figure 4. Cassettes used in transformation. Cassettes TD-GFP- H and TD-GFP-K were used in transformation of *N. glauca* and *N. tabacum* 'TN 90' with *A. tumefaciens* strain EHA105. Vectors are identical except for the *hpt* gene present in TD-GFP-H and the *npt II* gene present in TD-GFP-K**.**

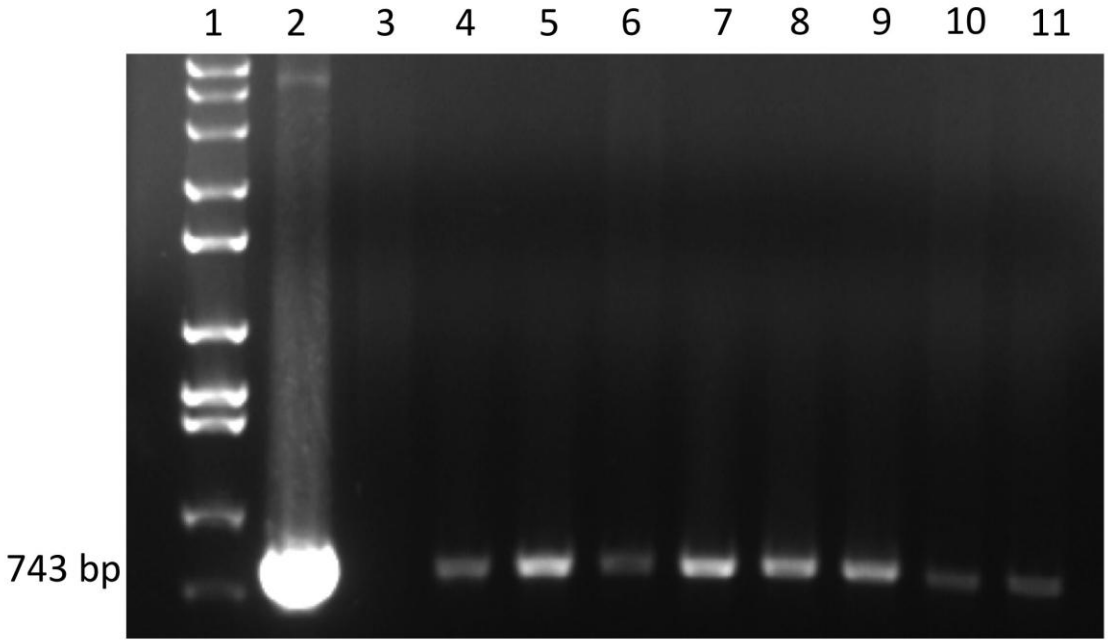

Figure 5. PCR analysis of T_0 TN 90 plants transformed with TD-GFP-H. Lane 1: DNA marker, Lane 2: purified mGFP5ER plasmid as a positive control. Lanes 3-11: genomic DNA from putative transgenic TN 90. The 743 bp band present in lanes 4 through 11 confirmed transgene presence in plants. All other transgenic events showed similar results.

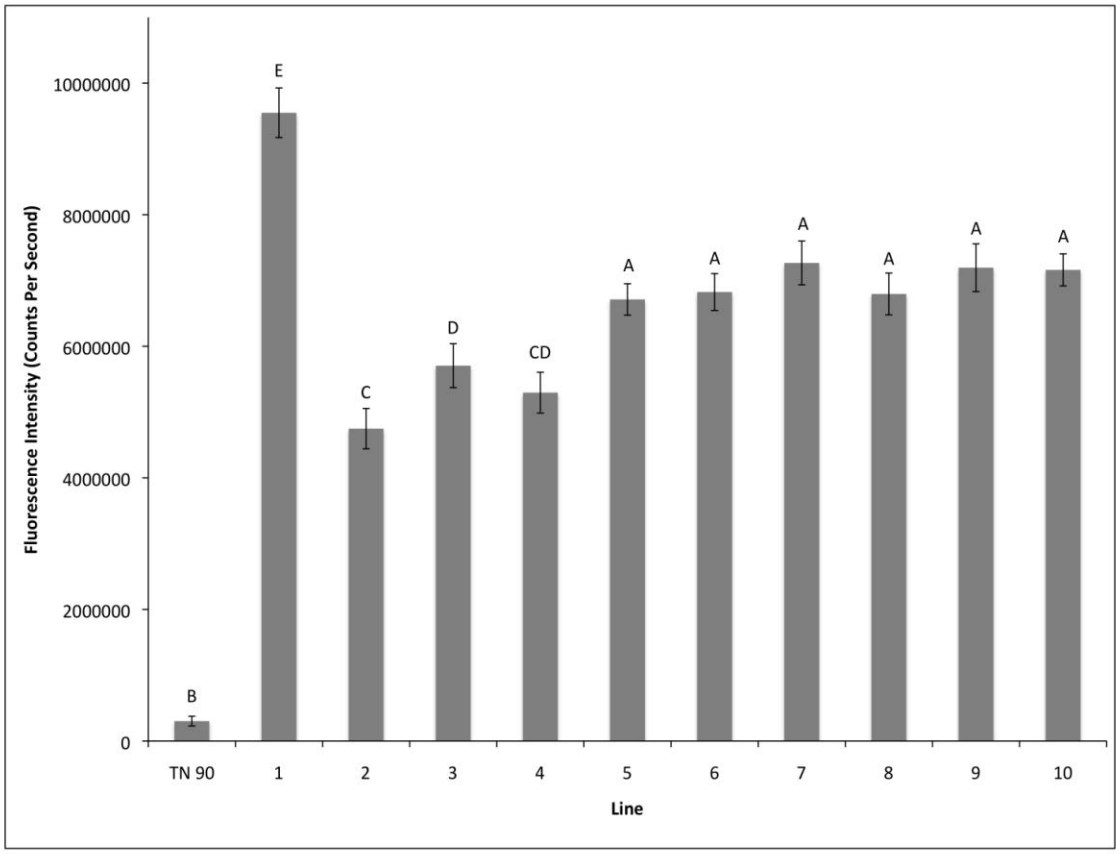

Figure 6. Average relative fluorescence intensity for TN 90 TD-GFP-H T_1 lines. Leaf tissue from a non-transgenic TN 90 line and ten transgenic T_1 lines were excited at 395 nm and measured at an emission of 509 nm with a spectrofluorometer. Fluorescence intensity values were normalized to an average measurement of TN 90 negative control plants outside the spectral range of GFP emission. For TN 90 n=2, TD-GFP-H lines1-8, and 10 n=34, line 9 n=33. Error bars represent the standard error from the mean and different letters indicate significant differences at $P < 0.05$.

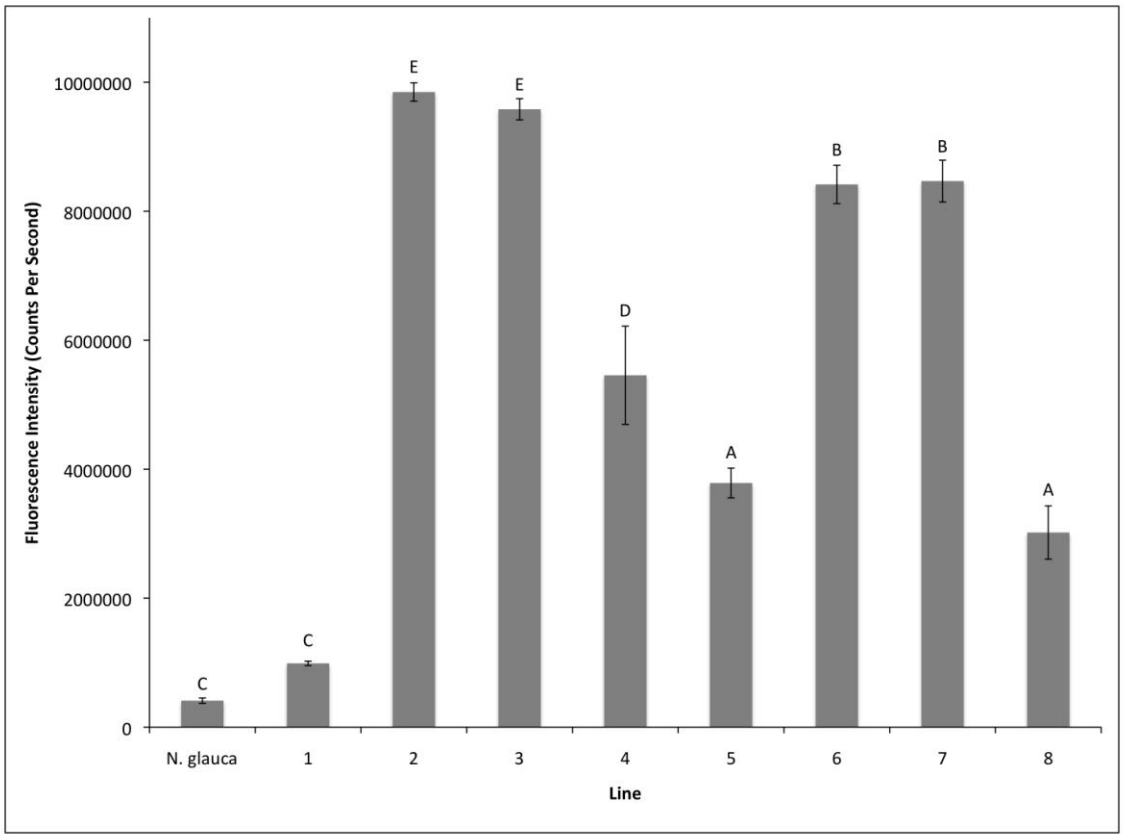

Figure 7. Average relative fluorescence intensity for *N. glauca* TD-GFP-K T_1 lines. Leaf tissue from a non-transgenic *N. glauca* line and eight transgenic T_1 lines were excited at 395 nm and measured at an emission of 509 nm with a spectrofluorometer. Fluoresence intensity values were normalized to an average measurement of *N. glauca* negative control plants outside the spectral range of GFP emission. For *N. glauca* n=2, TD-GFP-K lines 1 n=26, line 2 n=34, line 3 n=31, line 4 n=16, lines 5 and 6 n=33, line 7 n=28, and line 8 n=24. Error bars represent the standard error from the mean and different letters indicate significant differences at $P < 0.05$.

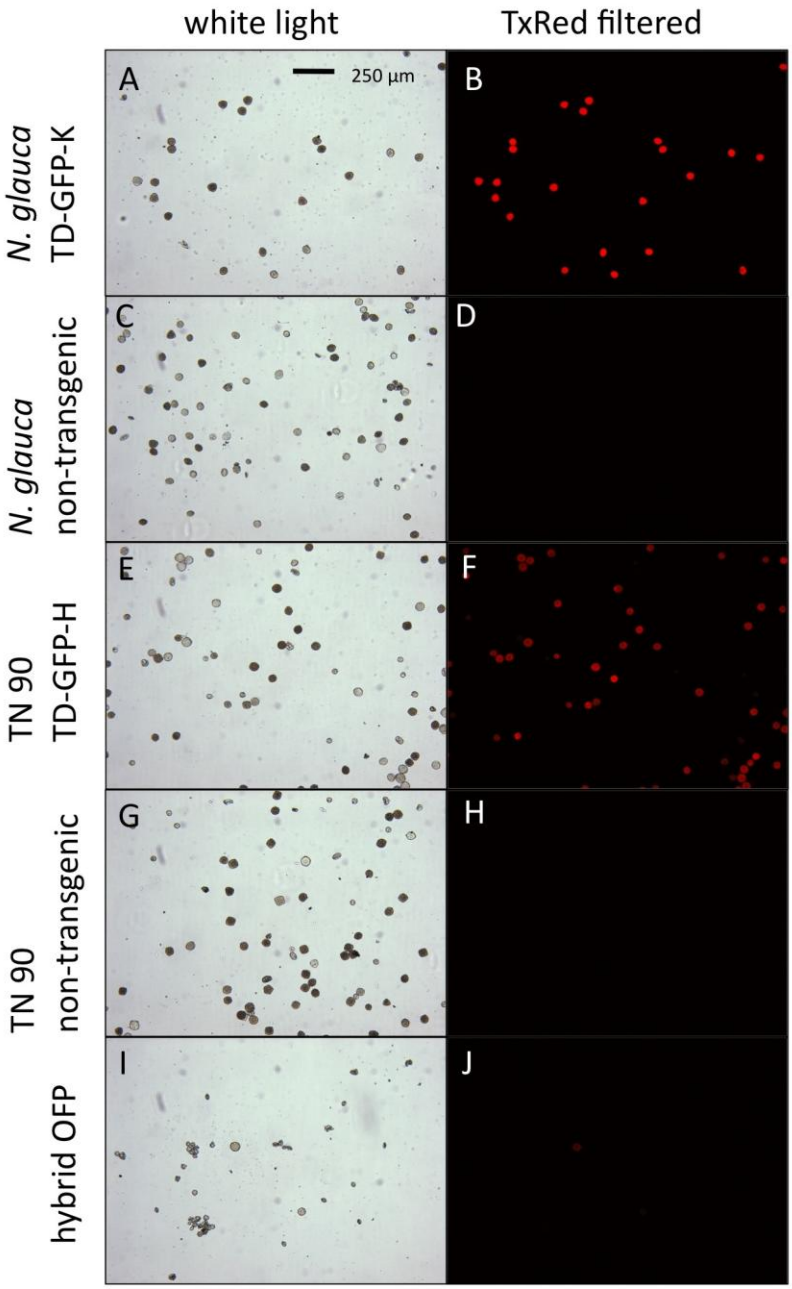

Figure 8. Fluorescent protein visualization in pollen. Orange fluorescent protein-tagged pollen as viewed under an epifluorescent microscope at 100×. *N. glauca* and TN 90 plants have been bred for homozygosity of tdTomato-ER, resulting in hybrid OFP plants. All white light images were captured at 80 ms exposure time. Panels (B) and (D) were captured at 50 ms exposure time. Panels (F) and (H) were captured at 80 ms exposure time. Panel (J) was captured at 180 ms exposure time.

Vita

John Hollis Rice was born in Oak Ridge, TN on May 31, 1987. He graduated from Oak Ridge Public Schools and graduated from Oak Ridge High School in 2005. In December 2009 he graduated from the University of Tennessee, Knoxville with a B.S. in Plant Sciences and began a post-baccalaureate internship under Dr. Neal Stewart. In August 2010 he started as a graduate research assistant pursuing a Master of Science degree in Plant Science.