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Developing biocontainment strategies to suppress transgene escape via pollen dispersal from transgenic plants

Hong Seok Moon
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To the Graduate Council:

I am submitting herewith a dissertation written by Hong Seok Moon entitled "Developing biocontainment strategies to suppress transgene escape via pollen dispersal from transgenic plants." I have examined the final electronic copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Plants, Soils, and Insects.

C. Neal Stewart, Jr., Major Professor

We have read this dissertation and recommend its acceptance:

Robert M. Augé, Arnold M. Saxton, Zong-Ming Cheng, Feng Chen

Accepted for the Council:
Dixie L. Thompson

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

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A Dissertation presented for the
Doctor of Philosophy Degree
The University of Tennessee, Knoxville

Hong Seok Moon
August 2011

Dedication

This dissertation is dedicated to my lovely wife, Inho Kim, and daughter, Hannah Kim Moon, my parents, Dr. Yang-Soo Moon and Mae-Ja Kang, and my parents-in-law, Jin-Won Kim and Myeong-Hee Mo, whose love, patience, and prayers allowed for its completion.

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Abstract

Genetic engineering is important to enhance crop characteristics and certain traits. Genetically engineered crop cultivation brings environmental and ecological concerns with the potential of unwanted transgene escape and introgression. Transgene escape has been considered as a major environmental and regulatory concern. This concern could be alleviated by appropriate biocontainment strategies. Therefore, it is important to develop efficient and reliable biocontainment strategies.

Removing transgenes from pollen has been known to be the most environmentally friendly biocontainment strategy. A transgene excision vector containing a codon optimized serine resolvase CinH recombinase (CinH) and its recognition sites *RS2* were constructed and transformed into tobacco (*Nicotiana tabacum* cv. Xanthi). In this system, the pollen-specific LAT52 promoter from tomato was employed to control the expression of CinH recombinase. Loss of expression of a green fluorescent protein (GFP) gene under the control of the LAT59 promoter from tomato was used as an indicator of transgene excision. Efficiency of transgene excision from pollen was determined by flow cytometry (FCM)-based pollen screening. While a transgenic event in the absence of CinH recombinase contained about 70% of GFP-synthesizing pollen, three single-copy transgene events contained less than 1% of GFP-synthesizing pollen based on 30,000 pollen grains analyzed per event. This suggests that CinH-*RS2* recombination system could be effectively utilized for transgene biocontainment.

A novel approach for selective male sterility in pollen was developed and evaluated as a biocontainment strategy. Overexpression of the EcoRI restriction endonuclease caused pollen ablation and/or infertility in tobacco, but exhibited normal phenotypes when compared to non-transgenic tobacco. Three EcoRI contained 0% GFP positive pollen, while GFP control plants contained 64% GFP positive pollen based on 9,000 pollen grains analyzed by flow cytometry-based transgenic pollen screening method. However, seven EcoRI events appeared to have 100% efficiency on selective male sterility based on the test-crosses. The results suggested that this selective male sterility could be used as a highly efficient and reliable biocontainment strategy for genetically engineered crop cultivation.

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Chapter 1: Introduction

Transgene introgression in crop relatives: molecular evidence and mitigation strategies

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Hong S. Moon's contribution was writing the transgene introgression management or mitigation strategies section and drafting the figures in the manuscript.

1.1 Abstract

Incorporation of crop genes into wild and weedy relative populations (i.e. introgression) has long been of interest to ecologists and weed scientists. Potential negative outcomes resulting from crop transgene introgression (e.g. extinction of native wild relative populations; invasive spread by wild or weedy hosts) have not been documented, and few examples of transgene introgression exist. However, molecular evidence of introgression from non-transgenic crops to their relatives continues to emerge, even for crops deemed low-risk candidates for transgene introgression. We posit that transgene introgression monitoring and mitigation strategies are warranted in cases where transgenes are predicted to confer selective advantages and disadvantages to recipient hosts. The utility and consequences of such strategies are examined, and future directions provided.

1.2 Introduction

Populations of wild relatives and weedy relatives of crop plants that experience potential gene exchange have been the focus of research on transgene introgression. Indeed, the suggestion of potential outcomes of transgene introgression provides the foundation for several recent scientific investigations in which events prior to introgression (e.g. pollination and hybridization) are subjects of interest (Guadagnuolo et al., 2006; Watrud et al., 2004; Mercer et al., 2007; Cao et al., 2009; Laughlin et al., 2009; Elling et al., 2010; Song et al., 2010). A continued increase in research and acreage dedicated to transgenic crops (Chapman & Burke, 2006; Lemaux, 2009) will likely lead to further examination of potential scenarios resulting from transgene introgression, the repercussions of which are often cast in the context of negative ecological effects (Figure 1-1). In one scenario, selective sweeps could lead to weed management problems

in co-existing transgene-introgressed weedy populations (Cao et al., 2009; Londo et al., 2010), as well as introgressed wild or weedy populations acting as sources of invasive individuals (Snow, 2002) via dispersal (Figure 1-1a,b). The invasiveness herein described might be accentuated by rapid evolution in the introgressed population (Ellstrand, 2003; Campbell et al., 2009). At another extreme, in a manner similar to that described in (Levin et al., 1996; Rhymer & Simberloff, 1996; Haygood et al., 2003), demographic swamping coupled with selection against the transgenes could lead to local extinction of native wild populations (Figure 1-1b). In addition, the introgression of transgenes into wild populations is of major concern in centers of diversity (Engels et al., 2006), because such a process could lead to gene pool modification, even in situations where selection on transgenic individuals is weak or nonexistent. Based on a lack of empirical evidence pertaining to the aforementioned potential negative outcomes, we posit that transgene escape and introgression will generally have little or no negative environmental or evolutionary consequences and only poses biosafety risks in specific cases, most notably when: (i) the associated traits confer novel or enhanced fitness or weediness, as increased weediness could necessitate new or increased weed control measures; and (ii) introgressed transgenes would confer a selective disadvantage in small wild relative populations existing in close proximity to the transgenic crop.

This review discusses recent molecular approaches to identify cases of introgression of crop alleles into wild and weedy relatives and also examines the few studies that have used molecular evidence to document actual transgene introgression, elaborating on why potential negative outcomes (Figure 1-1) have not been observed. We also review introgression management actions that target prerequisite conditions and transitions associated with the introgression process, and discuss the consequences of implementing such plans. Future research

directions are proposed that might assist with more effective documentation of transgene introgression and formulation of containment strategies.

1.3 The process of introgression and its documentation

Introgression can be defined as the permanent incorporation of genes from one population into the genome of another reproductively integrated population through a series of crossing and backcrossing events (Rieseberg & Wendel, 1993). With regard to crop plants and their wild and weedy relatives, others also stipulate that introgression does not include inadvertent and often genetically unstable chromosome transfer of genes (Chèvre et al., 1997). Introgression requires a set of initial conditions, followed by multiple processes occurring through time and space. In crop-to-wild and -to-weedy-relative systems, the introgression processes typically include hybridization and subsequent backcrossing events. If initial conditions are met, introgression might be possible; moreover, certain processes might be accentuated under variable initial conditions. For example, large crop populations (especially in the case of modern agronomic settings) can promote hybridization via copious pollen flow to individuals in smaller wild or weedy populations, especially if the related taxon is an obligate outcrosser, thereby making introgression more likely (Haygood et al., 2003; Wolf et al., 2001). Failure at pre- and post-initial-hybridization steps could preclude introgression (Stewart et al., 2003). Indeed, initial conditions and subsequent demographic steps encompass the targets of transgene containment strategies (see 'Transgene introgression management', below). In cases where initial conditions are met, monitoring and documenting subsequent steps, especially those involving hybridizing and backcrossing, is therefore necessary to definitively attribute the persistent presence of transgenic individuals in [formerly] wild or weedy populations to introgression.

The tactic used to document transgene introgression via molecular methods is usually different than that employed for non-transgenic crop-to-wild and crop-to-weed studies. As a single gene is often the subject of interest in transgene introgression, rather than a set of molecular markers widely dispersed over multiple chromosomes, molecular methods targeting diagnostic transgene sequences (or sequences of promoters or reporters), gene products, and their zygosity levels (homozygous versus heterozygous) are used to document initial steps (e.g. hybridization via transgenic pollen-mediated gene flow). Later, distinguishing between backcrossed transgenic progeny (i.e. actual introgression) and the presence of transgenic individuals due solely to seed dispersal will require nuclear and cytoplasmic diagnostic molecular markers (Reichman et al., 2006; Arnaud et al., 2009). Undoubtedly, complementing such work with data on selection assays and phenotypic/morphological corroborations will assist in definitively identifying cases of transgene introgression.

In principle, the above scenario of introgression has played out in non-transgenic crops and their wild and weedy relatives (Stewart et al., 2003). Recent work in this area places emphasis on the use of molecular markers to provide evidence of introgression (Table 1-1). Studies addressing and suggesting repercussions (e.g. fitness consequences) to introgression of nontransgenic crop genes in wild or weedy relative populations (Di Vecchi-Staraz et al., 2009; Snow et al., 2010) are rare; this may be due to assumptions that most domestication-associated genes would confer decreased fitness in wild populations (Stewart et al., 2003; Warwick & Stewart, 2005). Inferences of introgression in such cases are typically made on the basis of single "snapshots" of population genetic structure and the inclusion of alleles characterizing – though not necessarily diagnostic of – crop populations into wild or weedy populations. Studies utilizing Bayesian methods and admixture analyses have been useful in this approach, and have also

provided evidence for ruling out cases of recent introgression (Andersen et al., 2005; Konishi & Ohnishi, 2007; Li et al., 2010). However, long-term, multi-sample (i.e. longitudinal) efforts [e.g. Sørensen et al., 2007; Warwick et al., 2008) have been utilized rarely to document the multi-step process of introgression. Despite their shortcomings, the highlighted studies in Table 1-1 shed light on how selectively neutral transgenes could introgress into wild and weedy populations. It is also important to note that these examples of suggested introgression span the entire range of very low to high transgene introgression risk categories outlined in (Stewart et al., 2003) (even those crops posited as very low risk (Kuroda et al., 2006; Papa et al., 2005). Thus, although transgene introgression is likely to occur, automatic assumptions of negative outcomes should be tempered by the general lack of evidence of negative outcomes in cases of nontransgenic introgression.

Documentation of transgene introgression into wild or weedy populations has been even less substantiated. Arguably the most exemplary study involved quantifies the presence of transgene-affiliated herbicide resistance and species-specific amplified fragment length polymorphism (AFLP) markers in transgenic *Brassica napus* (crop) × *Brassica rapa* (weed) hybrids (parents of different ploidy levels and chromosome numbers) that have backcrossed over several generations with weedy plants in field margins (Warwick et al., 2008). By the end of the study, immunological testing using glyphosate-resistance test strips had confirmed a 1:1 segregation of the transgene conferring herbicide resistance in the offspring of an introgressed individual. Because this study of a natural weed population took place during a period of no herbicide resistance selective pressure, it illustrates a situation in which a transgene – originally presumed to be neutral in the absence of herbicide use (Warwick et al., 2009) – could introgress into a weed population. Similar results were found in an experimental system (e.g. greenhouse

and open-pollination) involving transgene introgression from wheat (*Triticum aestivum* L.; 2*n*=6x=42, AABBDD genome) into its weedy relative, jointed goatgrass (*Aegilops cylindrica* Host; 2*n*=4x=28, DDCC genome), in which the *bar* gene, along with wheat-specific sequence-characterized amplified regions, was molecularly documented in the progeny of a selfed backcross generation (Schoenenberger et al., 2006). Both examples profiled above involve interspecifc hybridization and introgression across a ploidy barrier, bringing into question the effectiveness of divergent ploidy levels as a mitigation strategy.

Rapid transgene spread has been documented recently for transgenic herbicide-resistant creeping bentgrass (*Agrostis stolonifera* L.) via sequence verification of the *CP4 EPSPS* transgene and analysis of nuclear internal transcribed spacer (ITS) and chloroplast *matK* gene trees in non-agronomic habitats, only two years after a confined experimental trial released the transgene in the environment (Reichman et al., 2006). The authors suggest that pollen-mediated intraspecific hybridizations, crop seed escape, and herbicide use all contributed to the spread of transgenic individuals into wild *Agrostis* populations. The final transgene introgression study having molecular evidence is the recent re-evaluation of the presence of transgenes (more specifically, the widely used constitutive CaMV 35S promoter region of the transgene of interest) in Mexican landrace populations of maize (*Zea mays* L.) (Piñeyro-Nelson et al., 2009a; Piñeyro-Nelson et al., 2009b; Schoel & Fagan, 2009); in this case, the presence of transgenes three years after the initial 2001 documentation (Quist & Chapela, 2001) of hybridization suggests that introgression could have occurred. We note here that the two previous examples of crop transgene introgression (involving *A. stolonifera* and *Z. mays*), although molecularly documenting transgenic individuals and hybrids in wild and weedy populations, did not

document both hybridization and backcrossing; hence, populations "infiltrated" by transgenic individuals via seed dispersal might be at the incipient stages of introgression.

The most severe ecological outcomes of transgene introgression (Figure 1-1) have not been observed. To the best of our knowledge, well-documented introgression of a transgene conferring a selective advantage into a wild population has yet to occur, and spatially new populations of invasive transgenic plants (wild or weedy) originating from transgene introgression have not been found. Thusfar, only experimental work has indicated that such scenarios are possible (e.g. backcrossed herbivore-resistant transgenic sunflower (*Helianthus annuus*) progeny exhibit higher fecundity measures than nontransgenic sunflower in settings mimicking wild populations subject to herbivore pressure (Snow et al., 2003)). Extinction of wild populations as the result of transgene introgression has not been documented. For weedy species, experiments have illustrated that herbicide-resistant and insecticide-resistant transgenic weed x crop hybrids can exhibit higher fitness than nontransgenic weeds in managed agronomic settings (Cao et al., 2009) and adjacent habitats receiving "herbicide drift" effects (Londo et al., 2010); whether such weeds are more likely to become invasive and spread throughout the landscape has not been documented. Owing to the series of steps and conditions necessary to arrive at the most severe outcomes of transgene introgression, such scenarios are highly unlikely. Transgene introgression management plans may therefore be most appropriate for cases in which significant fitness advantages and disadvantages conferred by the transgenic trait are postulated for a recipient wild or weedy relative population.

1.4 Transgene introgression management or mitigation strategies

Management strategies aimed at preventing transgene introgression are intimately tied with challenging the initial prerequisite conditions and subsequent events (Figure 1-2). Most of the strategies developed target pre-hybridization steps (Figure 1-2a); far fewer target post-hybridization events (Figure 1-2b, c; Table 1-1). In this section, transgene management strategies that have been integrated into transgenic crop plants are discussed, beginning with those whose actions serve to prevent hybridization, and concluding with those whose actions take effect post-hybridization. Potential negative consequences or pitfalls of the management strategies are also presented.

1.4.1 Male sterility

Pollen carrying a transgene is required in almost all transgene introgression models. Hence, transgene introgression could be completely prevented if pollen does not develop, and multiple methods have been used to decrease pollen fertility via genic or cytoplasmic male sterility. The first transgenic sterile male plant was generated by transforming tobacco plants with the chimaeric ribonuclease *TA29* gene (Mariani et al., 1990). Since then, several efforts have been aimed at developing genic male sterility in plants. These include using cytotoxic *barnase* gene expression in pollen or anthers of poplar (*Populus*) trees and *Kalanchoe blossfeldiana* (Wei et al., 2007; García-Sogo et al., 2010). Plant-derived cysteine proteases and a gibberellin-insensitive (*gai*) gene have also been used to induce genic male sterility in *Arabidopsis* (Konagaya et al., 2008), while inactivation of the *UDP-glucose pyrophosphorylase 1* (*UGPase1*) gene for flower development has resulted in a genic male sterile phenotype in rice (Woo et al., 2008). Such a strategy would be highly appropriate in crops whose primary economic purpose is not tied to

successful fruit or seed development (e.g. biomass crops). Since genic male sterility strategies inhibit development of anther or pollen, the lack of pollen could create negative impacts on pollen-feeding insects (Mlynárová et al., 2006).

Naturally occurring cytoplasmic male sterility (CMS), in which maternally inherited genes confer pollen-sterile plants, has been used heavily in plant breeding for hybrid seed production. More recently, genetically engineered CMS has been used in transgene biocontainment. This started with the successful genetic engineering of the tobacco (*Nicotiana tabacum* L.) chloroplast genome with the *phaA* gene coding for ß–ketothiolase, which is known to confer cytoplasmic male sterility (Ruiz & Daniell, 2005). Expression of an abnormal mitochondrial open reading frame *orf79* in rice has also resulted in CMS (Wang et al., 2006). Disruption of a nuclear gene *Msh1* that is responsible for the suppression of mitochondrial DNA rearrangement has caused heritable CMS in tobacco (*Nicotiana tabacum* cv. Xanthi) and tomato (*Solanum lycopersicum* cvs. Moneymaker and Rutgers) (Sandhu et al., 2007).

A potential drawback of using CMS as a biocontainment tool is the potential for transmission of the transgene from the cytoplasm to the nucleus. Transmission of paternal plastids and mitochondria from transplastomic pollen occurs at low frequency (10^{-4} to 10^{-5}), and even less frequent transmission is expected under field conditions (Svab & Maliga, 2007). Lower than 10^{-3} transmission rate of chloroplast DNA through pollen has been suggested as an acceptable level for release of transgenic crop plants for large-scale cultivation (Haywood et al., 2004). Lack of stability of the CMS system under different environments may be an issue, but the CMS in maize, especially as conferred by T and C type cytoplasm, has been shown to be stable under various environmental conditions in three different countries over multiple years (Weider et al., 2009). Moreover, pollen containment along with increased yield has been

documented in maize systems employing a combination of CMS hybrids and unrelated male-fertile maize plants (Munsch et al., 2010).

1.4.2 Delayed and decreased flowering

Synchronous flowering time, at least partially, between transgenic crops and wild relatives is a prerequisite condition for hybridization. The development of a different flowering time of transgenic crops to avoid synchronous flowering with wild or weedy relatives would halt hybridization, and a variety of gene-specific methodologies for shifting flowering time appears promising in this regard. Flowering time has been inhibited and delayed until vernalization treatment was applied by the overexpression of the *Flowering Locus C* (*FLC*) in *Arabidopsis* and *B. rapa* (Kim et al., 2007). Flowering was significantly delayed by a repressor of floral development *TFL1* from grapevine in tobacco (*Nicotiana tabacum* L.) and *Arabidopsis* (Boss et al., 2006). A putative CAATT-binding transcription factor has delayed flowering time in *Arabidopsis thaliana* under a long-day photoperiod (Cai et al., 2007). *FLC* overexpression in *Arabidopsis* resulted in not only delayed flowering time, but also increased biomass (Salehi et al., 2005); thus, this delayed flowering time strategy might be suitable for crops of primary interest in biomass, such as switchgrass (*Panicum virgatum* L.) for biofuel and bioproducts.

Delays in or prevention of flowering might be tied to important biochemical pathways (e.g. ascorbic acid (Barth et al., 2006)), and, further, the genes linked to synthesis of enzymes in these pathways could prove useful in pre-hybridization management strategies. For agronomic crops cultivated primarily for seed production, significantly delayed flowering could result in less seed production because of an inadequate flowering period. Since, flowers in a single individual plant generally do not develop at the same time, complete flowering delay without

overlapping time would require very significant delay for all flowers. A possibility of an overlap of flowering time could still exist between the earliest flower of a donor plant and the latest one of a recipient plant or vice versa.

1.4.3 Post-zygotic barriers to introgression

Even after successful hybridization between transgenic crops and wild or weedy relatives, undesired transgene introgression could be prevented if a transgene were inserted in a genomic region that would unlikely be introgressed into the genome of pollen recipients owing to linkage disequilibrium (LD). Transgene placement in loci, for example, that are located in crop genome regions conferring lower fitness and competitiveness would be negatively selected and unlikely to be transferred to a wild relative under selection pressure (Stewart et al., 2003). Many studies on LD in higher plants have been conducted (Gupta et al., 2005). It has been demonstrated that certain crop-specific alleles can introgress easily and persist in wild relatives, while other alleles cannot (Snow et al., 2010). Other studies have demonstrated preferred integration or insertion sites, including the existence of preferred DNA sequences for *Agrobacterium* T-DNA integration in *Arabidopsis* (Schneeberger et al., 2005), and the observation of biased transgene insertion into specific maize chromosomes using a site-specific recombination system-containing vector (Vega et al., 2008). However, LD does not only rely on the nature of T-DNA preference on integration sites. Advanced biotechnology allows targeted insertion of transgenes at pre-characterized loci (Li et al., 2009). Transgenes could be inserted at desired sites in a plant genome, because transgene insertion at a targeted locus is currently feasible using zinc-finger nuclease technology (Shukla et al., 2009; Cai et al., 2009). Indeed, site-specific integration of very large DNA fragments into any desired location has also been demonstrated by the expression of lambda-red

enzyme in *Escherichia coli* (Kuhlman & Cox, 2010). However, target-site-specific/LD-linked transgene introgression management strategies might not be constantly reliable owing to frequently occurring recombination in the genome.

Different ploidy levels could suppress transgene introgression via pollen movement (Sandhu et al., 2010). Intercrossing between individuals with different ploidy levels has resulted in dramatically reduced seed production with various phenotypic traits, including non-flowering hybrids in select grass species (Sandhu et al., 2009). While inter-ploidy hybrids (e.g. triploids resulting from crosses between diploid and tetraploid individuals) might be viable and contain the transgene, low hybrid fertility and aneuploidy resulting from backcrossing would lead to a low probability of introgression of the transgene into the wild population. However, a transgene conferring herbicide resistance in hybrids between tetraploid *B. napus* and diploid *B. rapa* has been successfully introgressed in backcrossed hybrids over several generations (Warwick et al., 2008). Also, the use of ploidy barrier as a transgene introgression management strategy is limited to cases where recombination is rare between different parental genomes (Stewart et al., 2003). Recombination and gene transfer, for example, has been shown to occur between A and C chromosomes in triploid hybrids of *B. napus* (AACC) and *B. rapa* (AA) (Leflon et al., 2006).

1.4.4 Transgene excision and mitigation

Transgene introgression could be effectively suppressed post-hybridization with the removal of the transgene from the pollen. Pollen-specific transgene excision using site-specific recombinases, such as Cre or FLP (Luo et al., 2007; Moon et al., 2010), is one method to create transgene-free pollen carrying only a non-coding recombination site. Efficient microspore-specific transgene excision has been demonstrated in tobacco (*Nicotiana tabacum* cv. Petit

Havana SR1) using Cre recombinase directed by a microspore-specific NTM19 promoter (Mlynárová et al., 2006). Other recombinases, including ParA and PhiC31, that have been shown to excise transgenes in plants, have the potential to be used for pollen-specific transgene excision [(Thomson et al., 2009; Kempe et al., 2010). This transgene excision in pollen might be considered as a side step of terminator technology. This strategy would allow continuous production of transgenic progeny seeds by using a pollen-specific promoter unlike the terminator technology that would not produce any seeds. Pollen would not carry any functional transgenes after transgene excision occurred; therefore, only half of the produced seeds would be transgenic by the presence of transgene in female gamete. This might be a possible pitfall of this trasngene excision strategy. However, a possible way to produce homozygous transgenic seeds for commercialization has been suggested with an incorporation of a conditionally expressed recombinase repression gene into the transgene excision strategy (Moon et al., 2010).

Alleviation of potential consequences of transgene introgression could be achieved by coupling a transgene with a mitigating gene, such as a dwarfing gene (Al-Ahmad et al., 2006), even after transgene introgression has successfully occurred in the wild relative genome. The mitigating gene should have positive or neutral effects to crops (e.g. increased biomass or seed production of dwarf plants in crop systems (Al-Ahmad et al., 2006)) and negative to weeds, because weeds would be rendered less competitive to compete for light (Gressel & Valverde, 2009). Transgenic *B. rapa* × *B. napus* hybrids containing a fitness-mitigating dwarfing gene has resulted in a significant decrease in the number of weedy progeny that persist through time under competitive conditions (Rose et al., 2009). Such an approach could constitute a *post de facto* mitigation of a potentially adverse transgene introgression in a wild or weedy population.

However, this transgene mitigation strategy would not be appropriate for gene transfer management from transgenic crops to non-transgenic crops growing in close proximity.

1.4.5 Selectively terminable transgenic lines

The creation of selectively terminable transgenic lines represents another strategy, as demonstrated in rice by the tagging of a gene of interest with an RNAi cassette that suppresses the bentazon detoxification gene *CYP81A6* (Lin et al., 2008). This has resulted in the creation of rice sensitive to a major herbicide, bentazon, to control weeds in a rice field (Beckie et al., 2010). Therefore, any possible hybrids outside of the field would be controlled by spraying bentazon during the conventional rice weed control process, even if transgene introgression were to occur in rice weedy relatives in or near the agronomic field.

1.5 Future directions

1.5.1 Documentation of the transgene introgression process

Research into transgenic crops is expected to increase dramatically: with the release of several new abiotic and biotic stress-tolerant transgenic crop lines and biofuel plant platforms. These traits will be the foci of future long-term monitoring programs because they have greater potential to alter plant fitness and to increase weedy or invasive tendencies (Warwick et al., 2009; Beckie et al., 2010) compared with traits in current commercial transgenic crops. Novel molecular strategies for monitoring and strategies for containment will also be foci of future studies. Monitoring approaches that survey transgenic crops and wild or weedy populations at critical steps along the introgression process could also provide empirical data for enhancement,

and evaluation and utilization, of population models (Thompson et al., 2003; Hooftman et al., 2005, 2007, 2008; Meirmans et al., 2009) of transgene introgression.

1.5.2 Novel transgene introgression management approaches

Several management strategies currently show promise for further development. For example, cleistogamy (i.e. a condition where flowers do not open and are instead self-pollinated in the bud) could be an effective strategy to prevent hybridization and transgene introgression. Increased utilization of cleistogamy is now possible in many agronomically valuable cereal crops by genetic engineering of class-B floral homeotic genes (Yoshida et al., 2007). Cleistogamous rice harboring such a missense mutation in the class-B MADS-box gene *SUPERWOMAN1* (*SPW1*) has been identified (Yoshida et al., 2007). Other possible management strategies of transgene introgression that should continue to be explored include the potential use of the *Ph*1 gene or molecular chaperone acting gene from wheat which is known to suppress recombination between homoeologous or homologous chromosomes. Prevention of transgene introgression into weedy relatives has been hypothesized using a transgene fused or linked with the wheat *Ph*1 gene (Weissmann et al., 2008).

It is clear from the empirical data reviewed in this article that many mitigation strategies, such as hybrid incompatibility and ploidy differences, earlier predicted to prevent transgene escape, are not individually foolproof. We should expect that even one in a thousand or one in a million probabilities will occur given the time and land area involved with agriculture. Therefore, it would be prudent to consider the incorporation of multiple biocontainment strategies within a transgenic crop. It will also be critical to evaluate the potential consequences of escape of the containment strategy and the likely effect on wild or natural weedy relatives.

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Appendix

Tables and Figures

Table 1-1 Recent (2005-2010) studies that provide molecular evidence of introgression from non-transgenic crops to their wild or weedy relatives.

Crop	Relative	Molecular marker[A]	Refs.
Cichorium intybus	*C. intybus*	AFLP	Sørensen et al., 2007
Glycine max	*Glycine soja*	SSR	Kuroda et al., 2006
Helianthus annuus var. *macrocarpus*	*Helianthus petiolaris*	RAPD	Gutierrez et al., 2010
Medicago sativa	*M. sativa*	AFLP, SSR	Greene et al., 2008
Oryza sativa	*Oryza rufipogon*	SSR	Song et al., 2006
Pennisetum glaucum	*P. glaucum*	SSR	Lewis, 2010
Phaseolus vulgaris	*P. vulgaris*	AFLP	Papa et al., 2005
Raphanus sativus	*Raphanus raphanistrum*	Allozyme	Snow et al., 2010
Sorghum bicolor	*Sorghum halepense*	RFLP	Morrell et al., 2005
Triticum aestivum	*Aegilops peregrine*	Fragment of noncoding locus	Weissmann et al., 2005
Vigna unguiculata	*V. unguiculata* ssp. *unguiculata* var. *spontanea*	RFLP	Feleke et al., 2006
Vitis vinifera	*V. vinifera* ssp. *silvestris*	SSR	Di Vecchi-Staraz et al., 2009
Zea mays	*Z. mays*	SSR	Bitocchi et al., 2009

[A]abbreviations: AFLP, amplified fragment length polymorphism; RAPD, randomly amplified polymorphism; RFLP, restriction fragment length polymorphism; SSR, simple sequence repeat.

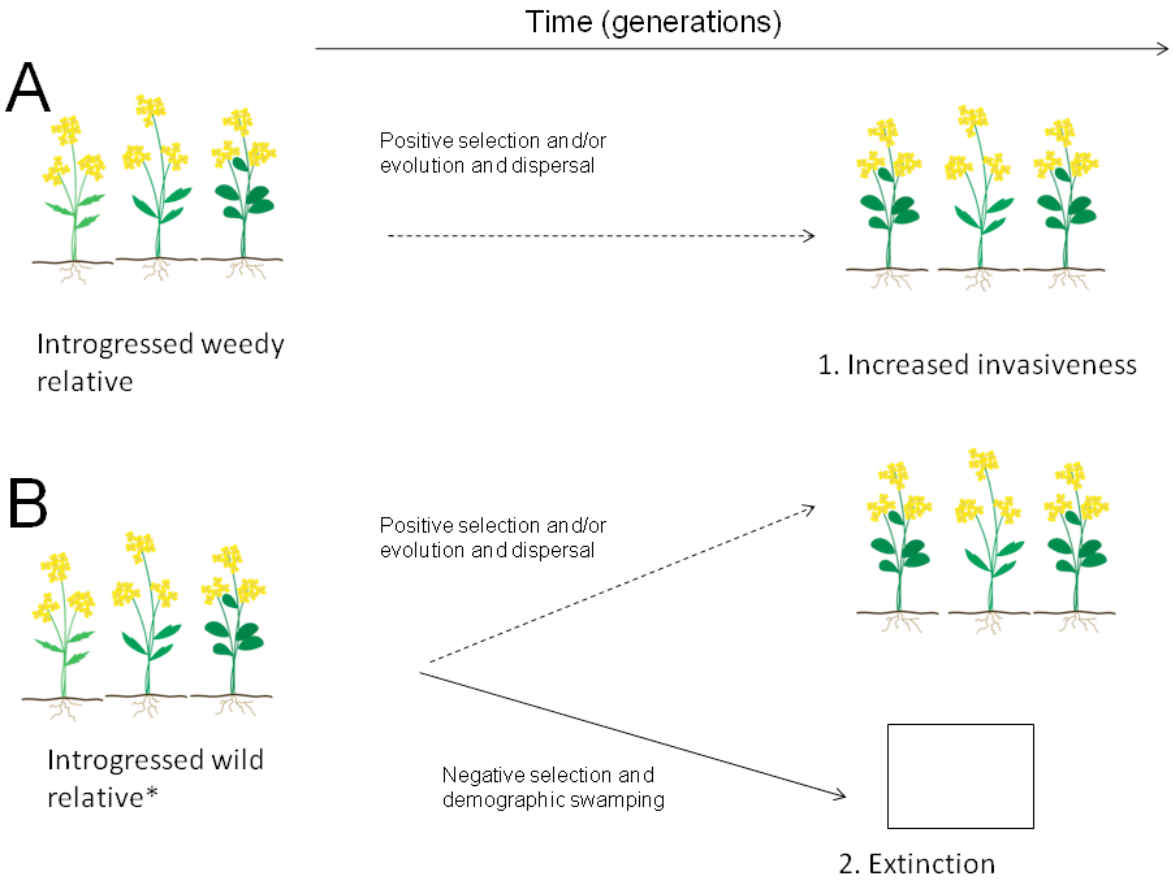

Figure 1-1 Two potential risks following transgene introgression from crops to their wild or weedy relatives.

(1) Invasive hybrid population from introgressed (A) weedy or (B) wild population (dashed arrow representing seed dispersal) brought about by positive selection and/or evolution; (2) extinction of wild relative population brought about by demographic swamping (e.g. copious pollen or seed dispersal from transgenic crops) and negative selection. We note that an introgressed co-existing weedy population may present problems for weed management when introgressed transgenes confer a selective advantage in the managed agronomic system (*), and that an introgressed wild population may also be of concern to managers of crop-wild relative genetic conservation (**).

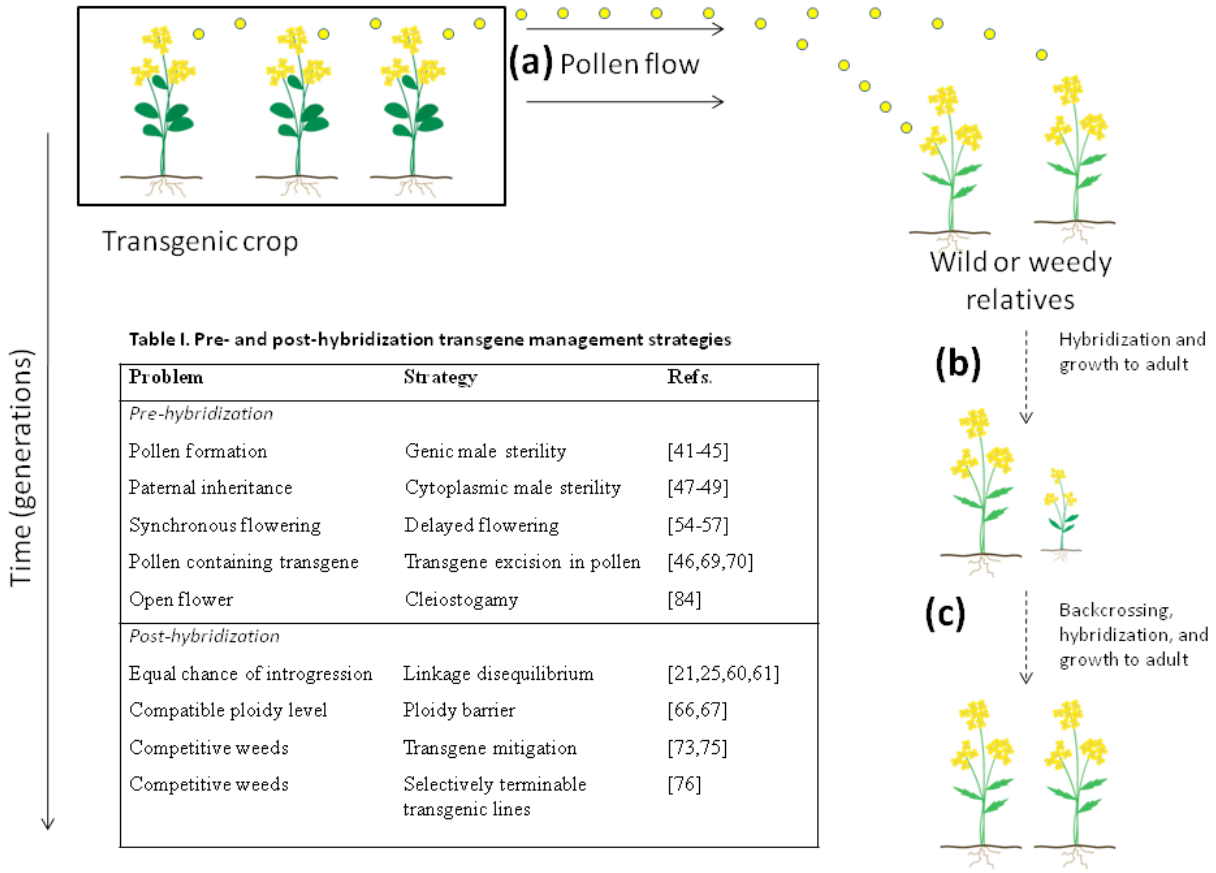

Table I. Pre- and post-hybridization transgene management strategies

Problem	Strategy	Refs.
Pre-hybridization		
Pollen formation	Genic male sterility	[41-45]
Paternal inheritance	Cytoplasmic male sterility	[47-49]
Synchronous flowering	Delayed flowering	[54-57]
Pollen containing transgene	Transgene excision in pollen	[46,69,70]
Open flower	Cleiostogamy	[84]
Post-hybridization		
Equal chance of introgression	Linkage disequilibrium	[21,25,60,61]
Compatible ploidy level	Ploidy barrier	[66,67]
Competitive weeds	Transgene mitigation	[73,75]
Competitive weeds	Selectively terminable transgenic lines	[76]

Figure 1-2 Stages along the portrayed transgene introgression pathway where transgene management strategies can operate.

Pre-hybridization strategies are aimed at preventing cross-pollination from occurring, and typically target the transgenic crop (a). Post-hybridization strategies are aimed at decreasing the fitness of resulting transgenic hybrids (b and c). Pictured is the scenario of transgene mitigation, wherein a transgene-linked dwarfing gene results in shorter plants that are selected against in the wild/weedy environment. All mentioned strategies are incorporated into the transgenic crop.

Chapter 2: Keeping the genie in the bottle: transgene biocontainment by excision in pollen

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Hong S. Moon was the principal author of the manuscript.

2.1 Abstract

Gene flow from transgenic plants is an environmental and regulatory concern. While biocontainment might be achieved using male sterility or transgenic mitigation tools, we believe that perhaps the optimal solution might be to simply remove transgenes from pollen. Male-sterility might not be ideal for many pollinators, and might not be implementable using standardized genes. Transgenic mitigation might not be useful to control conspecific gene flow (e.g., crop to crop), and relies on competition and not biocontainment per se. Site-specific recombination systems could allow highly efficient excision of transgenes in pollen to eliminate, at least minimize, unwanted transgene movement via pollen dispersal. There are other potential biotechnologies, such as zinc finger nucleases, that could be also used for transgene excision.

2.2 Introduction

Transgenic plants have played important roles in solving current agricultural problems, and hold even greater prospects of alleviating poverty and malnutrition in developing countries. For example, Golden rice containing high levels of β-carotene could be a great help for the people with vitamin-A deficiency (Al-Babili & Beyer, 2005). Currently over 3 billion people are under micronutrient malnourishment (Welch & Graham, 2004). Micronutrient deficiencies negatively impact human health and cause subsequent societal problems; nearly two-thirds of childhood deaths worldwide are directly caused by malnutrition (Welch & Graham, 2004). Transgenic plants have demonstrated benefits including higher yields, enhanced nutrients, and easier pest control (James, 2008). The Green Revolution has boosted crop yield in many parts of the world but Africa has not realized its benefits. In Africa, impediments such as insufficient water for irrigation and nutrient-depleted soils have resulted in low yields and often crop failure with

conventional plant varieties (Frison, 2008). Biotechnology has the potential to trigger drastically improved agriculture in Africa by adding traits, such as drought-, salt- or heat- tolerance traits.

Regardless of their potential benefit, biotechnology has not been fully exploited in very many crops, even in developed countries, because of regulatory and environmental concerns about gene flow. Following the example above, the large-scale deployment of Golden rice has largely been hampered because of concerns about gene flow to neighboring farms that do not currently contain transgenic plants and interference with existing vitamin A supplementation (Mayer, 2005). The concern that Golden rice could be grown in a country lacking sufficient biosafety regulations and monitoring capabilities coupled with potential gene flow from transgenic rice to weedy rice has stymied its cultivation (Lu & Snow, 2005). Ingo Potrykus, a principal developer and advocate of Golden rice, considers this long delay of its cultivation to be a serious moral downfall (Korth, 2008). In late 2008, Rockefeller Foundation promised its financial support for the deregulation process of Golden rice cultivations in several developing countries (Miller, 2009). It seems, however, that gene flow remains to be a significant regulatory hurdle.

In theory, gene flow could be prevented or rendered a negligible risk if strategies were realized that could contain transgenic traits within cultivated transgenic fields. Uncontrolled transgene escape to non-transgenic crop fields or sexually compatible wild relatives is a particularly important issue if transgene introgression is probable, or even possible, within a crop-wild system (Stewart et al., 2003). One especially problematic class of transgenes are those used for plant-made pharmaceuticals (PMP) that are expressed in transgenic food crops or species that are prone to gene flow; i.e., those that are outcrossers or with sexually-compatible wild relatives (Stewart et al., 2003). Trace adventitious presence of PMP transgenes in food

processed from other non-PMP transgenic crops is not acceptable by either regulators or food companies (Stewart, 2008). In 2002, highly stringent regulatory standard was applied to the biotechnology company ProdiGene for the presence of noncompliant PMP gene in the experimental field trials (Spök et al., 2008). Therefore, PMPs will most likely be the subject of even higher regulatory scrutiny with regards to gene flow than non-PMP transgenic crops with input traits. These and other biotechnological applications beg for effective methods for biocontainment.

Removal of transgenes from pollen and/or seeds could minimize gene flow problems. Transgene movement from transgenic to non-transgenic plants typically occurs most frequently via pollen dispersal (Conner et al., 2003). Therefore, for most plants, the first line of containment would be pollen, the long-distance vector for hybridization and introgression. To prevent pollen dispersal, formation of sexual reproductive organism can be simply suppressed under field conditions for some transgenic crops by harvesting leaves prior to flowering (Verma & Daniell, 2007). However, this is not practically useful method for most crop species because no seed production will be significantly disadvantageous. There are a number of biological transgene biocontainment tools that could eliminate, or at least minimize, unwanted transgene escape from transgenic to non-transgenic plant populations including wild-relatives or potential negative consequences of transgene flow (Daniell, 2002). These include male sterility and transgenic mitigation, while perhaps the most effective method would be the removal of transgenes from pollen using site-specific DNA recombinases.

While pollen is considered to be the primary agronomy-based vehicle for long-range gene dispersal, it is not the only one. Transgenic seeds can be dispersed as volunteers in the next season, during harvest, transport, and sometimes also mediated by animals. Compared with

pollen dispersal, seed dispersal is more predictable because it is most likely caused by human-mediated dissemination, which can be can be decreased with improved shipment and handling procedures (Stewart, 2004). However, transgene movement via pollen dispersal mediated by insects or wind is almost inevitable without an appropriate pollen biocontainment procedure in place.

2.3 Male sterility and chloroplast transformation as potential biocontainment tools

Male-sterility is one of the most commonly used transgene biocontainment systems in commercial fields. Mariani et al. (1990) generated male-sterile tobacco and canola using a mechanism that prevented pollen formation through the expression of chimaeric ribonuclease genes (Barnases). Male sterile plants are able to act as maternal parents and are fertilized with pollen from outside of the field. Hybrid seeds from crossing between male-sterile plants and wild relatives can acquire fertility restoration in successive generations via the Barstar gene (Mariani et al., 1992). Male sterility, however, might negatively affect many pollinators that acquire food and nutrients from pollen. For example, the survival of pollen beetle to adulthood was shown to be reduced in the absence of pollen compared to wild-type flowers (Cook et al., 2004). Cytotoxicity of Barnase gene expression resulted in ablation of tapetal cells and embryos of plants (Mariani et al., 1990; Kuvshinov et al., 2001). Furthermore, since Barnase toxins were shown to be cytotoxic in animal and human cell line models, their cell-specific expression to plants parts that are not consumed is required (Prior et al., 1996). However, even low amounts of cytotoxic genes, such as Barnases in non-targeted plants parts caused by leaky expression might negatively affect plant growth (Skinner et al., 2000). Considering the cytotoxicity and potentially

unregulated expression of Barnase that could result in cell death (Ramos, 2005), male sterility might thus not be the best choice for transgene biocontainment.

Cytoplasmic male sterility (CMS) is another method to effectively contain genes though maternal inheritance, which has been demonstrated by in proof-of-principle experiments in transgenic tobacco (Ruiz & Daniell, 2005). However, gene transfer from the cytoplasm to the nucleus occurs at high frequency (Martin, 2003; Stegemann et al., 2003). High transfer rate of integrated DNA from the cytoplasm to nucleus that would result in termination of maternal inheritance might not be an appropriate characteristic for reliable biocontainment systems (Huang et al., 2003). Fertility of cytoplasmic male sterility in *Petunia* could been restored by a nuclear gene expression (Bentolila et al., 2002). Also, abnormal morphology of flower part and poor nectar production in hybrid plants have been reported as unexpected consequences of some CMS systems (Jagannath et al., 2002). CMS systems have to surmount these downfalls to effectively play roles as transgene biocontainment systems.

Plant plastids and their genomes are exclusively maternally inherited in many crop species, thus transplastomic approaches could be effective in biocontainment of male gametophyte-mediated transgene flow. However, maternal inheritance is not universal, which limits the use of plastid transformation for pollen-targeted biocontainment (Hagemann, 2004). More than one-third of the species in angiosperm does not have a trait of strict maternal inheritance (Maliga, 2004). Also, efficient tissue culture and selection protocols that are required in order to obtain homoplastomic plants have not been established for most monocotyledonous species plants (Verma & Daniell, 2007; Maliga, 2004). It has been suggested that additional methods should be paired with plastid transformation to achieve complete transgene containment (Wang et al., 2004; Daniell, 2007).

2.4 Genetic engineering for transgene removal from pollen

With regards to pollen biocontainment, transgene removal is an alternative to male-sterility, which as mentioned above can be leaky, and to chloroplast transformation, for which maternal inheritance is typical (no transgenes in pollen), but nevertheless is rather difficult to accomplish in many plant species. One suggested possibility for this was the transgene removal using site-specific recombination as this simply cuts the transgenes from pollen (Keenan & Stemmer, 2002). In this approach, the entire transgenic construct could be flanked with recognition sites for the site-specific recombinase gene, introduced under the control of a tightly-regulated pollen-specific promoter (Fig. 2-1). Upon expression of the recombinase in pollen, the entire transgenic cassette is excised, leaving only a short recognition site in the mature pollen (Fig. 2-1). Similarly, zinc-finger nucleases (ZFNs), which can be specifically designed to bind and cleave target DNA sequences, could also be used to excise transgenes from pollen (Kim et al., 1996; Bibikova et al., 2002; Tovkach et al., 2009) (Fig. 2-2).

In contrast to male-sterility, a transgene removal strategy allows for normal production of pollen and fertilization, thus not adversely affecting the many flower-feeding herbivores. Concerns about re-integration of the excised transgene with reversible recombination systems including Cre-*lox* and FLP-*FRT* into pollen genome could be resolved with newly discovered non-reversible recombination systems, such as ParA-*MRS* or CinH-*RS2* (Thomson & Ow, 2006). The potential of this approach has already been demonstrated with the successful removal of integrated transgenes in plants, in particular selectable marker genes, using site-specific recombination systems (Dale & Ow, 1991; Schaart et al., 2004; Gilbertson, 2003). Furthermore, Luo et al. (2007) achieved dramatically increased efficiency of transgene removal in tobacco

when they combined *loxP-FRT* recognition sites with pollen-specific expression of either Cre or FLP recombinases compared to non-fused recognition sites of Cre-*lox* or FLP-*FRT* recombination system. Based on screening over 25,000 progeny per transgenic event, several transgenic tobacco events showed complete transgene excision from their pollen (Luo et al., 2007). Here, co-expression of both Cre and FLP recombinases actually decreased the efficiency of transgene removal, which might originate from a competition of the two recombinases to bind to adjacent recognition sites (Luo et al., 2007).

Transgene removal within a particular organ or tissue is made possible by judicious selection of tissue-specific promoters. Several pollen-specific promoters, such as LAT59 and LAT52 promoters from tomato (Twell et al., 1990; Twell et al., 1991), ZM13 promoter in maize (Hamilton et al., 1998), and DEFH125 promoter in *Antirrhinum* (Lauri et al., 2006) have been characterized as being only activated in pollen cells, with non-detectable activity in other tissues or developmental stages. A site-specific recombinase or ZFN driven by a pollen- or microspore-specific promoter might also be useful for transgene removal from pollen (Mlyunarova et al., 2006). Availability of several pollen-specific promoters from various sources might provide more chances to use the transgene removal system in many other crop species.

2.5 Regulatory and economic considerations

Recently, there has been a trend to decrease the amount of transgenic DNA in plants to the extent, which is absolutely necessary to deliver a trait, a development that has been embraced by both companies and regulators. The introduction of additional transgenes as means for biocontainment would thus run counter to this trend, except that biocontainment itself might be considered a valuable trait. From an economic perspective, sufficient benefits with regard to significant

biosafety gains or sustainability would be required to outweigh the additional costs for discovering and licensing of promoters and genes required for transgene removal. Such a transgene removal system that requires initial investment would likely be first deployed in those crops that are the greatest risks with regard to introgression to weedy wild relatives, such as sorghum (Stewart et al., 2003) and switchgrass (Stewart, 2007). However, once the system is established, transgene removal system in other marketable crops would be significantly cost effective compared to the cost for extensive monitoring and clean-up of accidental transgene contaminants. From a regulatory perspective, it is currently uncertain which decrease in transgene flow would constitute an acceptable risk. In addition, the components required for site-specific recombination would need to undergo a risk assessment analysis for various ecological and food safety parameters. In pollen, very little foreign DNA would remain after excision events as for example, transgene removal using the Cre-*lox* recombination system would leave just a single 34 bp *loxP* site in the pollen genome.

There are several choices of either well- or partially characterized transgene removal systems. The well-characterized Cre-*lox* that is derived from phage P1 and the yeast-derived FLP-*FRT* systems are both reversible, which potentially allows the transgene to reenter into the genome, although re-integration of the excised products has not been reported, likely because transgene excision is the preferable reaction in this system (Hare & Chua, 2002).

Non-reversible site-specific recombination systems are also available, such as ParA-*MRS* and CinH-*RS2*, which are both derived from the serine resolvase family of recombinases (Thomson & Ow, 2006). Transgene removal in plants by ParA recombinase that was derived from bacterial plasmids RK2 and RP4 has been shown to be precisely site-specific to excise an embedded sequence between the recognition sites (Thomson et al., 2009). CinH recombinase

derived from *Acetinetobacter* plasmids pKLH2, pKLH204, and pKLH205 has shown a site-specific gene excision function in yeast, but it has not yet been deployed in plants (Kholodii, 2001).

2.6 Perspectives

Transgene removal from pollen using site-specific recombination system could be an effective tool for transgene biocontainment; however, no system has so far been tested under agronomic conditions, or even in the field. Any transgene biocontainment system for commercial field application would likely be required to not be leaky and have no pleiotropic effects. As Luo et al. (2007) were able to achieve complete transgene excision from pollen using fusion recognition sites of *loxP-FRT*, it appears feasible to employ site-specific recombination as a transgene biocontainment strategy. Further experiments, including those in field settings are needed to increase sample sizes and confidence limits, and also to test for reversal in the bidirectional recombination systems Cre-*lox* and FLP-*FRT*. Non-reversible recombination systems, such as ParA-*MRS* and CinH-*RS2*, with their longer recognition site sequences might provide more reliable transgene removal while removing the possibility of potential transgene re-integration.

Homozygous transgenic seeds could not be produced with transgene removal using a site-specific recombination system. This could be disadvantageous for seed-propagated plants for commercial purposes. If a transgene removal system is completely efficient, transgenic seed production would rely on the presence of transgenes in eggs; i.e., the transgenic female parent (Fig, 2-3). Seeds from transgenic plants containing the transgene-removal trait by site-specific recombination in their pollen would either be hemizygous for transgenic trait or non-transgenic. Practically speaking, half of the seeds containing no transgenic traits could be eliminated for

commercial purposes by soaking seeds in a selection agent or by post-germination selection (Conner & Christey, 1997). However, homozygous transgenic seeds could be produced if a conditionally expressed recombinase repression gene is incorporated into the transgene removal system (Conner & Christey, 1997; Li et al., 2007). In this case, expression of the recombinase gene will be conditionally suppressed in pollen and seed at generations, in which transgenes need to be maintained, e.g., in breeding stock (Fig. 2-3) (Li et al., 2007).

Our view on future perspectives on commercial use is cautiously optimistic. Transgenic tobacco plants with lab-effective site-specific recombination system containing the fused *loxP/FRT* recognition sites (Luo et al., 2007) are currently being tested under agronomic conditions to test the efficacy of transgene removal in pollen in the field. We are also testing multiple systems in *Brassica napus* (canola); again to be challenged under field conditions. If one or more systems perform as well in the field as they do under more controlled conditions, they could then be good candidates in a commercially-vectored system and applied to transgenic crops that could otherwise be delayed by regulatory issues. Of special interest is application to crops never before considered for transgenic release such as outcrossing grasses for bioenergy productions in the foreseeable future. Transgene removal from pollen using site-specific recombination may be the best choice as an environmentally friendly biocontainment strategy with high efficiency.

2.7 Acknowledgements

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Appendix

Figures

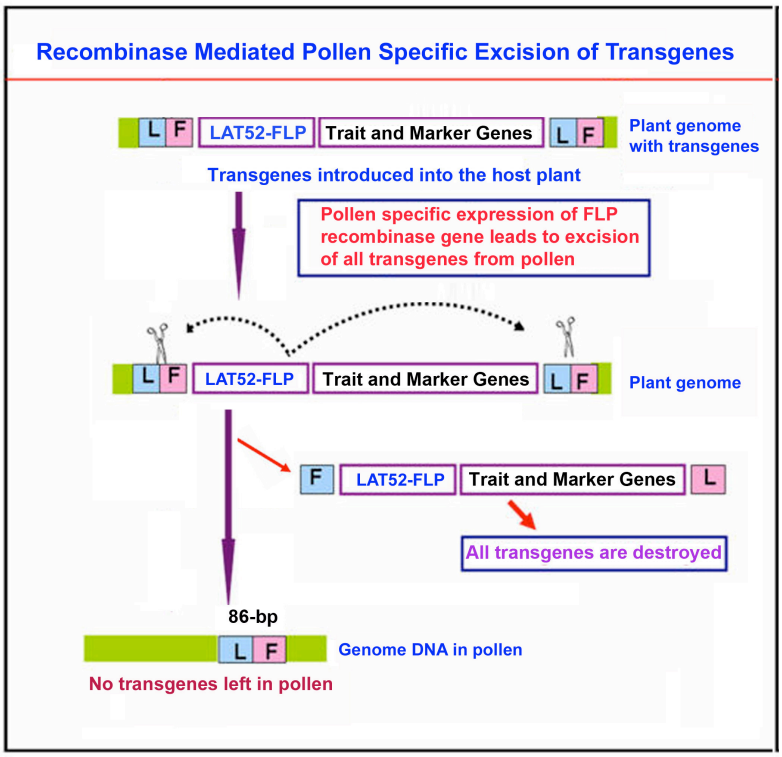


Figure 2-1 Principle of the gene excision of transgenes from pollen.

Here, L represents the *loxP* recognition sequence from the phage Cre/*lox* system and F represents the *FRT* recognition sequence of the yeast FLP/*FRT* system. LAT52 is a pollen-specific gene promoter from tomato (Twell et al., 1990; Belostosky & Meagher, 1996). FLP is a DNA recombinase from the FLP/*FRT* system. Expression of FLP under the control of the LAT52 promoter leads to deletion of all transgenes between the two LF (*loxP-FRT* fusion) sites, including the recombinase gene in pollen specifically. The excised gene sequences will be destroyed by non-specific nucleases present in the cell. This figure is reprinted from Reference 52 with permission of the copyright holders.

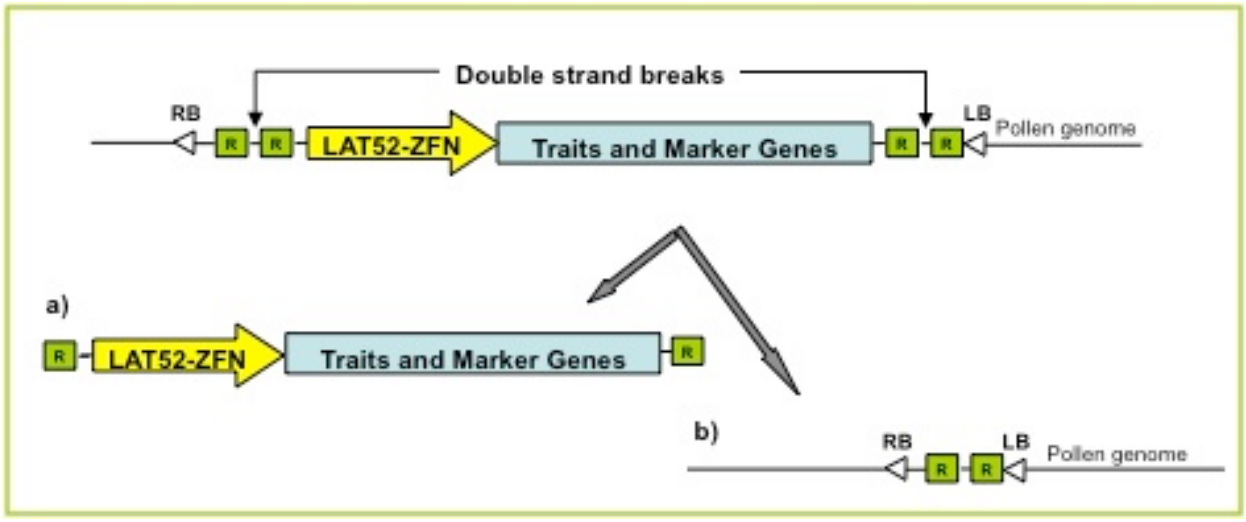
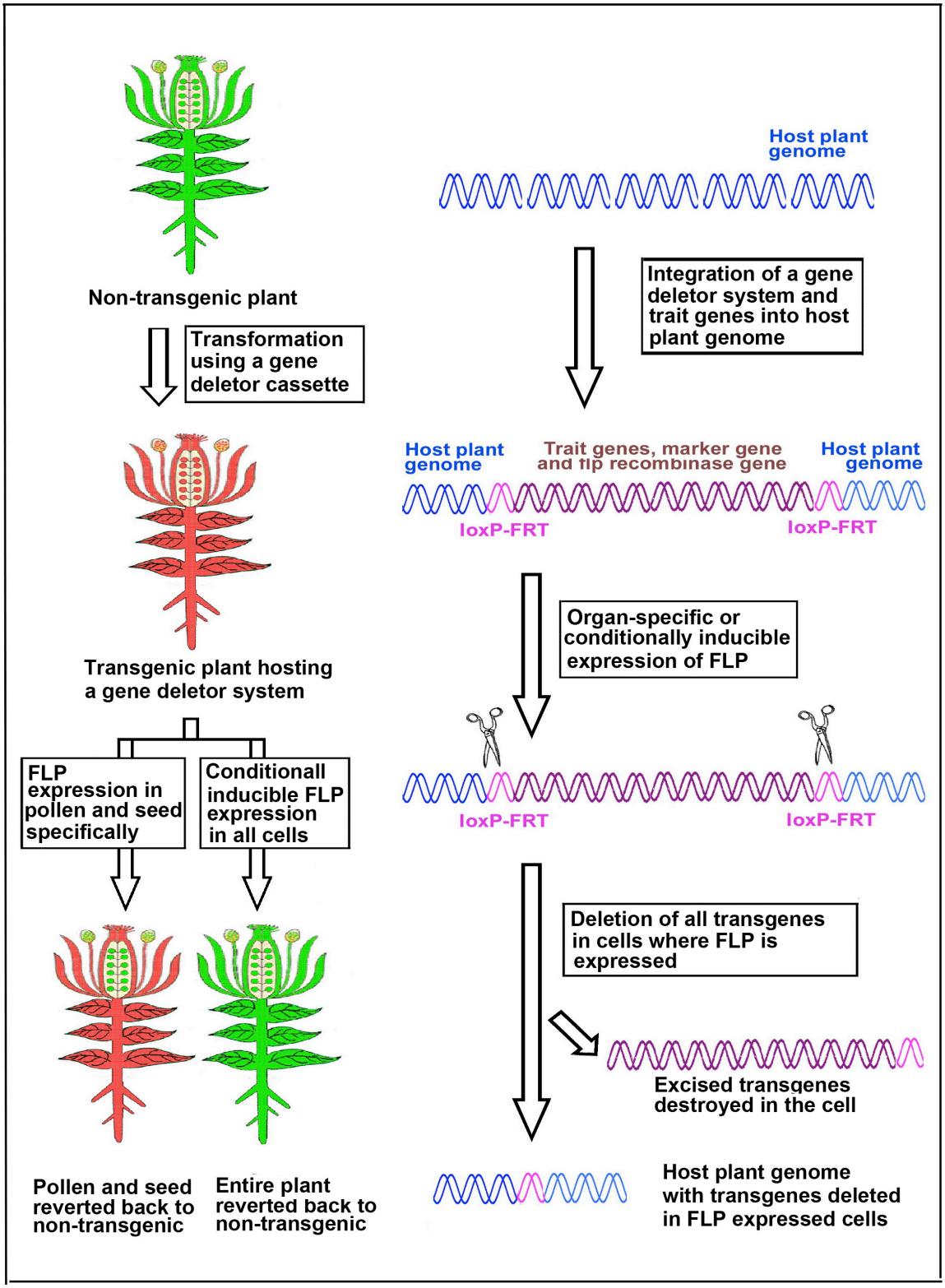


Figure 2-2 Zinc finger nuclease (ZFN)-mediated transgene excision from pollen.

ZFN expression under the control of a pollen-specific promoter LAT52 (LAT52) creates a double strand break in the spacer region between two adjacent ZFN recognition sites (R) forming one set of ZFN sites. This results in (i) one DNA fragment containing the functional transgenes including trait and marker genes that have been excised from pollen genome and which are destroyed in the cell; and (ii) the pollen genome with only one set of two adjacent ZFN recognition sites, which by itself, is non-functional.

Figure 2-3 Schematic illustration biocontainment using a gene deletor system.

The left half of the illustration shows how the use of a gene deletor system can be used to produce non-transgenic pollen, seed or plants from a transgenic plant. The right half shows that, if all transgenes, such as trait genes, marker gene and FLP or Cre recombinase gene, are inserted into the two *loxP-FRT* sites (86 bp in length), these DNA sequences should be deleted from cells in which the recombinase is expressed. When a pollen- and seed-specific gene promoter is used to control recombinase expression, all functional transgenes should be deleted from these specific organs. When a conditionally inducible gene promoter, such as a chemically inducible or high-temperature inducible gene promoter, is used to control recombinase expression, all functional transgenes should be deleted throughout the plant on application of the inducer. This figure is modified from Figure 5 in Luo et al. (2007), which is used with the permission of the copyright holder.


Non-transgenic plant
Transformation using a gene deletor cassette
Transgenic plant hosting a gene deletor system
FLP expression in pollen and seed specifically
Conditionall inducible FLP expression in all cells
Pollen and seed reverted back to non-transgenic
Entire plant reverted back to non-transgenic
Host plant genome
Integration of a gene deletor system and trait genes into host plant genome
Host plant genome
Trait genes, marker gene and flp recombinase gene
Host plant genome
loxP-FRT
loxP-FRT
Organ-specific or conditionally inducible expression of FLP
loxP-FRT
loxP-FRT
Deletion of all transgenes in cells where FLP is expressed
Excised transgenes destroyed in the cell
Host plant genome with transgenes deleted in FLP expressed cells

Chapter 3: An efficient and rapid transgenic pollen screening and detection method using flow cytometry

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Hong S. Moon designed, executed and analyzed the experiments and drafted the manuscript.

3.1 Abstract

Pollen is a major vector from which transgenes flow from transgenic plants to non-transgenic plants or wild relatives. Assaying for transgenic pollen provides valuable information and essential data for the study of gene flow and assessing the effectiveness of transgene containment and is also useful in ecological and population biology research. Most studies have employed microscopic screening methods or progeny analysis to estimate the frequency of transgenic pollen. Microscopic methods are time-consuming and laborious when large numbers of pollen grains must be analyzed, which is certainly the case in looking for rare transgenic pollen grains amongst predominantly non-transgenic pollen. Progeny analysis requires collection and screening of next generation seeds to infer pollen flow, which is neither time sensitive or completely accurate. Thus, there is an urgent need for the development of a simple, rapid, and high throughput analysis method for transgenic pollen analysis. In this study, our objective was to determine the accuracy of using flow cytometry technology for transgenic pollen quantification in the relevant case where transgenic pollen is not frequent. A suspension of non-transgenic tobacco pollen was spiked with known amount of verified transgenic tobacco pollen synthesizing low or high amounts of green fluorescent protein (GFP). The flow cytometric method detected approximately 75% and 100% of pollen grains synthesizing low and high amounts of GFP, respectively. Our data indicate that this flow cytometric method would be useful for the study of gene flow and assessment of transgene containment and is rapid, able to count 5000 pollen grains per minute-long run.

3.2 Introduction

Gene flow from transgenic crops to non-transgenic wild relatives continues to be a pertinent subject in biotechnology risk assessment. Transgenes can be vectored in pollen or seed and less frequently asexually, but most regulatory and ecological concern is centered on pollen-dispersed transgenes because of the potential for long-distance pollination (Rieger et al., 2002). In addition to risk assessment and gene flow studies, pollen also has been studied for tracking the movements of insects in local landscapes as well as long-distance moth migration routes (Silberbauer et al., 2004; Westbrook et al., 1997). To study pollen movement, several different direct pollen labeling or tagging methods have been developed. Microtags that are small self-adhesive microfilms have been used in orchid to mark pollinium, a coherent mass of pollen that is transferred during pollination as a single unit, and to also monitor the stigma to measure pollen transfer (Nilsson et al., 1992). The microtag method is limited to a few species including orchids that have pollinia for pollination. Radioactive labels including iodine (^{131}I), phosphorous (^{32}P), and carbon (^{14}C) were used to isotopically tag pollen to track individual pollen grains (Reinke & Bloom, 1979). However, releasing radioactive labels in the environment is less than ideal. Marker genes including β-glucuronidase (GUS) and green fluorescent protein (GFP) are valuable tools to visualize pollen grains for tracking movement. GFP-tagged transgenic pollen grains can be differentiated from non-transgenic pollen using epifluorescence microscopy (Hudson et al., 2001; Moon et al., 2006). Indirect methods of monitoring pollen movement, such as the screening of herbicide/antibiotic resistant progeny seeds or reporter gene expression in progeny plants has been used in many previous studies (Scheffler et al., 1993; Lavigne et al., 1998; Halfhill et al., 2004; Manshardt et al., 2007). However, progeny analysis does not provide direct

evidence of pollen movement and is time-consuming, requiring a generation to pass prior to estimation (Moon et al., 2007).

Transgene containment and unintended movement of transgenes are of great interest to regulators and scientists. Various biological transgene containment methods have proven to be effective in some plant species (Mariani et al., 1990; Ruiz & Daniell, 2005; Luo et al., 2007). Transgene removal systems are considered to be among the most environmentally friendly and potentially effective biocontainment strategy (Moon et al., 2010). Transgene removal systems have shown efficient removal of transgenes in pollen and/or seeds in tobacco (Luo et al., 2007). Luo et al. (2007) screened and detected GUS-positive progeny seedlings from self-pollinated transgenic plants or cross-pollinated plants between the transgenic plants with non-transgenic plants. This progeny analysis was time-consuming and laborious. Direct pollen analysis for marker genes such as GFP would be much faster than progeny analysis because it can be done in the current generation of plants to be assayed and pollen itself is analyzed. However, even when using GFP, assaying large numbers of pollen grains is tedious using epifluorescence microscopy. Pollen has been screened and quantified using three major methods including manual counting by human eye, image analysis, and electronic or laser-based particle counters (Costa & Yang, 2009). Pollen screening by human eye is normally performed using microscopy. This manual pollen screening is labor intensive, time-consuming, and error-prone from fatigue and other human error (Costa & Yang, 2009). Transgenic pollen screening via microscopy is subjective and inaccurate data could be collected because of heterogeneous synthesis levels of the fluorescent protein. There are several image processing analyzers that can be utilized for pollen screening and quantification (Costa & Yang, 2009; Fronseca et al., 2002). However, no image processing analyzer has the proven capability to distinguish transgenic pollen from non-

transgenic pollen. Electronic particle counters may underestimate actual number of pollen grains from pollen clumping and random dust particles (Kearns & Inouye, 1993). A laser-optics instrument has been developed for automatic counting of airborne pollen in the field (Kawashima et al., 2007). This airborne pollen counting system had a number of problems including misrecognition of non-pollen particle and no distinction of different kinds of pollen when they had similar measuring characteristics (Kawashima et al., 2007).

Flow cytometry (FCM) is a powerful laboratory laser-based technology that is used in a broad range of applications including ploidy analysis, estimation of nuclear DNA content, cell-cycle analysis, cell counting, fluorescent protein expression analysis and cell sorting (Galbraith, 1990; Sandoval et al., 2003). The possibility of the use of FCM was suggested in automated scoring of pollen mutants (Pinkel, 1981), Clumps of pollen grains are always problematic for currently available pollen screening methods (Kearns & Inouye, 1993). However, FCM allows users to digitally distinguish and exclude large particles including pollen clumps during assays. In many experiments, researchers are interested in finding a few transgenic pollen grains in a pool of mostly non-transgenic grain population. Here, we examined whether an FCM method can be useful in transgenic pollen assessment to count the frequency of GFP-positive pollen grains.

3.3 Materials and methods

3.3.1 Vector construction

ParA_Drec and CinH_Drec vectors (Fig. 3-1) were constructed as control vectors for ParA and CinH recombination vector, respectively (unpublished). These vectors are identical except for

the binding sites of corresponding recombinases. Recombinase ParA or CinH was excised from the respective recombination vector. Each vector contained the *eGFP* gene driven by the pollen specific *LAT59* promoter (Twell et al., 1990) that we used in previous studies (Hudson et al., 2001; Moon et al., 2006). A glufosinate ammonium-resistance gene, Bar, was used as a selectable marker and driven by nopaline synthase (*NOS*) promoter.

3.3.2 Plant transformation

Agrobacterium-mediated tobacco (*Nicotiana tabacum* L.) transformation was performed using published methods (Horsh et al., 1985). In short, the vectors were mobilized into *Agrobacterium tumefaciens* strain EHA105 by freeze-thaw method and transgenic plants were produced containing the constructs of interest. Tobacco seeds were sterilized with 10 % bleach and 70 % ethanol. Sterilized seeds were grown on MS medium (Murashige & Skoog, 1962) containing B5 vitamins. Tobacco leaves were cut into 1 -1 .5 cm^2 and co-incubated in *Agrobacterium* for 30 min. Infected leaf explants were placed on antibiotic-free DBI medium containing 1mg/L of indoleacetic acid for tobacco shoot organogenesis and co-cultivated for 48 hr. The explants were then transferred to selective DBI medium containing 5 mg/L glufosinate ammonium and 400 mg/L timetin. Regenerated shoots were removed from each callus and moved to MS media for root development. All cultures were maintained at 24 ± 2 °C under a 16/8 h light/dark photoperiod. Rooted shoots were transplanted to soil and acclimated for 2 weeks. More than 10 T_0 events for each vector construct were regenerated and confirmed by polymerase chain reaction (PCR) and pollen was screened using epifluorescence (FITC filtered) microscopy (Olympus BX51 model) with blue light excitation at 200x

magnification. T_1 seeds that were produced by self-pollination were harvested and selected on MS medium containing glufosinate ammonium at 5 mg/L. Selected T_1 seedlings were grown in the greenhouse and homozygous lines were selected by screening pollen using epifluorescence microscopy. One high-synthesis GFP event from the CinH_Drec vector and one low-synthesis event from the ParA_Drec vector construct were selected based on visual screening and homozygous T_1 plants were grown in the greenhouse. T_2 seeds from selected homozygous lines were harvested and confirmed by progeny analysis using herbicide-containing selection media.

3.3.3 Pollen preparation

Eight plants of each non-transgenic, high and low GFP synthesis tobacco events were grown in the greenhouse. Each plant type was grown at 2 m distance from other plant types to prevent potential cross-contamination. Pollen grains were collected from mature flowers by tapping pollen from flowers into 1.5 ml tubes. Collected pollen was frozen in a -80°C freezer immediately after collection. At the time of assay, 1 ml of sterile water was added into each tube containing pollen and the tubes were shaken in a mixer (Eppendorf 5432 mixer) for 10 min to suspend pollen in the water. The pollen suspension was filtered with a 132 μm pore nylon mesh (Sefar Nitex 03-132/43, Sefar filtration Inc., Depew, NY, USA) to remove anthers and clumps of pollen that could clog the fluidic system of FCM. Filtered pollen was transferred into 5 ml polystyrene round bottom tubes (BD falcon, San Jose, CA, USA) for FCM experiments. The concentrations of pollen samples were determined by measuring optical density (OD) with a spectrophotometer (Nanodrop 2000,

Thermo Scientific, Wilmington, DE, USA). For each FCM experiment, 7 μl of respective transgenic pollen sample was added into 693 μl of non-transgenic pollen. Each FCM experiment was repeated 3 times.

3.3.4 FCM parameters

Pollen suspensions were analyzed by using a LSR II flow cytometer (BD Biosciences, San Jose, CA, USA) with the photomultiplier tube voltages set at 200 eV for forward scatter channel (FSC), 190 eV for side scatter channel, and 468 eV for FL1 channel. The flow cytometer is equipped with a coherent sapphire 20 mW solid state 488 nm laser for the FL1 channel. GFP fluorescence was detected by using 505LP dichroic mirror and 530/30 bandpass filter. The threshold was set at 72,500 on forward scatter channel to exclude relatively small (likely immature) pollen grains in the suspension. The flow rate was set to 1.25 μl/second. Data, obtained by counting 5,000 particles for each sample, were analyzed using DiVa software (BD Biosciences, San Jose, CA, USA) and CyflogicTM software (CyFlo Ltd, Finland).

3.4 Results and discussion

One expects variable amounts of GFP to be synthesized among different transgenic events, but relatively homogeneous synthesis levels among pollen grains within any single transgenic event (Moon et al., 2007). With respect to GFP synthesis, one high and one low event were chosen to test the sensitivity and robustness of FCM for transgene assessment. All T_2 seeds from the

selected high and low GFP synthesis events were positive for the glufosinate ammonium selection, which confirmed both selected events were homozygous for the GFP gene. The variation of GFP synthesis of the transgenic pollen grains was confirmed by using the epifluorescence microscopy (Fig 3-2A-C) and by the FCM analysis (Fig. 3-2D-F). GFP synthesis levels between two events were observed under microscopy (Fig. 3-2B and C) and measured by FCM (Fig. 3-2E and F).

The non-transgenic pollen sample, which should not have any transgenic pollen, contained a small percentage of fluorescent particles (Fig. 3-2D; Fig. 3-3A and D). The fluorescent particles in the non-transgenic pollen suspension could be autofluorescent dust particles with similar size of pollen grains. Another possibility is that transgenic pollen grains in the previous measurement were retained in the fluidic system and carried over into the measurement of non-transgenic pollen. These are two potential problems of the proposed method. In addition to these possibilities, non-transgenic pollen might be contaminated with GFP synthesizing pollen during pollen collection process. Extensive flushing of the fluidic system in FCM between measurements reduced the number of GFP positives in non-transgenic pollen sample (data not shown). However, fluorescent particles in non-transgenic pollen could not be completely removed. Optical density at 600 nm (OD600) of non-transgenic, low GFP synthesis, and high GFP synthesis pollen suspensions were 0.081, 0.027 and 0.036, respectively. Since transgenic pollen suspensions were diluted 100 times (v/v) with non transgenic pollen suspension, the ratios of high and low GFP synthesis pollen to non-transgenic pollen were expected to be 3.6×10^{-2}(OD600 for high GFP) $\times 100^{-1}$ (100 times dilution) $\times 100 / 8.1 \times 10^{-2}$ (OD600 for non-transgenic) = 0.44% and 2.7×10^{-2} (OD600 for low GFP) $\times 100^{-1} \times 100 / 8.1 \times 10^{-2} = 0.33\%$, respectively. By using the FCM-based method, the small number of transgenic pollen in the

mixture was detected as GFP positive particles (Fig. 3-3B and C). The detection rates of low and high GFP synthesis pollen were 0.253% (SD=0.025) and 0.463 % (SD=0.050), respectively (Fig. 3-3D), indicating that approximately 75% (0.25/0.33 × 100 = 75.75%) of low GFP pollen and 100% (0.46/0.44 × 100 = 104.54%) of high GFP pollen were detected by this method. Slightly higher than 100% transgenic pollen detection was resulted with high GFP pollen sample. This anomaly might be explained by inhomogeneous pollen suspension from heavy individual tobacco pollen grains that tend to sink immediately after homogenization of pollen suspension by vortexing. Concentrations of transgenic pollen samples that were added to non-transgenic pollen might be slightly different, even though the samples were consistently pulled from the middle of the tube immediately after vortexing.

The lower detection rate for low GFP synthesis event pollen was expected because there was a large fluorescence overlap between non-transgenic pollen and low GFP synthesis transgenic event (Fig. 3-2D and E). – i.e., about 25% of low GFP pollen fell into the range of non-transgenic pollen population, presumably because of low fluorescence. Conversely, high GFP event transgenic pollen grains that have more than 99% of pollen grains in a range of GFP positive fluorescence (Fig. 3-2F) were accurately quantified by the FCM-based method. Since expression of *gfp* is positively correlated with that of the gene of interest (Halfhill et al., 2003), high GFP synthesis events would most likely be selected for use in field trials.

FCM is a high throughput analysis system and is capable of analyzing thousands of particles per second. Five thousand pollen grains were analyzed per each run within a minute. No other pollen screening methods are able to count this many pollen grains within a short time with high accuracy. This feature of rapid analysis by FCM is advantageous because, if the analysis

takes hours to complete, pollen in liquid solution would tend to germinate and GFP synthesis would be obscured because of GFP dilution in the cytoplasm (Hudson & Stewart, 2004).

In addition to counting a genetically-encoded fluorescent protein, this FCM technique could be adaptable to pollen or spores that have been tagged using other methods, such as painted, immunologically-tagged, or natural pigment variants. It should be possible to also sort on the marker to recover pollen of interest for other purposes.

3.5 Concluding remarks

Rapid detection and counting of relatively rare transgenic pollen in a mixture of predominantly non-transgenic pollen is necessary for efficient and reliable transgenic pollen screening and detection. FCM-based transgenic pollen screening is not only less laborious than other methods, but also extremely fast and accurate for high GFP-synthesis events. Transgene containment strategies and other transgene flow studies can be accurately and efficiently analyzed with this FCM-based method. Since high levels of expression of fluorescent marker genes would be expected in transgenic pollen containing a transgene removal system in the fields, the efficiency of the transgene removal system could be determined in a fast and accurate manner by analyzing large numbers of pollen using FCM. Also, if floating pollen grains in the air need to be analyzed for transgenic pollen tracking, collected pollen could be effectively analyzed for transgenic pollen detection by the FCM-based method. Even though transgenic plants do not have a built-in fluorescent marker for pollen, fluorescently labeled surface markers such as an antibody or quantum dot could possibly be employed and utilized for transgenic pollen detection. Fluorescent background normalization to control for non-pollen fluorescent particles could be

accomplished in a number of ways including cell sorting, where the small contaminant could be visually verified under microscopy. Alternatively, Even though it is not used in this study, highly autofluorescenced non-pollen particles that were possibly in non-transgenic pollen can be identified with a ImageStream (Amnis Corp., Seattle, WA, USA) that captures images of each cell in a stream of fluid in a FCM.

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Appendix

Figures

Figure 3-1 Vector construct used for plant transformation.

The pollen-specific *LAT59* promoter (*LAT59*) [21] controls the expression of *eGFP* gene. *Bar* gene was under the control of nopaline synthase promoter (*NOS* P). LB: left border, RB: right border, RS: recognition site of respective recombinase ParA or CinH, *35S* T: *35S* terminator, *NOS* T: nopaline synthase terminator.

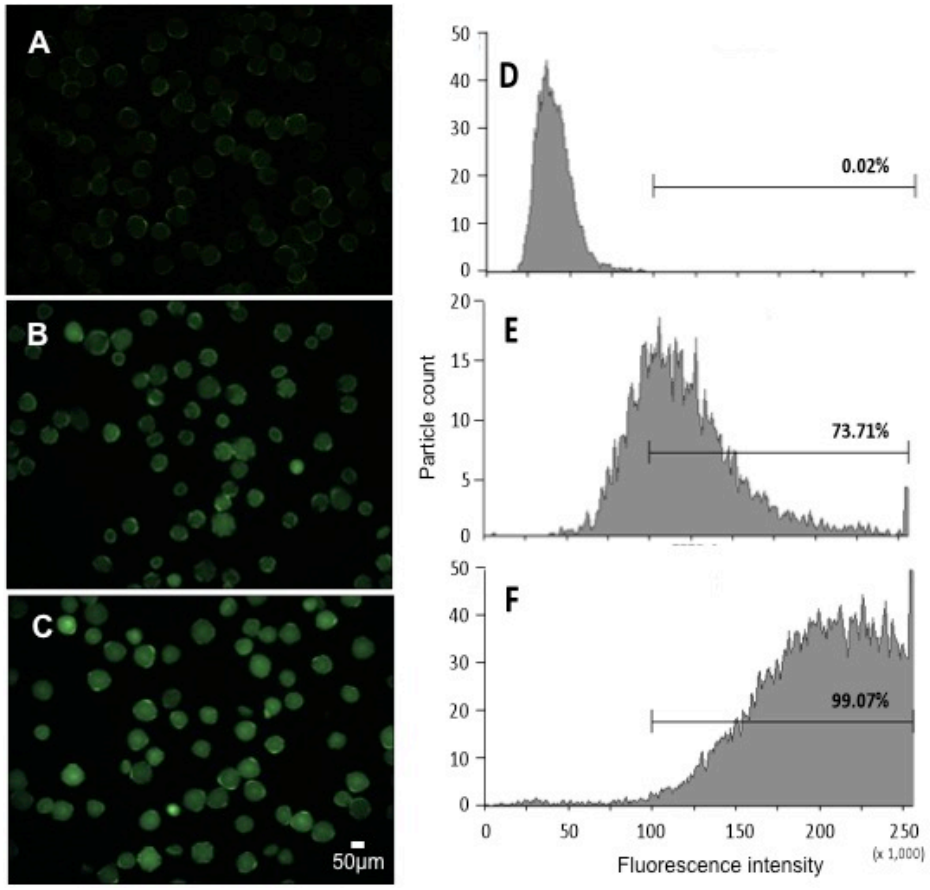

Figure 3-2 Different GFP synthesis levels of pollen grains under epifluorescence microscopy and flow cytometric measurements.

All images were taken under a FITC filtered epifluorescence microscopy with blue light and 3 s exposure time at 200x magnification. (A-C) Microscopic images of pollen grains. All microscopic images were taken under blue light at 200x magnification. (A) Non-transgenic pollen, (B) Low GFP event pollen, (C) High GFP event pollen, (D-F) Flow cytometric measurements of pollen, (D) Non-transgenic pollen, (E) Low GFP event pollen, (F) High GFP event pollen. Each bar in (D, E, F) represents a range of GFP positive fluorescence that is differentiated from non-transgenic pollen autofluorescence. Percentage of particles in a range of GFP positive fluorescence (D, E, F) in each sample. Fluorescence is in arbitrary units.

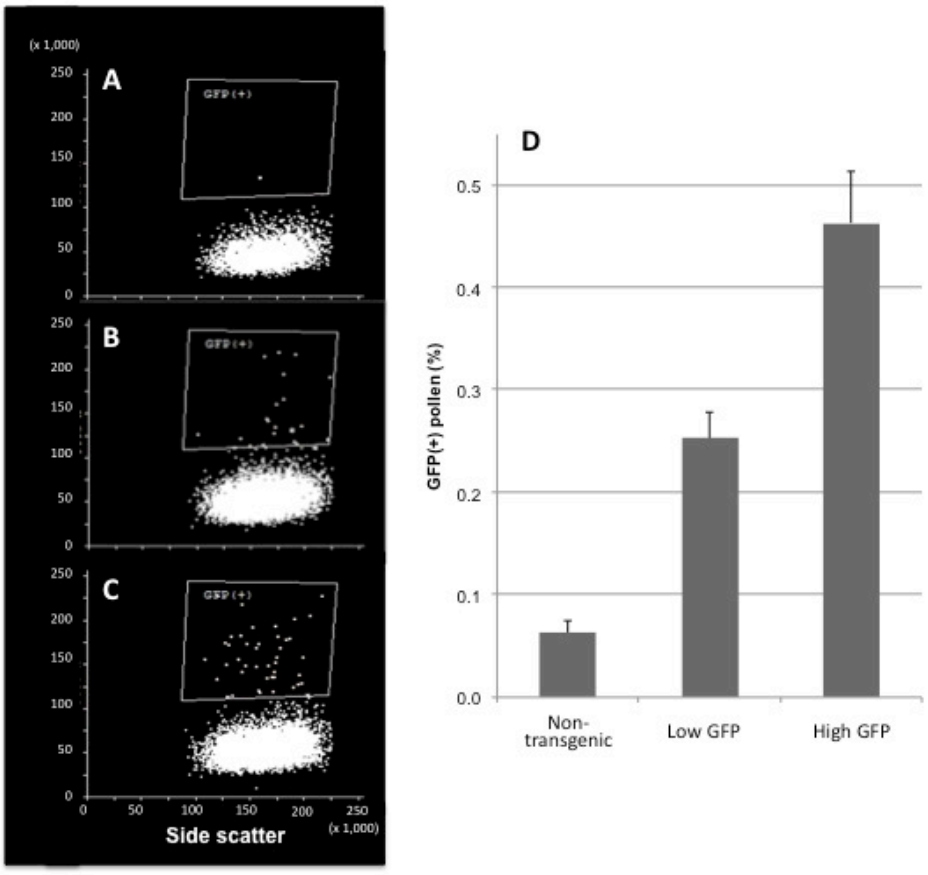

Figure 3-3 Detection of transgenic pollen grains with FCM.

(A) Non-transgenic pollen, (B) low GFP pollen grains in a sample of otherwise non-transgenic pollen, (C) high GFP pollen grains in a sample of otherwise non-transgenic pollen, Boxes and dots in (A, B, C) represent a range of GFP positive fluorescence and the detected individual pollen grains, respectively. Side scatter on the X axis refers to cell complexity or granularity. Fluorescence and side scatter are quantified in arbitrary units. (D) Percentage of detected transgenic pollen grains in non-transgenic pollen. Each bar represents mean ± standard deviation (SD) of triplicate measurements. GFP (+) percentage is a percentage of detected GFP positive pollen out of total pollen. There are significant differences among the means (t-test, $p<0.005$).

Chapter 4: Transgene excision in pollen using a codon optimized serine resolvase CinH-*RS2* site-specific recombination system

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Hong S. Moon designed, executed and analyzed the experiments and drafted the manuscript.

4.1 Abstract

Transgene escape, a major environmental and regulatory concern in transgenic crop cultivation, could be alleviated by removing transgenes from pollen, the most frequent vector for transgene flow. A transgene excision vector containing a codon optimized serine resolvase CinH recombinase (CinH) and its recognition sites *RS2* were constructed and transformed into tobacco (*Nicotiana tabacum* cv. Xanthi). CinH recombinase recognized 119 bp of nucleic acid sequences, *RS2,* in pollen and excised the transgene flanked by the *RS2* sites. In this system, the pollen-specific LAT52 promoter from tomato was employed to control the expression of CinH recombinase. Loss of expression of a green fluorescent protein (GFP) gene under the control of the LAT59 promoter from tomato was used as an indicator of transgene excision. Efficiency of transgene excision from pollen was determined by flow cytometry (FCM)-based pollen screening. While a transgenic event in the absence of CinH recombinase contained about 70% of GFP-synthesizing pollen, three single-copy transgene events contained less than 1% of GFP-synthesizing pollen based on 30,000 pollen grains analyzed per event. This suggests that CinH-*RS2* recombination system could be effectively utilized for transgene biocontainment.

4.2 Introduction

The transfer of transgenes conferring traits including herbicide and insect resistance from transgenic plants to sexually compatible non-transgenic plants has occurred via several vectors such as pollen, seeds, and propagules. Uncontrolled transgene escape that has been an important issue to environmentalists, regulators, scientists, and farmers could result in undesirable consequences such as creation of more invasive or competitive weeds, and admixture in crops that are intended to be non-transgenic. Transgene transfer through pollination via mediators

including wind, insects, and birds is possible between transgenic and non-transgenic plants, including many commercially cultivated crop species (Stewart et al., 2003). Transgene escape has occurred between two different types of transgenic *Brassica napus* (canola) by outcrossing with each other under field conditions (Beckie et al., 2003; Anno et al., 2006) and canola to its wild relative *Brassica rapa* (Chevre et al., 2000; Halfhill et al., 2004). Transgene presence in native Mexican landrace populations of maize (*Zea mays* L.) has been documented with molecular evidence (Piñeyro-Nelson et al., 2009).

Transgene containment strategies have been proposed to suppress or eliminate unintentional transgene escape from transgenic plant populations. Two categories of transgene contaminant strategies exist: physical and biological. Physical transgene containment strategies include trap crops, spatial isolation, fences, the removal of flowers, or even cultivation underground (Ingram, 2000; Morris et al., 1994; Whittington, 2006 http://www.associatedcontent.com/ article/27102/underground_farming.html?cat=15). Most physical transgene containment strategies are not considered to be very effective. Underground cultivation, suggested for pharmaceutical production, is limited by the number of available abandoned mines (Whittington, 2006).

Several biological transgene containment strategies—biocontainment— such as male sterility, maternal inheritance, transgenic mitigation, and transgene excision, have been the subject of research for many years. Male sterility was successfully achieved using chimaeric ribonuclease genes (Barnase) in tobacco and canola (Mariani et al., 1990). The drawbacks of this approach, including fertilization with non-transgenic plants as a maternal parent and potential negative effects on pollen-feeding pollinators including pollen beetles make this strategy inappropriate for widespread use in commercial fields (Daniell, 2002; Cook et al., 2004).

Transgenes targeted to the maternally inherited chloroplast genome has been used as a containment strategy in some plant species (Daniell et al., 1998; Iamtham & Day, 2000; Ruf et al., 2001). Despite maternal inheritance of the chloroplast transgenes in many species, low levels of transgenes can be transmitted via pollen in some species (Haygood et al., 2004; Svab & Maliga, 2007). Also, commercial utilization requires maternally inherited chloroplast genes be engineered to homoplasmy, which appears to be very difficult in most of crop species. Chloroplasts in one-third of angiosperm species are not maternally inherited, thus this strategy could not be used in these species (Maliga, 2004). A mitigating gene linked to a transgene can be used to reduce the competitiveness of transgene that could be introgressed weeds (Al-Ahmad et al., 2004, 2006). However, this mitigation strategy might not be effective for transgene escape within the same plant species (Moon et al., 2010).

Since most transgene dispersal occurs by pollen movement, biocontainment targeting pollen has attracted considerable attention (Gray & Raybould, 1998). A transgene excision strategy, where transgenes are excised from the pollen genome, appears to be one of the most attractive transgene biocontainment strategies (Luo et al., 2007; Moon et al., 2010). Unlike male sterility, the pollen reproductive process in plants would be uninterrupted using the transgene excision strategy. Therefore, seeds would be produced normally and pollen-feeding insects would not suffer from a lack of food. Several site-specific recombination systems have shown to effectively remove a transgene from transgenic plant, including pollen and seed genomes (Mlynárová et al., 2006; Luo et al., 2007; Kobertekh et al., 2010). Recently, zinc-finger nuclease (ZFN)-mediated transgene excision has been demonstrated in transgenic tobacco containing a reporter gene flanked by ZFN cleavage sites by crossing with a tobacco plant carrying a ZFN gene (Petolino et al., 2010). Most site-specific recombinase-mediated transgene excision occurs

by activating a transgenically-encoded recombinase with an inducible or tissue-specific promoter (Fig. 4-1). When expressed, the recombinase recognizes its recombination sites that flank all functional transgene cassettes and excises the sandwiched transgene between two recombination sites in the target cells, in this case, pollen. Once excised from genomic DNA, all transgenes are degraded and no longer functional (Fig. 4-1). Some small components of the integrated transgene such as the left and right border and one recombination site would remain in the pollen genome after transgene excision (Fig. 4-1). Site-specific recombination is involved in many natural biological functions including DNA transposition, integration of viral DNA into a host chromosome, excision or inversion of DNA segment, and regulation of gene expression (Grindley et al., 2006). Several fully reversible site-specific recombination systems including Cre-*lox*, FLP-*FRT*, Gin-*gix* and R-*RS* have shown transgene excision in transformed plants (Dale & Ow, 1990; Lyznik et al., 1993; Maeser & Kahmann, 1991; Onouchi et al., 1991). Cre-*lox* mediated-transgene excision by chemical induction has been demonstrated in *Arabidopsis* (Zuo et al., 2001). Recombination by the yeast FLP recombinase at two *FRT* sites resulted in the deletion of the embedded sequence between the sites (Lloyd & Davis, 1994; Rao et al., 2010). A mutant Gin recombinase mediated deletion of the DNA sequences between the specific recombination sites called *gix*; however, wild-type Gin recombinase failed to rearrange the DNA sequences (Klippel et al., 1993). The DNA segment between the recombination sites has shown to be excised by activation of the R recombinase in *Arabidopsis* and rice (Onouchi et al., 1995; Nakagawa et al., 2001). Luo et al. (2007) reported that highly improved efficiencies of transgene excision using Cre or FLP recombinase have been achieved by the fusion of *loxP* and *FRT* recognition sites. Transgenes in pollen and/or seeds were excised by Cre or FLP recombinase with the fused *loxP-FRT* recognition sites in several transgenic tobacco events, whereby a 100%

excision efficiency was achieved in some events based on the analysis of 25,000 progeny seedlings (Luo et al., 2007). However, the progeny seeds were produced by crossing without emasculation of maternal non-transgenic tobacco plants. Therefore, large portion of the seeds might come from self-fertilization of the maternal plants.

The serine resolvase family site-specific recombinases, including ParA and CinH recombinases, have shown efficient excision of plasmid DNA in transformed fission yeast (*Schizosaccharomyces pombe*)(Thomson & Ow, 2006). ParA recombinase was effective in catalyzing DNA excision in transgenic *Arabidopsis thaliana* plants (Thomson et al., 2009). An intervening transgene was excised by *Streptomyces*-derived phiC31-mediated recombination in transgenic *Arabidopsis* and wheat (Thomson et al., 2010; Kempe et al., 2010). CinH recombinase from *Acetinetobacter* plasmids pKLH2, pKLH204, and pKLH205 has shown activity in site-specific recombination in yeast, but this system has yet to be characterized in plants (Kholodii, 2001; Thomson & Ow, 2006). Here, we demonstrated transgene excision in pollen by the plant codon usage optimized CinH recombinase for use in transgene biocontainment. This is the first report of the CinH-*RS2* system-mediated recombination in transgenic plants.

4.3 Materials and methods

4.3.1 Vector constructs

CinH and CinH_Drec vectors (Fig. 4-2A, B) were constructed for plant transformation. Plasmids containing the CinH recombinase optimized for codon usage in plants and the CinH recombination site (*RS2*),

5’- CGTTACTTTGGGGTATACCCTAAAGTTACAATATAAAAGTTCTTAAAACT

ATGTAACATTTAAATGATTTTTAACCATATATAACATGTAACTTTGATAT TTAAAGTTTATAATTTACG-3', were constructed. The pLAT59-12 plasmid containing the LAT59 promoter (Genbank accession X56488) and tomato genomic DNA were gifts from the McCormick Lab (Plant Gene Expression Center, Albany, CA, USA). pPK100 containing the *tevL-eGFP* cassette was a gift of Patrick Gallois (University of Manchester, Manchester, UK). The two flanking CinH recognition sites, *RS2*, one designed for the right border (BamHI-BglII–*RS2*-SpeI) and the other designed for the left border (EcoRI-ApaI-*RS2*-PmeI-AatII-SacI) were PCR amplified and cloned in pGemT Easy to yield pBioC#461 and pBioC#464 respectively. Primers used were

(attRB1: 5'-ggatccccagatctCGTTACTTTGGGGTATAC-3', attRB2: 5'-gcgttaacactagtCGTAAATTATAAACTTTAAATATC-3' and attLB1: 5'-gaattcgggcccCGTTACTTTGGGGTATAC-3', attLB2: 5'-gagctcgacgtcgtttaaacCGTtAATTATAAACTTTAAATATC-3'). Enzyme sites underlined.

The LAT52 promoter (Twell et al., 1990) was PCR amplified from *Solanum lycopersicum* gDNA using primers (Lat52(F): 5'-gtttaaacgacgtcCCTATACCCCTTGGATAAG-3', Lat52(R) 5'-ggcgcgccTTTAAATTGGAATTTTTTTTTTGGTGTGTGTAC-3') cloned in pGemT Easy to yield pBioC#499. The PmeI to AscI promoter fragment was cloned in place of the CaMV35S promoter of pC35S CinH to yield pBioC535 (pLAT52::CinH). The NOS terminator of pCambia 0390 (http://www.cambia.org) was removed by inserting a SpeI site using PCR (primers - 0390F: 5'-aactactagtgtttgacaggatatattggc-3'; 0390R: 5'-aacgtcagaagccgactgcac-3'); the amplified fragment was cloned in pGemT Easy, digested with SpeI and SphI and inserted in place of the original fragment. The resulting plasmid was then digested with PstI and BamHI for ligation of the PstI to BamHI *Bar*-selection cassette of pGreen0229 (http://www.pgreen.ac.uk) to yield

pBioC#538. Then the BglII to SpeI fragment of pBioC#461 containing *RS2* designed for the right border was cloned in pBioC#538 to yield pBioC#542. As a preparative step, pPK100 was digested with NotI and BamHI, filled with dNTPs using T4 polymerase and religated to yield pRC10. Then pRC10 was digested XhoI and EcoRI and filled with primers (CPe1: 5'-TCGAcatcgatcacgtgc-3', CPe2: 5'-aattgcacgtgatcgatg-3') to yield pRC28 with a PmlI site. The HindIII to PmlI fragment of pRC28 containing the CaMV35S promoter was replaced with the HindIII to SmaI LAT59 promoter fragment of pLAT59-12 to yield pBioC#401.

The *RS2* EcoRI to SacI fragment of pBioC#464 designed for the left border was cloned into pBioC#401 digested with EcoRI and SacI to yield pBioC#472 (*RS2*, linker, LAT59::*eGFP*). The fragment from PmeI to SacI of pBioC#535 (pLAT52::CinH) was then cloned in pBioC#472 to yield pBioC#545 (*RS2*, LAT52::CinH, LAT59::*eGFP*).

Finally, the ApaI to HindIII fragment of pBioC#472 or pBioC#545 was cloned into pBioC#542 to yield CinH_Drec (Fig. 4-2B) (Moon et al., 2011) and CinH vectors (Fig. 4-2A).

4.3.2 Plant transformation

Each CinH or CinH_Drec vector was transformed into *Agrobacterium tumefaciens* strain EHA105. *Agrobacterium*-mediated tobacco (*Nicotiana tabacum* L.) transformation was performed using a standard protocol (Horsch et al., 1985). All cultures were maintained at 24 ± 2 °C under a 16/8 h light/dark photoperiod. Rooted shoots were transplanted to soil and acclimated for 2 weeks. T_0 transgenic events from tissue culture were confirmed by polymerase chain reaction (PCR) and grown in the greenhouse. Pollen grains collected

from the T_0 events were screened to confirm transgenicity under epifluorescence microscopy as described in the next section.

4.3.3 Microscopic visual assay

Pollen grains from two plants of each CinH_Drec T_1 event were collected to confirm transgenicity and to estimate GFP synthesis level. CinH T_1 event pollen grains were screened to estimate the frequency of GFP synthesizing pollen. Pollen was collected by tapping flowers so that pollen fell into a 1.5 ml microfuge tube and was then suspended in sterile water. The microfuge tubes were immediately shaken in a mixer (Eppendorf 5432 mixer) for 10 min to minimize clumping of pollen. The pollen suspension was taken from the tube and placed on a slide glass and covered with a glass cover slip. Pollen screening was performed under an epifluorescence (FITC filtered) microscopy (Olympus BX51 model) with blue light excitation at 200x magnification. QCapture software (Qimaging, Surrey, BC, Canada) was used to acquire pollen images.

4.3.4 Progeny analysis

T_1 seeds were produced by self-pollination of T_0 events. Progeny analysis with the harvested T_1 seeds was performed on MSO medium containing glufosinate ammonium at 5 mg/L. After 10-12 days of seed placement on the media, the total number of germinated seeds and number of negative and positive seedlings for glufosinate ammonium selection were recorded. Chi-square goodness of fit test was used to analyze fitness of the data.

4.3.5 Southern blot analysis

Positive T_1 seedlings for glufosinate ammonium selection were grown in the greenhouse. PCR and pollen screening under epifluorescence microscopy were performed to confirm transgenicity. Genomic DNA was extracted from 2 g of macerated snap-frozen leaf tissue using a modified CTAB extraction method (Stewart & Via, 1993). Resulting genomic DNA was purified by repeated phenol chloroform extractions. Ten micrograms of genomic DNA was digested to completion with HindIII. Control plasmid DNA from the binary vector CinH was also digested with HindIII. Resulting fragments were separated on a 1.2% agarose gel. Fragments were transferred to Zeta-Probe GT membrane (Bio-Rad, Hercules, CA, USA) by high salt Southern transfer (Brown, 2001). A PCR product containing the full-length open reading frame of *cinH* was radioactively labeled with α-^{32}P dCTP using Prime-It II Random Primers Labeling Kit (Stratagene, La Jolla, CA, USA). The 0.5kb *cinH* ^{32}P-labeled probe was produced by GoTag® Green master mix (Promega, Madison, WI, USA) using primers 5'-CTACGTTCGTGTTTCATCGG-3' and 5'-CTGGATATGCCGAACGCTTT-3'. Labeled probe was purified using mini Quick Spin DNA columns (Roche Diagnostics, Indianapolis, IN, USA). Southern blots were hybridized with labeled probe in ULTRAhyb hybridization buffer (Ambion, Austin, TX, USA) and washed according to the manufacturer's protocol. Blots were visualized by exposure to phosphor-imaging screens (Storage Phosphor Screen GP, Eastman Kodak, Rochester, NY, USA) and scanned using Personal FX (Bio-Rad, Hercules, CA, USA). Image analysis was undertaken using Quantity One software (Bio-Rad, Hercules, CA, USA).

4.3.6 Pollen collection

All plants including non-transgenic tobacco, CinH_Drec-1 T_1 event, and CinH T_1 events were grown in the greenhouse. Four plants per each type were grown with 2 m distance from other plant types to prevent potential cross-contamination. Pollen collections were conducted for 10 days during flowering period by tapping the mature flowers each into a 1.5 ml microfuge tube and immediately freezing at -80°C. At the time of assay, 1 ml of sterile water was added into each tube containing pollen grains and the tubes were shaken in a mixer (Eppendorf 5432 mixer) for 10 min. Pollen suspensions were filtered with a 132 μm pore nylon mesh (Sefar Nitex 03-132/43, Sefar filtration Inc., Depew, NY, USA) to remove non-pollen debris such as anthers and clumps of pollen that may clog the fluidic system of the flow cytometer. Filtered pollen suspension was transferred into 5 ml polystyrene round bottom tubes (BD falcon, San Jose, CA, USA) for FCM analysis.

4.3.7 Flow cytometry (FCM) analysis

FCM-based transgenic pollen analysis was performed using methods described in Moon et al. (2011). Briefly, a LSR II flow cytometer (BD Biosciences, San Jose, CA, USA) was used to detect GFP fluorescence in pollen grains with the voltage settings of 200 eV for forward scatter channel, 190 eV for side scatter channel, and 468 eV for FL1 channel. Data were obtained by counting 30,000 pollen grains for each sample and analyzed using DiVa software (BD Biosciences, San Jose, CA, USA) and CyflogicTM software (CyFlo Ltd, Finland).

4.4 Results

4.4.1 Regeneration of transgenic tobacco events

Twenty independent transgenic events were generated containing the CinH vector (Fig. 4-2A). Of the nine CinH_Drec events, one high GFP-synthesizing event was selected by visual screening of pollen grains under a blue-light microscopy. T_1 seeds from PCR-confirmed T_0 events were selected on selective media containing glufosinate ammonium 5 mg/L. Selected T_1 seeds were germinated and plants grown in the greenhouse. Visual screening of pollen from the selected CinH_Drec T_1 event allowed differentiation between different zygosity status including homozygous, hemizygous, and nullizygous for the transgene (data not shown).

4.4.2 Progeny analysis

Progeny analysis was performed with all twenty CinH events. Non-transgenic tobacco seeds did not survive on glufosinate ammonium selection media (Fig. 4-3A), while all non-transgenic seeds survived on non-selection media (Fig. 4-3B). T_1 seeds from CinH transgenic events were segregated for glufosinate ammonium resistance (Fig. 4-3C, D). Surviving seeds were a mixture of hemizygous and homozygous seeds for the transgene. For CinH events, should the transgene excision system completely excise all DNA between the *RS2* sites in pollen, transgenic DNA would exist only in maternal reproductive cells. Pollen that failed to excise transgene by CinH-*RS2* recombination system retain *eGFP* expression cassette, and exhibit GFP fluorescence. The expected progeny segregation ratio for completely effective pollen transgene excision for a single locus would be 1:1 for transgenic: non-transgenic T_1 individuals. However, all 20 CinH events significantly differed from the expected 1:1 ratio (Table 4-1). This could be due to

incomplete excision in pollen and/or the presence of multiple transgenic loci segregating in the female gametes. Eight events with relatively small deviations from the expected ratio, CinH-2, 3, 4, 6, 9, 10, 20, and 21, were selected for further analysis. In addition to these 8 events, 2 more CinH events, CinH-19 and 22, that had very small numbers of non-transgenic seedlings were also selected for further analysis.

4.4.3 Microscopic visual assay of pollen

Four T_1 plants per each event were grown in the greenhouse and had confirmed transgenicity by PCR (data not shown). Phenotypic differences were not found between transgenic tobacco events and non-transgenic tobacco. Pollen grains from CinH or CinH_Drec T_1 transgenic events were not phenotypically different from non-transgenic tobacco pollen under white light. However, the CinH_Drec event had large numbers of GFP positive pollen grains under blue light, while non-transgenic tobacco had no green-fluorescent pollen grains. Compared to the CinH_Drec event, CinH T_1 events had significantly less or lacked GFP positive pollen in the microscopic images (Fig. 4-4). Elimination of GFP expression would be expected from transgene excision of the *eGFP* marker occurring in pollen via site-specific recombination (Fig. 4-1). Therefore, the loss of green florescence serves as an indicator of transgene excision in pollen.

4.4.4 Southern blot analysis

Ten CinH events were subject to Southern blot analysis to confirm transgene integration and copy number. As expected, genomic DNA from non-transgenic tobacco and the CinH_Drec event did not reveal hybridizing bands with the *cinH*-specific probe. Five CinH events, CinH-2,

4, 9, 10, and 21, exhibited a single hybridization band which indicated a single copy of transgene integration (Fig. 4-5). The remainder of the events appeared to contain multiple transgene copies in their genomes (Fig. 4-5). Southern blot analysis also confirmed that the 10 selected CinH events were independently transformed events.

4.4.5 Flow cytometry (FCM) analysis

FCM analysis was performed to determine efficiency of CinH-*RS2* recombination-mediated transgene excision in large numbers (ca., 30,000) of pollen. FCM has been shown to be fast and effective in discriminating between GFP-synthesizing transgenic pollen and non-expressing pollen (Moon et al., 2011). In this study, FCM was used to develop a system for GFP expression in pollen. Here, GFP positive particles were detected in non-transgenic pollen sample at a very low rate (0.02%), even after extensive flushing of FCM fluidic system between measurements (Fig. 4-6). GFP positive pollen ratio ranged from 0.46 – 17.48 % for single transgene copy-inserted events (Fig. 4-6). Three events, CinH-4, 10, and 21, had 0.93%, 0.47%, and 0.46 % of GFP positive pollen, respectively, out of 30,000 pollen grains analyzed (Fig. 4-6). The CinH_Drec-1 event had more than 50% GFP positive pollen, which is consistent with the expectations of collecting pollen from a mixture of hemizygous and homozygous T_1 plants.

4.5 Discussion

Transgene excision via site-specific recombination could be an effective transgene biocontainment strategy when gene flow from pollen is the main concern for transgenes leaving the field of interest (Luo et al., 2007; Moon et al., 2010). A codon optimized serine resolvase

recombinase CinH recognizes specific 119 base sequence recombination sites and excised embedded functional transgenes including the *eGFP* gene from the pollen genome. An absence of GFP expression served as an indicator of transgene excision. Three independent CinH tobacco T_1 events exhibited less than 1% GFP positive pollen, based on FCM analysis of 30,000 pollen grains. As compared to a control CinH_Drec event, which had GFP expression in 70% of pollen, CinH events had a significant decrease in percentage of GFP positive pollen. This indicated that transgenic events containing CinH recombinase efficiently excised transgenes from the pollen genome. Unlike the tyrosine family of recombinases including Cre and FLP recombinases, CinH is an irreversible recombinase that prevents possible re-insertion of excised transgenes into the genome. CinH recombinase has superior specificity by recognizing a 119 nucleic acid base sequence known as *RS2* (NCBI ref# AF213017). As compared to Cre and FLP recombinases, which have 34 bp recombination sites, CinH has recombination sites of 119 bp that should provide high specificity to the site-specific recombination, this negating the risk of illicit recombination within plant genomes. One *RS2* recombination site would remain in the pollen genome after transgene excision occurs. However, this single recombination site in the absence of a recombinase and another *RS2* sites should not lead to recombination in pollen genome.

None of CinH transgenic events had the expected 1:1 segregation ratio. Since none of the CinH events demonstrated complete transgene excision in pollen, deviation from the expected 1:1 T_1 segregation ratio of transgenic to non-transgenic would most likely be due to incomplete excision in T_1 pollen. Sample size of progeny analysis might need to be larger to make a confident conclusion. In addition, to acquire more reliable progeny analysis data with this transgene excision system, transgenic plants should be crossed with emasculated or male-sterile non-transgenic maternal plants. Transgene excision system with a fused recombination sites –

loxP/*FRT* – has shown high efficient transgene excision in pollen (Luo et al., 2007). However, the efficiency of transgene excision was estimated by reciprocal cross with non-transgenic tobacco plants without emasculation. The efficiency might not reflect the fact that large portion of progeny seeds could come from self-fertilization of non-transgenic tobacco. Field experiments with larger sample sizes should be useful to better define real-world utility.

Various efficiencies of recombination systems could be optimized by choice of promoter (Kopertekh et al., 2010). The tomato-derived LAT52 pollen-specific promoter has shown high specificity to pollen (Luo et al., 2007), and indeed, transgenes were efficiently excised, although the efficiency of this promoter in tobacco could possibly be overestimated due to non-emasculated maternal plants. Reverse transcriptase (RT)-PCR has revealed high specificity of the LAT52 promoter to pollen, when other plant parts including root, stem and leaf were subject to RT-PCR (Luo et al., 2007). This indicates that CinH-*RS2* recombination system coupled with the LAT52 pollen-specific promoter could be a strong candidate to excise transgenic DNA for the transgene biocontainment strategy, but perhaps higher expression is needed for complete excision.

FCM analysis of pollen detected fluorescent signal even in non-transgenic pollen sample at a very low level (0.02%). There are several possible explanations of GFP positive particles in non-transgenic pollen sample. Carry-over of GFP positive pollen from previous measurements, bright autofluorescent non-pollen particles in similar size range, or contamination of transgenic pollen during pollen collection process could contribute to fluorescent signal detection (Moon et al., 2011).

Different levels of GFP fluorescence in pollen among the independently transformed events might be expected. GFP fluorescence levels could not be determined due to a small

number of GFP positive pollen grains in the CinH events. It could be possible to underestimate the frequency of GFP positive pollen with the FCM-based method for some low GFP fluorescence events. However, a significantly low GFP fluorescence event was detected with the accuracy of 75% using the FCM-based method (Moon et al., 2011). Therefore, maximum inaccuracy of possible low GFP expressing events would not be more than 25% of measured percentage. Analysis of large number of pollen grains would provide comparable data to other small number sampled studies, even though there is a possible underestimation with the minimum accuracy of 75%.

FCM analysis provided fast and efficient detection of GFP synthesizing transgenic pollen. Most transgene excision studies have reported their results based on small numbers of samples analyzed. This FCM-based transgenic pollen screening method allowed analysis of large numbers of pollen samples in short time. FCM is technically capable of analyzing thousands of particles per second based on the sample concentrations. Data was acquired at a speed of 10,755 pollen grains per minute on one sample that had optical density 0.336 at 600 nm. FCM analysis could eliminate laborious progeny analysis and microscopic screening and the inherent errors in manual counting to analyze efficiency of transgene excision in pollen (Moon et al., 2011).

GFP-synthesizing pollen was an efficient marker for transgene excision. Transgene excised pollen was visualized under microscope and easily indentified in the FCM by the absence of GFP expression in pollen. Most transgene excision studies in plants have confirmed transgene excision using molecular biological techniques including PCR (Woo et al., 2009; Rao et al., 2010; Thomson et al., 2010), However, each pollen grain is considered as a single event of the transgene excision system in pollen. PCR seems to be unfeasible for transgene excision system in pollen due to the technical challenge of DNA isolation from a single pollen grain. In

this study, loss of GFP expression in pollen served as an effective indicator of transgene excision in pollen. Non-GFP synthesizing pollen grains are the mixture of non-transgenic segregant pollen and transgene excised pollen grains. It is impossible to distinguish the transgene excised pollen from non-transgenic segregant pollen. However, the efficiency of the transgene excision system is determined based on the number or percentage of GFP synthesizing pollen grains. Unlike β-glucuronidase (GUS), GFP gene does not require chemical treatments for visualization of transgene excision. Therefore, GFP synthesizing pollen or transgene-excised pollen can be sorted and utilized for further research.

Efficiency of CinH-*RS2* recombination system-mediated transgene excision is comparable to other recombination systems including Cre-*lox* and FLP-*FRT* systems (Luo et al., 2007; Verweire et al., 2007). The FLP-*FRT* system using the pollen-specific LAT52 promoter has shown efficiency of transgene excision from pollen ranging from 0 to 99% with a 32 % average among events (Luo et al., 2007).

The Cre-*lox* recombination system under the control of a chemical-induced promoter has shown a wide range of transgene excision efficiency in different plant species including *Arabidopsis*, rice, and tomato (Zuo et al., 2001; Sreekala et al., 2005; Zhang et al., 2006). This suggested that transgene excision efficiency could be influenced by different genomes. Therefore, this CinH-*RS2* recombination system should be tested in various plant species to acquire species-specific recombination efficiency before the practical employment of the system for crops.

Variable transgene excision efficiencies among transgenic events in this system were most likely the result of position effects of transgene insertion in the genome. It is feasible that an insertion site that enabled higher gene expression with stability across generations could be located to improve efficiency. If so, it is also technically possible to use ZFN-mediated targeting

to place a transgene at the target locus (Reviewed in Weinthal et al., 2010). Based on high efficiency of transgene excision in pollen, this CinH-*RS2* recombination system would be a good candidate for highly efficient transgene excision system by itself or coupling with other site-specific recombinases or zinc-finger nucleases.

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Appendix

Tables and Figures

Table 4-1 Segregation analysis of T_1 progeny

	Total germinated	Total transgenic (T)	Total non-transgenic (N)	Observed ratio(T:N)	X^2 value under the 1:1 expected ratio
CinH-2	370	250	120	2.1 : 1	45.6*
CinH-3	224	150	74	2.0 : 1	25.7*
CinH-4	387	250	137	1.8 : 1	32.9*
CinH-5	258	222	36	6.2 : 1	134.0*
CinH-6	190	151	39	3.9 : 1	66.0*
CinH-7	295	280	15	18.7 : 1	238.0*
CinH-9	412	289	123	2.3 : 1	66.8*
CinH-10	294	213	81	2.6 : 1	59.2*
CinH-11	338	333	5	66.6 : 1	318.2*
CinH-12	311	290	21	13.8 : 1	232.6*
CinH-13	312	229	83	2.8 : 1	68.3*
CinH-14	317	236	81	2.9 : 1	75.7*
CinH-15	337	315	22	14.3 : 1	254.7*
CinH-16	325	317	8	39.6 : 1	293.7*
CinH-17	329	248	81	3.1 : 1	84.7*
CinH-18	282	204	78	2.6 : 1	56.2*
CinH-19	729	716	13	55.1 : 1	677.9*
CinH-20	226	139	87	1.6 : 1	11.9*
CinH-21	472	288	184	1.6 : 1	22.9*
CinH-22	250	243	7	34.7 : 1	222.7*

***Pr > x^2 <0.0001**

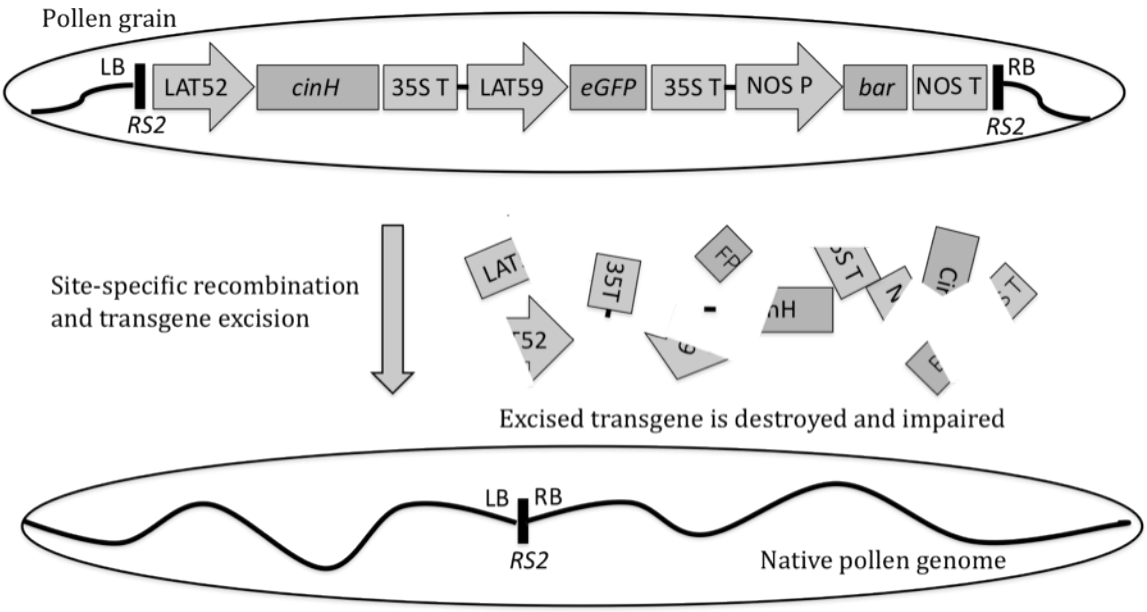

Figure 4-1 Schematic illustration of CinH recombinase-mediated transgene excision in pollen.

Pollen specific promoter LAT52 drives CinH recombinase in pollen. CinH recombinase recognizes 119 bp sequence sites, *RS2*, all functional transgenes flanked by the *RS2* sites excised leaving only one *RS2* site and T-DNA borders in the pollen genome. LAT52: Pollen-specific LAT52 promoter, *cinH*: Codon optimized CinH recombinase gene, 35S T: 35S terminator, LAT59: Pollen-specific LAT59 promoter, *eGFP*: Enhanced GFP gene, NOS P: Nopaline synthase promoter, *bar*: Herbicide resistant bar gene, NOS T: Nopaline synthase terminator, *RS2*: CinH recombinase recognition site.

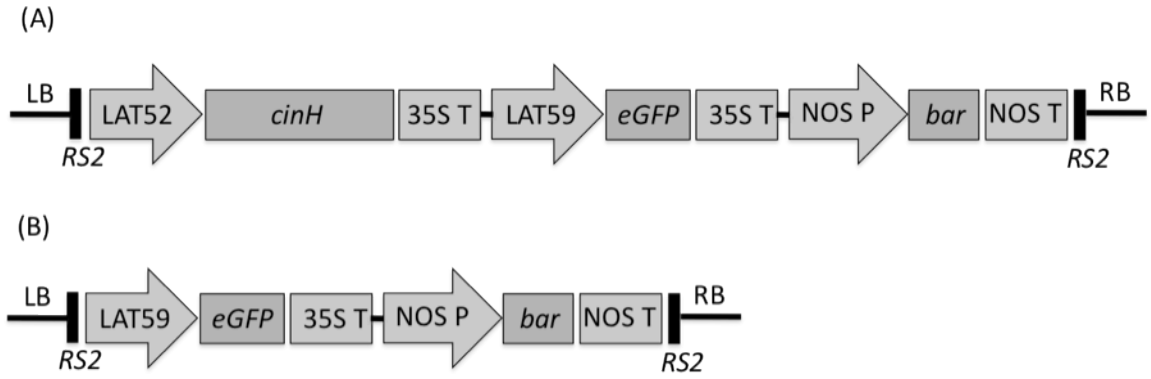

Figure 4-2 CinH and CinH_Drec vector constructs.

(A) CinH recombinase is under the control of pollen-specific LAT52 promoter. Enhanced GFP gene is driven by pollen-specific LAT59 promoter. Bar gene confers resistance to herbicide glufosinate ammonium. (B) CinH_Drec vector was constructed from the CinH vector by removing CinH recombinase cassette. LAT52: Pollen-specific LAT52 promoter, *cinH*: Codon optimized CinH recombinase gene, 35S T: 35S terminator, LAT59: Pollen-specific LAT59 promoter, *eGFP*: Enhanced GFP gene, NOS P: Nopaline synthase promoter, *bar*: Herbicide resistant bar gene, NOS T: Nopaline synthase terminator, *RS2*: CinH recombinase recognition site, LB: Left border, RB: Right border.

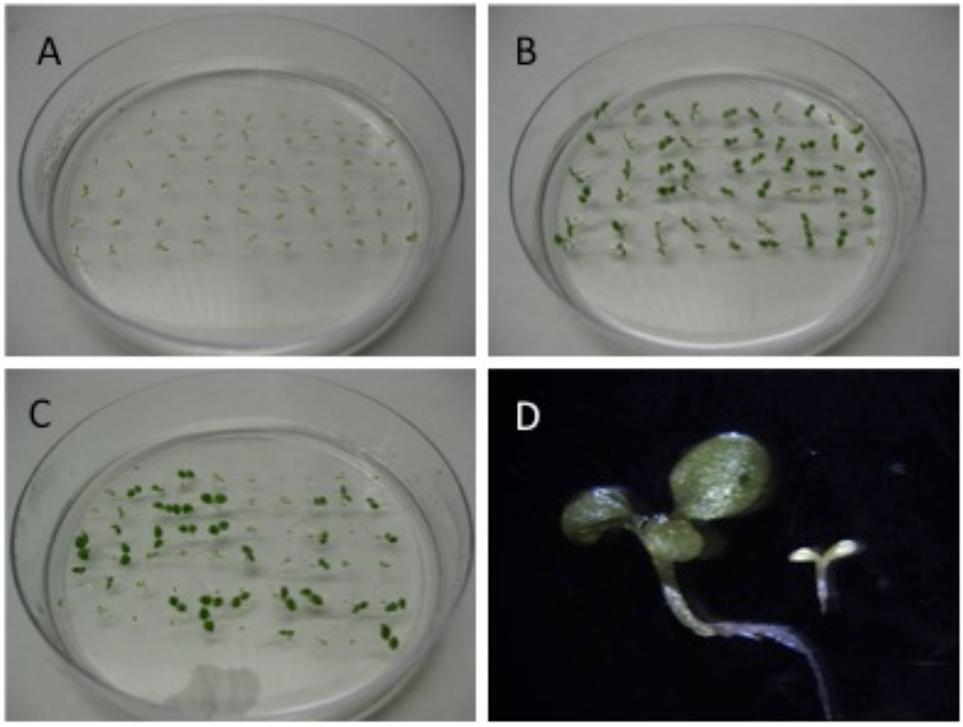

Figure 4-3 T_1 progeny selection on selection media containing glufosinate ammonium.

(A) Non-transgenic tobacco (Xanthi) seeds did not survived on selection media containing 5mg/L of glufosinate ammonium. (B) Non-transgenic tobacco seeds on non-selection media. (C) CinH event seeds were selected on the herbicide selection media. (D) Representation of living and dead CinH seedlings.

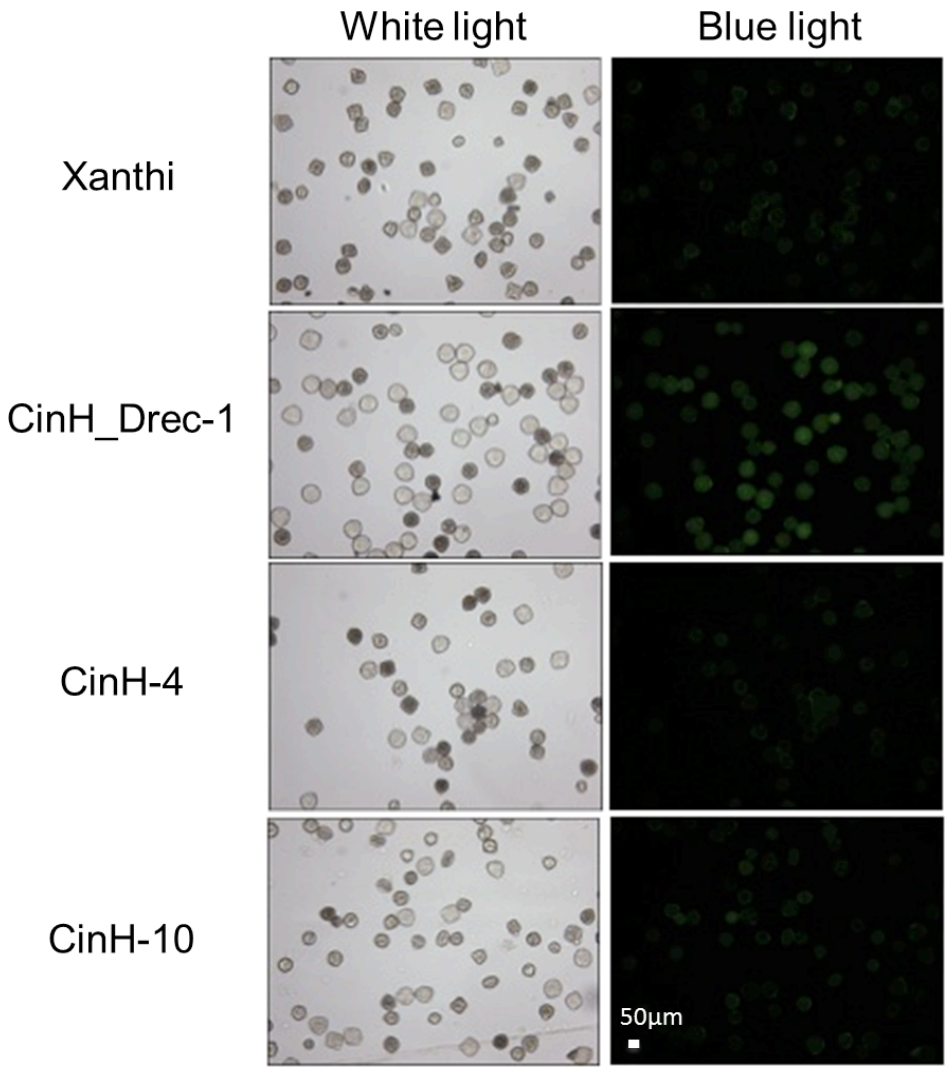

Figure 4-4 Microscopic images of pollen grains.

Pollen grains from non-transgenic tobacco (Xanthi), CinH_Drec event, and 2 CinH events were collected and screened under the FITC filtered epifluorescent microscopy. Left panel images were taken under white fluorescent light with 1.67 ms exposure time. Right panel images were taken under blue light with 3 s exposure time. All images were taken at 200x magnification.

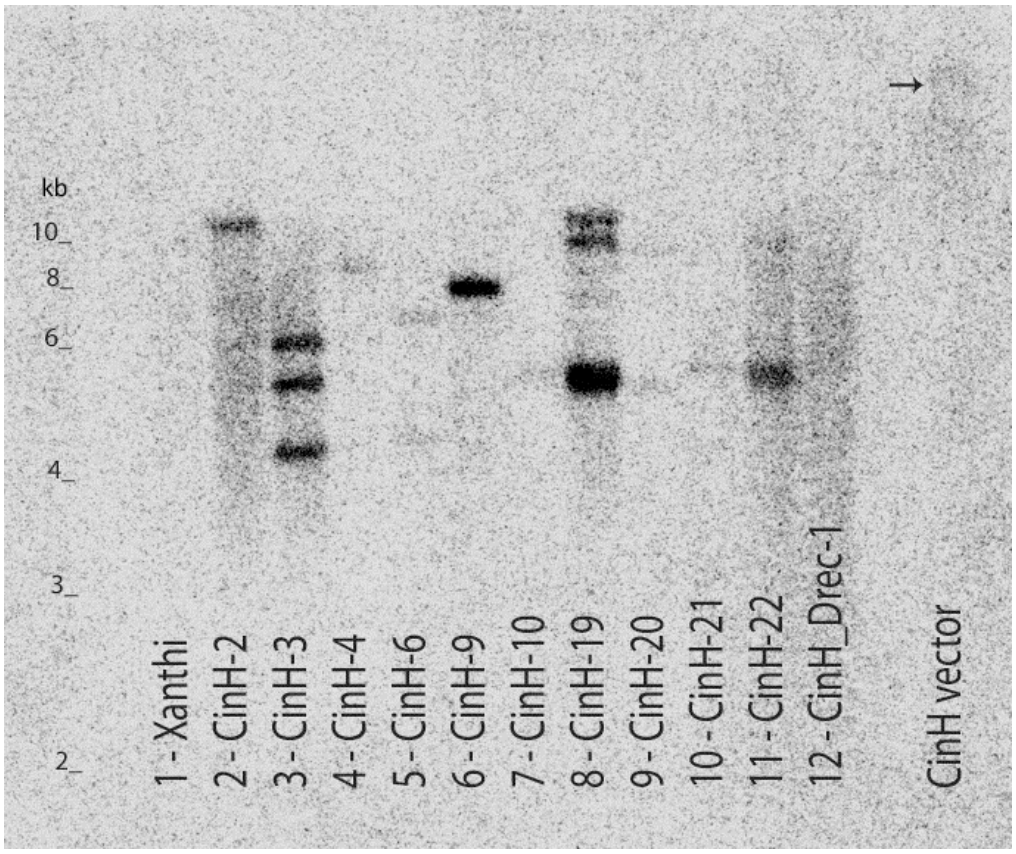

Figure 4-5 Southern blot analysis of T_1 CinH transgenic events.

Genomic DNA from non-transgenic tobacco (Xanthi), 10 CinH events (CinH-2, 3, 4, 6, 9, 10, 19, 20, 21, 22), and CinH_Drec event (CinH_Drec-1) were digested with HindIII and hybridized to *cinH* probe. HindIII digested binary CinH vector used for tobacco transformation is shown in CinH vector lane. Relative migration distances of DNA ladder fragments are shown in the left lane.

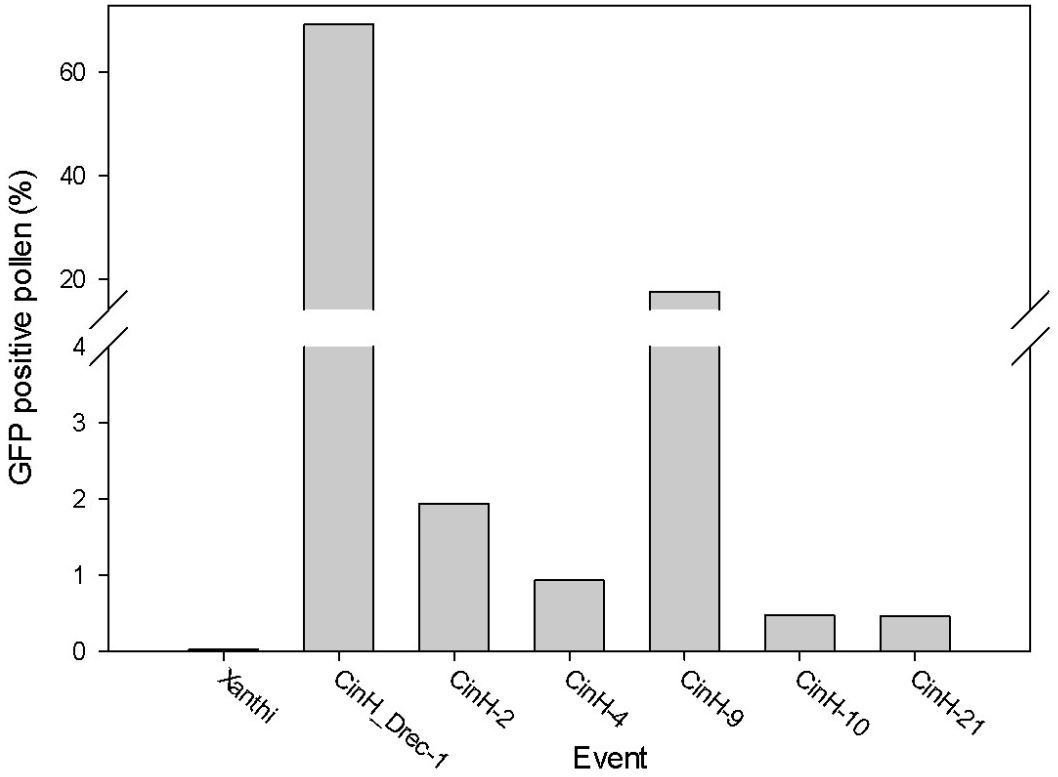

Figure 4-6 Percentage of GFP positive pollen in single transgene copy integrated CinH transgenic events.

Loss of GFP expression served as an effective indicator for transgene excision. Each event including non-transgenic tobacco contained GFP positive pollen as follows. Non-transgenic tobacco (Xanthi) - 0.02%, CinH_Drec - 69.36%, CinH-2 - 1.94%, CinH-4 - 0.93%, CinH-9 - 17.48%, CinH-10 - 0.47%, CinH-21 - 0.46%. Percentage of GFP positive pollen was based on 30,000 pollen grains analyzed.

Chapter 5: Selective male sterility by overexpression of EcoRI restriction endonuclease in tobacco

(This chapter is prepared to be submitted to Plant Physiology with the following authors: Hong S. Moon, Jason M. Abercrombie, Laura L. Abercrombie, Shigetoshi Eda, C. Neal Stewart, Jr.)

Hong S. Moon designed, executed and analyzed the experiments and drafted the manuscript.

5.1 Abstract

Genetic engineering is likely to be important for enhancing crop characteristics and other traits. The potential of transgene flow from genetically engineered crops to non-transgenic crop populations or wild relatives, important environmental and regulatory considerations, must be carefully addressed and prevented. Therefore, it is important to develop efficient and reliable biocontainment strategies. Since most transgene flow occur through pollen dispersal, a novel approach for selective male sterility in pollen was developed and evaluated as a biocontainment strategy. This system is composed of an expression of EcoRI restriction endonuclease driven by a tomato pollen-specific promoter (LAT52) in a proof-of-concept transgenic tobacco system to enable transgenic pollen ablation and/or infertility through the destruction of pollen genome. Overexpression of the EcoRI restriction endonuclease caused pollen ablation and/or infertility in tobacco, but did not negatively affect other parts of the plant. Transgenic EcoRI events overexpressing the EcoRI restriction endonuclease exhibited normal phenotypes when compared to non-transgenic tobacco. Three EcoRI events produced 0 % GFP positive pollen, while GFP control plants contained 64% GFP positive pollen based on 9,000 pollen grains analyzed by flow cytometry-based transgenic pollen screening method. However, seven EcoRI events appeared to have 100% efficiency on selective male sterility based on the test-crosses. Transgenic pollen was successfully ablated and/or infertile when the EcoRI restriction endonuclease was overexpressed in pollen. The results suggested that this selective male sterility could be used as a highly efficient and reliable biocontainment strategy for genetically engineered crop cultivation.

5.2 Introduction

Developing containment strategies has been considered as a crucial step for genetically engineered (GE) crop cultivation. Most physical containment strategies including building fences, setting isolation distances, and manually removing flowers appeared to have very limited use (Moon et al., 2010). Biological strategies have been considered as more efficient and reliable methods to contain unwanted transgene escape from GE crops. Male sterility has been most extensively studied among many other biocontainment strategies and commercially utilized. Male sterility was initially used in plant breeding for the production of F_1 hybrids. In the reproductive cycles of higher plants, viable pollen is required for successful pollen germination, tube growth, and eventual double-fertilization via transmission of the sperm cells to the ovule. Transgene escape and introgression through pollen could be completely prevented if pollen were rendered nonviable. Multiple methods have been used to decrease pollen fertility via genic or cytoplasmic male sterility. Disrupting pollen development through genetic engineering has been suggested for suppressing transgene escape and introgression (Daniell, 2002; Feil et al., 2003). For example, many male sterile plants have been genetically engineered using constructs that disrupt the tapetum, a layer of cells found within the pollen sac, essential for pollen development (reviewed in Daniell, 2002). The first transgenic male sterile plant was generated by genetic engineering of tobacco plants with the chimaeric ribonuclease gene (Mariani et al., 1990). Most genic male sterile plants have been achieved by using tapetum-specific promoters to drive the expression of toxic bacterial genes (e.g. *Barnase* from *Bacillus amyloliquefaciens*, diphtheria toxin A), resulting in no pollen formation (Hird et al., 1993; Koltunow et al., 1990; Lee et al., 2003). Since then, several genetic engineering efforts have been aimed at developing genic male sterility in plants. These include using cytotoxic *barnase* gene expression in pollen or anthers of

poplar (*Populus*) trees and *Kalanchoe blossfeldiana* (Wei et al., 2007; García-Sogo et al., 2010). Since genic male sterility strategies inhibit development of anther or pollen, the lack of pollen could create negative ecological impacts on pollen-feeding insects (Mlynárová et al., 2006). Another way to generate male sterile plants is cytoplasmic male sterility (CMS) (Chase, 2006). CMS that blocks the production of functional pollen resulted from mutations in plant mitochondrial genome (Hanson & Bentolila, 2004). CMS plants are thought to have utility for limiting transgene flow via pollen dispersal (Feil et al., 2003). More recently, genetically engineered CMS has been developed for transgene biocontainment (Ruiz & Daniell, 2005). This started with the successful genetic engineering of the tobacco (*Nicotiana tabacum* L.) chloroplast genome with the *phaA* gene coding for ß–ketothiolase, which is known to confer cytoplasmic male sterility (Ruiz & Daniell, 2005). A potential drawback of using CMS as a biocontainment tool is the potential for transmission of the transgene from the cytoplasm to the nucleus. Transmission of paternal plastids and mitochondria in crosses involving parents with an alien cytoplasm occurs at low frequency (10^{-4} to 10^{-5}), and even less frequent transmission is expected under field conditions (Svab & Maliga, 2007). Also the loss of fertility in a CMS breeding plant population could eventually be restored under natural conditions (Schnable & Wise, 1996). Plastid transformation is a method of male sterility using maternal inheritance feature. Maternally inherited plant plastid genome in most crop species provides several advantages in genetic engineering such as high level of transgene expression and express of multiple operons in the genome (Maliga, 2004). Since plastids are not maternally inherited in some plant species, the use of plastid-based male sterility may be limited to certain plant species (Hagemann, 2004). Despite its potential for biocontainment, plastid transformation has only been successfully established in limited numbers of plant species.

Pollen ablation has been demonstrated by expression of the diphtheria toxin gene under the control of the LAT52 pollen-specific and putative pectin esterase promoter in tobacco (Twell, 1995; Uk et al., 1998). Transgenic events containing single copy of the diphtheria toxin A-chain (DTx-A) gene have shown 50% aborted pollen and 50% normal pollen as expected (Uk et al., 1998). However, there could be concerns about the expression of such toxin genes that might negatively affect the pollinators or even human consumers.

Restriction endonucleases are typically classified into three classes (I, II, and III) based on their enzymology and cofactor requirements (Wilson, 1988). Type II restriction endonucleases are the best understood, but unique among the classes in that they consist of separate endonuclease and methylase enzymes. For decades, molecular biologists have relied on the utility of type II restriction endonucleases for routine DNA manipulation in the laboratory. Among the type II systems, the EcoRI restriction endonuclease is one of the most studied and well characterized. This restriction enzyme recognizes the nucleotide sequence 5'-GAATTC-3', requires Mg^{2+}, functions as a homodimer, and creates a double-strand break at the site (Wilson, 1988). Barnes and Rine (1985) demonstrated nuclear entry and the resultant cell death associated with EcoRI expression in yeast, *Saccharomyces cerevisiae*. Induced expression of the EcoRI restriction endonuclease was lethal to transformed *Escherichia coli* containing a plasmid carrying the EcoRI gene and suppressed the growth of the cells (Gholizadeh et al., 2010). The expression of any type II restriction endonucleases in plants has not been reported. Here, we report first overexpression of the EcoRI restriction endonuclease in plants, particularly in pollen, resulting in pollen ablation and/or infertility. This pollen ablation and/or infertility by overexpression of the EcoRI could be used as a biocontainment strategy to prevent pollen-mediated transgene escape and introgression.

5.3 Materials and methods

5.3.1 Vector constructs and tobacco transformation

A vector carrying a translational fusion of the EcoRI restriction endonuclease and *G3GFP* gene under the control of the pollen-specific LAT52 promoter was constructed with a R4 Gateway Binary Vector (Nakagawa et al., 2008) by using a site-specific multisite Gateway® cloning strategy. The pollen-specific promoter LAT52 derived from tomato has shown to direct high levels of pollen-specific transgene expression with undetectable levels of expression in all other tissues in several dicotyledonous plants including tobacco (Twell et al., 1990). A plasmid vector carrying the EcoRI restriction endonuclease gene was kindly provided by Linda Jen-Jacobsen at the University of Pittsburg. The EcoRI gene was cloned into TOPO vector (pcr8-GW-TOPO, Invitrogen, Carlsbad, CA, USA) without the stop codon to enable a reporter gene fusion construct. The LAT52 promoter was cloned into Multisite Gateway® vectors pENTR P4-P1r (Invitrogen, Carlsbad, CA, USA). All cloned DNA fragments were confirmed for correct orientation using restriction digests and sequence confirmed at the University of Tennessee molecular biology core facility. Tobacco was transformed with *Agrobacterium tumefaciens* strain EHA105 using an existing tobacco transformation protocol (Horsch et al., 1985). Regenerated events were grown in the greenhouse and T_1 seeds were collected from self-fertilized events. Collected T_1 seeds were surface-sterilized and screened on the medium containing hygromycin 50 mg l^{-1}.

5.3.2 Polymerase chain reaction (PCR)

Positive T_1 seedlings for hygromycin selection were transplanted in soil and grown in the greenhouse. Two individual plants were grown per each independent event. Genomic DNA was extracted from leaf tissue samples using an existing protocol (Stewart &Via, 1993). The EcoRI gene was amplified with a set of primers (Forward: 5'-ATGTCTAATAAAAAACAGTCAAATA-3'; Reverse: 5'-CTTCTTAGATGTAAGCTGTTC-3') to confirm transgenicity of selected individuals. The reactions were repeated through 40 cycles of 94 °C for 1 min, 50 °C for 30 s, and 72 °C for 1 min.

5.3.3 Microscopic analysis

Pollen samples from each EcoRI T_1 event were visualized to estimate the frequency of GFP positive pollen. Pollen grains from two plants of each EcoRI T_1 event were collected for visual confirmation under an epifluorescence microscopy. Pollen was directly collected from flowers by tapping into a 1.5 ml microfuge tube and was then suspended in sterile water. The microfuge tubes were immediately shaken in a mixer (Eppendorf 5432 mixer) for 10 min to minimize clumping of pollen. The pollen suspension was taken from the tube and placed on a slide glass and covered with a glass cover slip. Pollen screening was performed under an epifluorescence (FITC filtered) microscopy (Olympus BX51 model) with blue light excitation at 200x magnification. QCapture software (Qimaging, Surrey, BC, Canada) was used to acquire pollen images.

5.3.4 Pollen viability analysis

Pollen viability was analyzed by potassium iodide-iodine (IKI) stain method (Mulugeta et al., 1994). One gram of potassium acetate and 0.5 g of iodide were dissolved in sterile H_2O to make IKI staining solution. Fresh pollen grains were collected as previously described. IKI staining solution was added into a 1.5 ml tube containing collected pollen grains. After 30 minutes, 50 μl of pollen grains were sampled on a slide glass and covered with a glass cover slip. Pollen viability was determined under a microscopy (Olympus BX51 model) with white light at 100x magnification.

5.3.5 Flow cytometry (FCM)-based transgenic pollen screen

All plant types including EcoRI T_1 events, non-transgenic Xanthi, and GFP control (CinH_Drec event in Moon et al., 2001b) were grown in the greenhouse. Two individual plants per each event were grown with 2 m distance from other plant types to prevent potential cross-contamination. Pollen collections were conducted for 10 days during flowering period. Collected pollen samples were immediately frozen in liquid nitrogen and stored at -80°C. At the time of assay, 1 ml of sterile water was added into each tube containing pollen grains and the tubes were shaken in a mixer (Eppendorf 5432 mixer) for 10 min. Pollen suspensions were filtered with a 132 μm pore nylon mesh (Sefar Nitex 03-132/43, Sefar filtration Inc., Depew, NY, USA) to remove non-pollen debris such as anthers and clumps of pollen that may clog the fluidic system of the flow cytometer. Filtered pollen suspension was transferred into 5 ml polystyrene round bottom tubes (BD Falcon, San Jose, CA, USA) for FCM analysis. FCM-based transgenic pollen analysis was performed using an

existing method described in Moon et al. (2011b). Data were obtained by counting 3,000 pollen grains with 3 technical replicates. Acquired data were analyzed using DiVa software (BD Biosciences, San Jose, CA, USA) and CyflogicTM software (CyFlo Ltd, Finland).

5.3.6 Test-cross with male sterile tobacco

Male sterile (MS) tobacco cv. TN90 seeds were planted as pollen recipient individuals for test-cross. Two individual plants per each EcoRI event were selected on the medium containing hygromycin 50 mg l^{-1} and transplanted in soils. Non-transgenic Xanthi and GFP control tobacco were also planted. When the EcoRI events and MS-TN90 were flowering, nine PCR-confirmed EcoRI events were crossed with male-sterile tobacco (cv. TN-90). Non-transgenic Xanthi and GFP control were also crossed with male-sterile tobacco. Manual crosses between the EcoRI events and MS-TN90 were performed to produce seeds. When seed pods on MS-TN90 were mature, seeds were separately harvested from respective MS-TN90 and cleaned. Cleaned seeds were surface-sterilized with 10 % bleach and 70 % EtOH. Seeds were plated on the medium containing hygromycin 50 mg l^{-1}. Fourteen days after plating the seeds, numbers of total germinated, hygromycin positive and negative seedlings were recorded.

5.4 Results

5.4.1 Transgenic tobacco generation and phenotype

A transformation vector with a translational fusion of the EcoRI restriction endonuclease and *G3GFP* gene under the control of the pollen-specific LAT52 promoter was constructed by using

a site-specific multisite Gateway® cloning strategy (Fig. 5-1). Nine independent transgenic EcoRI events were generated via *Agrobacterium*-mediated transformation method. Transgenicity of T_0 and selected T_1 events were confirmed by PCR (Fig. 5-2). Selected transgenic seedlings on the medium containing hygromycin 50 mg l^{-1} were transferred to the greenhouse and grown in soil. Non-transgenic tobacco Xanthi and GFP control plants were grown along with the transgenic events. Throughout the life cycles of the plants, no phenotypic differences between non-transgenic Xanthi and transgenic EcoRI events were observed (Fig. 5-3). Transgenic EcoRI events had normal flowers including petals, sepals, stamens, and carpels (Fig. 5-3C,D). All T_0 EcoRI events produced their T_1 progeny seeds through self-fertilization.

5.4.2 Pollen viability

Pollen viability was tested by IKI staining method. Freshly collected pollen grains were stained and screened under an epifluorescence microscopy with 100x magnification. The pollen viability of transgenic EcoRI events was not significantly different from non-transgenic Xanthi and GFP control, except EcoRI-2 event (Fig. 5-4). Only the EcoRI-2 event had statistically different pollen viability compared to non-transgenic Xanthi.

5.4.3 Microscopic and FCM analyses

Visual confirmation of transgenic pollen ablation and/or infertility was performed by microscopic analysis. Based on the microscopic pollen images, EcoRI events had significantly less or no GFP positive pollen grains compared to the GFP control (Fig. 5-5). To accurately analyze large numbers of pollen grains, FCM-based transgenic pollen screening method was

used. All EcoRI events had less that 0.5% of GFP positive pollen grains, while GFP control had 64% of GFP positive pollen (Fig. 5-6). Since GFP control plants were mixed population of hemizygous and homozygous for GFP transgene, it was expected to have over 50% of GFP positive pollen grains. Non-transgenic Xanthi contained 0% of GFP positives as expected. Three EcoRI events, EcoRI-5, 7, and 8, had 0% of GFP positives (Fig. 5-6). This result suggests that these events could have perfect selective male sterility.

5.4.4 Test-cross

Theoretically, if transgenic pollen were successfully ablated or infertile, there would be no homozygous lines produced in T_1 generation. Positive T_1 seedlings of each EcoRI event were selected on hygromycin selection medium and grown in soils. No seeds were produced from MS-TN90 plants that have not been crossed with other tobacco. Except EcoRI-4 and 9 events, all other EcoRI events had 100% hygromycin negative seeds from the test-crosses (Table 5-1). All test-crossed EcoRI seeds, except the EcoRI-4 and 9 events, were dead on the selective medium (Fig. 5-7). This suggests that pollen from these EcoRI events excluding the EcoRI-4 and 9 events did not carry transgenes. This result indicates that transgenic pollen was successfully ablated and/or infertile by overexpression of the EcoRI restriction endonuclease in pollen. This approach could be used as an effective biocontainment strategy.

5.5 Discussion

Transgenic pollen was successfully ablated and/or rendered infertile by overexpression of the EcoRI restriction endonuclease in pollen. The type II restriction endonuclease EcoRI was

used to destruct nuclear genomic DNA in pollen. Pollen specific LAT52 promoter drove a translational fusion of the EcoRI and GFP gene. Based on the FCM-based transgenic pollen screening result, three events, EcoRI-5, 7, and 8 had 100% efficiency on selective male sterility. However, further confirmation with the test-cross showed that most of the EcoRI events had higher efficiency on selective male sterility compared to the ones from the FCM-based pollen analysis. Seven independent EcoRI events had 100% efficiency on selective male sterility. This suggests that this selective male sterility approach could result in a perfect prevention of transgenic pollen-mediated transgene escape. This approach could be used as a highly efficient biocontainment strategy. Complete genic or cytoplasmic male sterility could cause environmentally negative impacts on pollen-feeding insects. This selective male sterility approach could be considered as the most environmentally friendly strategy unlike other complete male sterility approaches since it could serve as a food source for pollen-feeding insects by producing plenty of non-transgenic pollen. There might be concerns regarding potential negative effects of restriction endonuclease on insect pollinators. If transgenic pollen were completely ablated, insect pollinators would not be exposed to restriction endonuclease. In case transgenic pollen rendered infertility while being formed, there might be an environmental safety issue. However, restriction endonuclease activity is not only related to the presence or absence of the respective restriction sites in chromosome (Tartarotti et al., 2000). The restriction endonuclease EcoRII did not attack satellite heterochromatin in cytological preparations of mouse chromosomes, even though it showed a capability of cleavage with extensively purified mouse satellite DNA (Southern, 1975). Chromatin structures of different species can be an important factor that affects the activity of the restriction endonucleases in eukaryotic chromosomes (Gosalvez et al., 1989; Petitpierre et al., 1996).

Cytotoxic genes were used in most male sterility studies to prevent pollen formation. Using these cytotoxic genes might be a concern of potential toxicity to non-targeted organisms or cells. This potential toxicity would not be a problem if the EcoRI restriction endonuclease that is not toxic to cells were used for male sterility.

One of the most environmentally friendly strategies, transgene excision in pollen, is being debated on its effectiveness and sustainability because it leaves some parts of T-DNA after transgene excision occurs. In case of site-specific recombinased-mediated transgene excisions, several parts of T-DNA including left and right borders and a recognition site of site-specific recombinase that have been integrated into plant genome through genetic engineering would remain in pollen genome. Transgene excision in pollen is arguable because it technically does not result in completely transgene-free pollen. However, this selective male sterility would not produce any pollen that carries a small portion of T-DNA even if those are not functional. This approach could be an ideal biocontainment strategy for environmental and regulatory concerns on transgenic crops.

Non-transgenic Xanthi contained 0% GFP positive pollen from the FCM-based pollen analysis unlike the previously reported studies (Moon et al., 2011a; 2011b). It suggested that GFP positive pollen contamination in non-transgenic pollen reported in the previous studies could come from cross-pollination between transgenic events and non-transgenic Xanthi in the greenhouse.

Based on the result of test-cross, seven independent events had 100% efficiency on selective male sterility. While only three events demonstrated 100% efficiency from the FCM-based transgenic pollen screening method. Since FCM-based transgenic pollen screening method relies on GFP positive pollen, it is possible that previously synthesized GFP was exhibited in

pollen, so it was considered as a GFP positive. However, it became infertile due to the destruction of pollen genome afterwards. In this case, FCM-based transgenic pollen screening method could possibly underestimate the efficiency of transgenic pollen ablation and/or infertility.

Pollen recipient male-sterile (MS) tobacco was an effective tool for an initial screening the efficacy of events in the greenhouse. To acquire accurate progeny data for biocontainment strategies including transgene excision approach, emasculated tobacco plants might be required as pollen recipient plants (Moon et al., 2011a). No seeds were produced from male-sterile tobacco plants that have not been crossed with other tobacco. It assures that all produced seeds were resulted from a cross between respective transgenic EcoRI events and MS tobacco.

If transgenic pollen grains are not fertile owing to the destruction of pollen genome, EcoRI events should have higher percentage of non-viable pollen than non-transgenic Xanthi. On the other hand, if there is no significant difference in pollen viability between EcoRI events and non-transgenic Xanthi, pollen would most likely being ablated. Once transgenic pollen is ablated, collected pollen from EcoRI event that is assumed to be non-transgenic would not be apparently different from that of non-transgenic Xanthi. The result of pollen viability test indirectly indicates that pollen ablation might occur, since most of the events had similar percentage of non-viable pollen with non-transgenic Xanthi. However, direct evidence such as pollen count is required to more clearly address this question. From the results, it is clear that pollen from transgenic plants overexpressing the EcoRI restriction endonuclease in pollen would not carry transgenes. These results indicate that overexpression of the EcoRI restriction endonuclease could cause transgenic pollen ablation and/or infertility. This selective male

sterility could be used as an efficient and reliable biocontainment strategy to eliminate pollen-mediated transgene escape and introgression.

5.6 Acknowledgements

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Appendix

Tables and Figures

Table 5-1 Segregation of test-crossed progeny for hygromycin selection. Positive refers to the numbers of surviving seedlings on selection medium, while negative refers to the numbers of dead seedlings. Efficiency refers to transgenic pollen ablation or infertility ratio. 100% efficiency means 100% of transgenic pollen were successfully ablated or infertile, so no pollen carried the transgene and passed it to the progeny.

Event	Medium	Germinated	Positive	Negative	Efficiency (%)
Xanthi	MSO	92	N/A	N/A	N/A
Xanthi	MSO+Hyg	93	0	93	N/A
GFP control	MSO+Gluf	75	57	18	N/A
EcoRI-1	MSO+Hyg	326	0	326	100
EcoRI-2	MSO+Hyg	304	0	304	100
EcoRI-3	MSO+Hyg	251	0	251	100
EcoRI-4	MSO+Hyg	329	7	322	97.9
EcoRI-5	MSO+Hyg	365	0	365	100
EcoRI-6	MSO+Hyg	318	0	318	100
EcoRI-7	MSO+Hyg	152	0	152	100
EcoRI-8	MSO+Hyg	247	0	247	100
EcoRI-9	MSO+Hyg	287	1	286	99.7

Figure 5-1 T-DNA of the tobacco transformation vector.

The EcoRI restriction endonuclease was fused with the green fluorescent protein (GFP) gene for the purposes of monitoring expression and estimating efficacy of the pollen ablation system. The fused genes were under the control of the pollen-specific LAT52 promoter. LAT52: Pollen-specific LAT52 promoter; NOS t: Nopaline synthase terminator; NOS p: Nopaline synthase promoter; *HPT*: Hygromycin phosphotransferase; LB: Left border; RB: Right border.

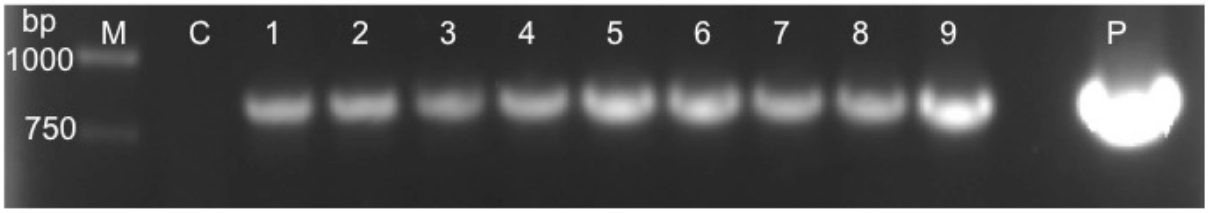

Figure 5-2 Transgenicity confirmation by polymerase chain reaction in T_1 generation events.

The EcoRI gene was amplified in all independently transformed events, while no amplification was observed in non-transgenic Xanthi. M: DNA size marker; C: non-transgenic Xanthi; 1-9: Transgenic events carrying the EcoRI gene; P: Tobacco transformation plasmid vector containing the EcoRI gene driven by the LAT52 promoter.

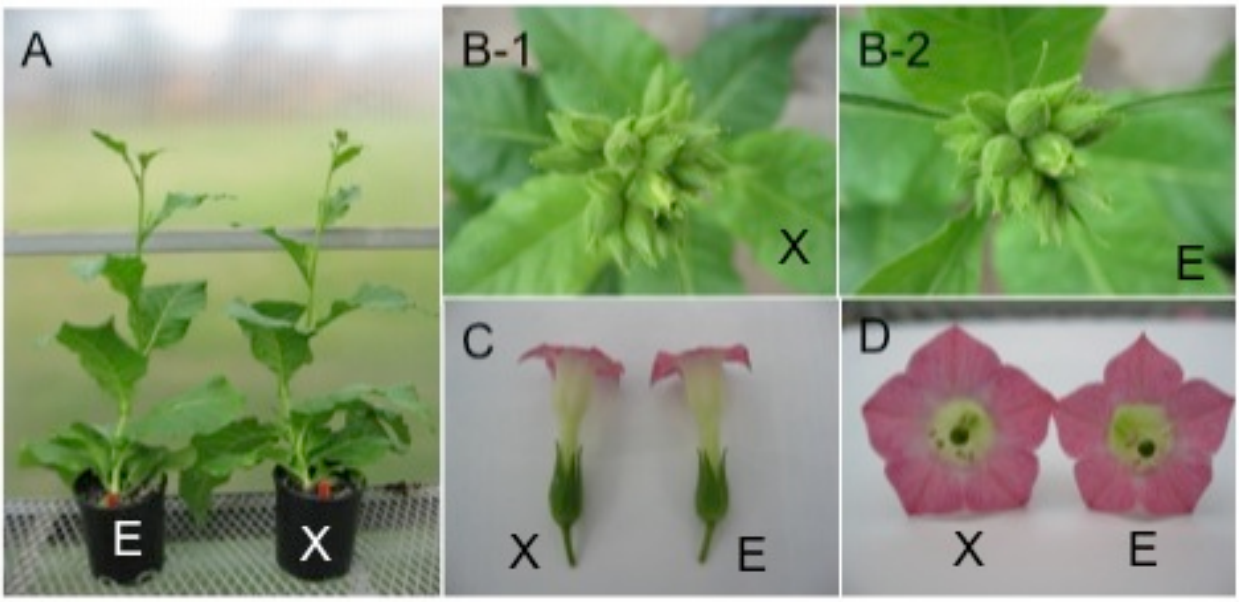

Figure 5-3 Phenotype comparison of non-transgenic Xanthi and T_1 trasngenic event overexpressing the EcoRI gene.

A: No phenotypic differences were observed between transgenic EcoRI event and non-transgenic Xanthi. B-1 and B-2: Flower buds of Xanthi and EcoRI-7, respectively. C: Lateral view of flowers. D: Front view of flowers. X: Non-transgenic tobacco cv. Xanthi; E: EcoRI-7 event.

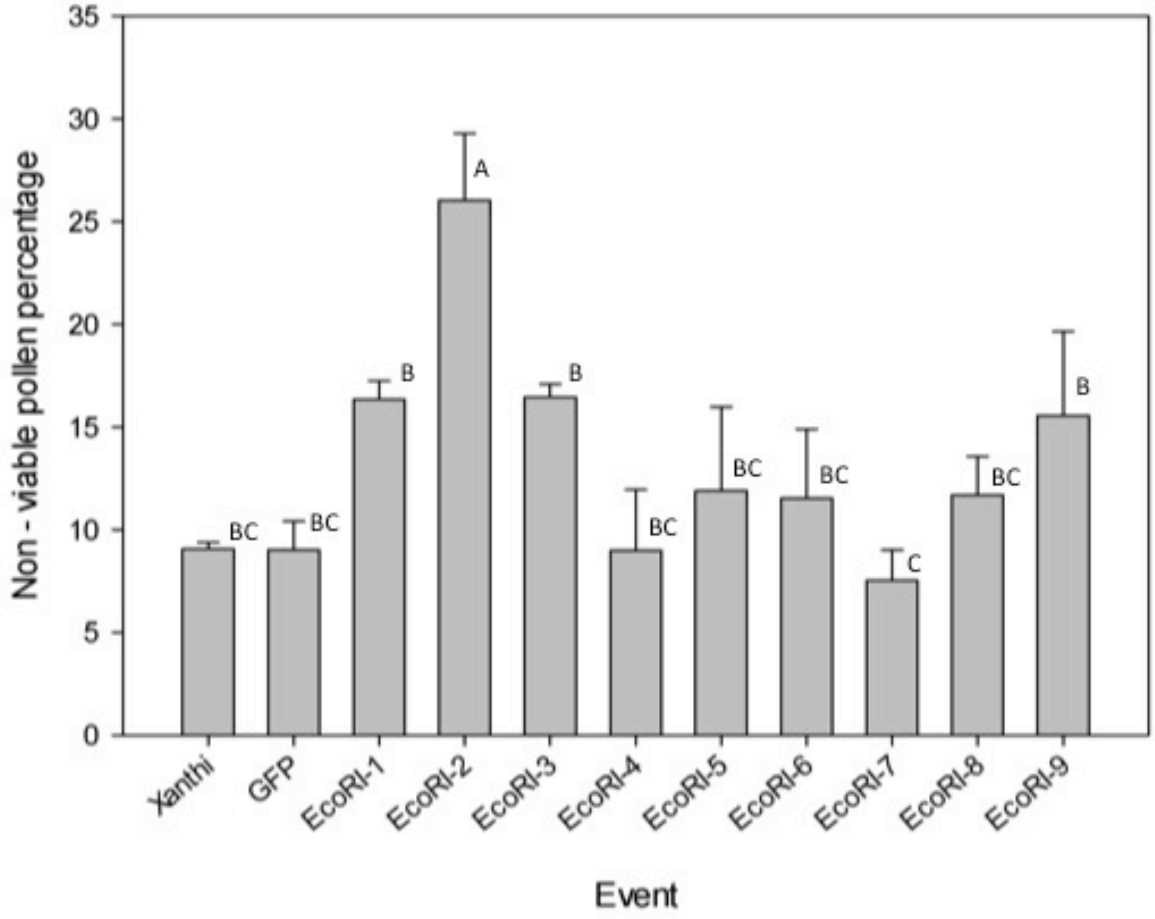

Figure 5-4 Viability of pollen from transgenic EcoRI events.

Potassium iodide-iodine (IKI) stain method (Mulugeta et al., 1994) was used to evaluate pollen viability. Only EcoRI-2 had significantly higher percentage of non-viable pollen when compared to other EcoRI events and control plants. Error bars represent standard deviation of the means.

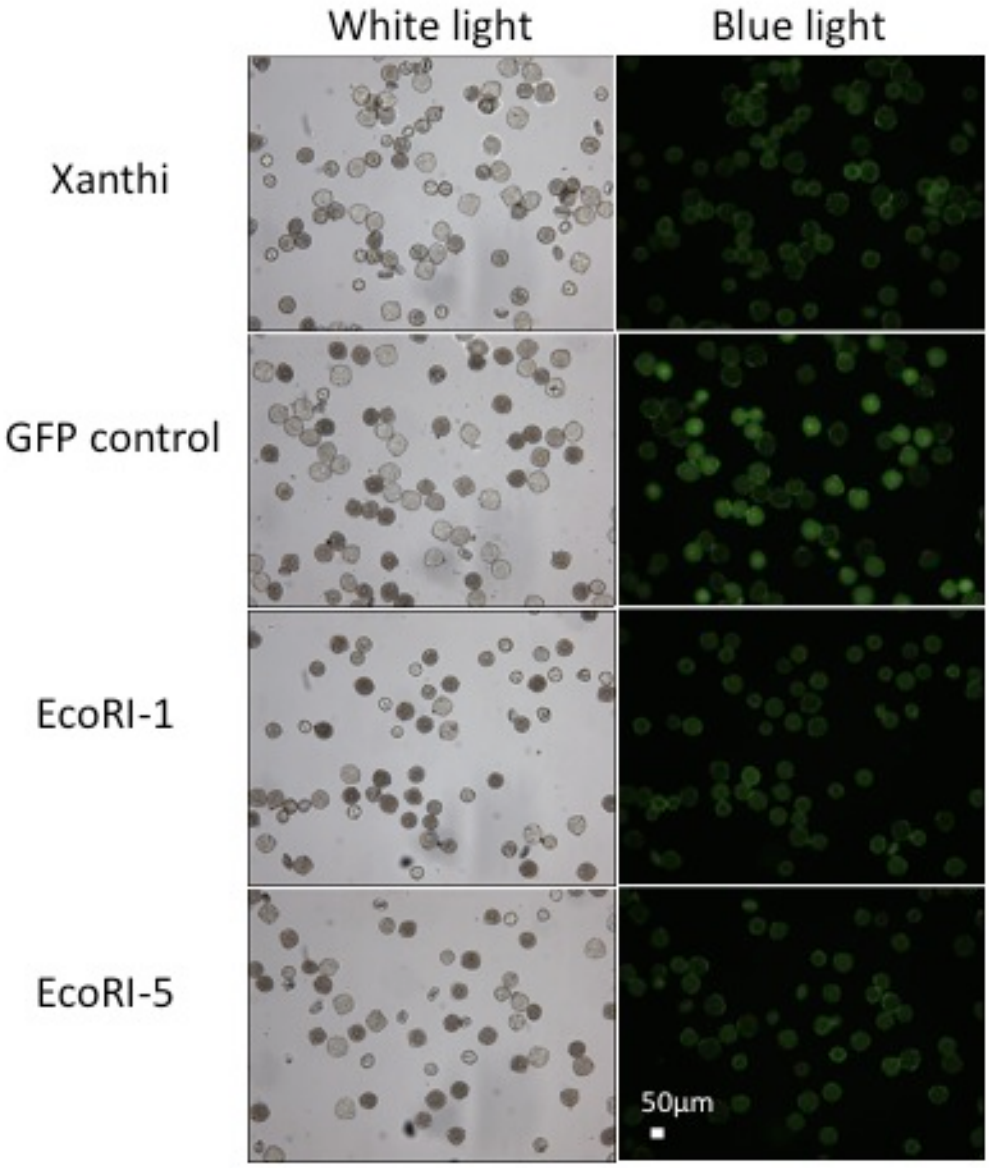

Figure 5-5 Microscopic images of pollen from transgenic EcoRI events.

Transgenic EcoRI events were transformed with a vector containing the LAT52 pollen-specific promoter, the *EcoRI* gene, and *GFP* gene as a visual marker. All images were taken under a FITC filtered epifluorescence microscopy (Olympus BX51 model) with white and blue light excitation at 200x magnification. Exposure time was 16.7 ms and 3 s for white light and blue light, respectively.

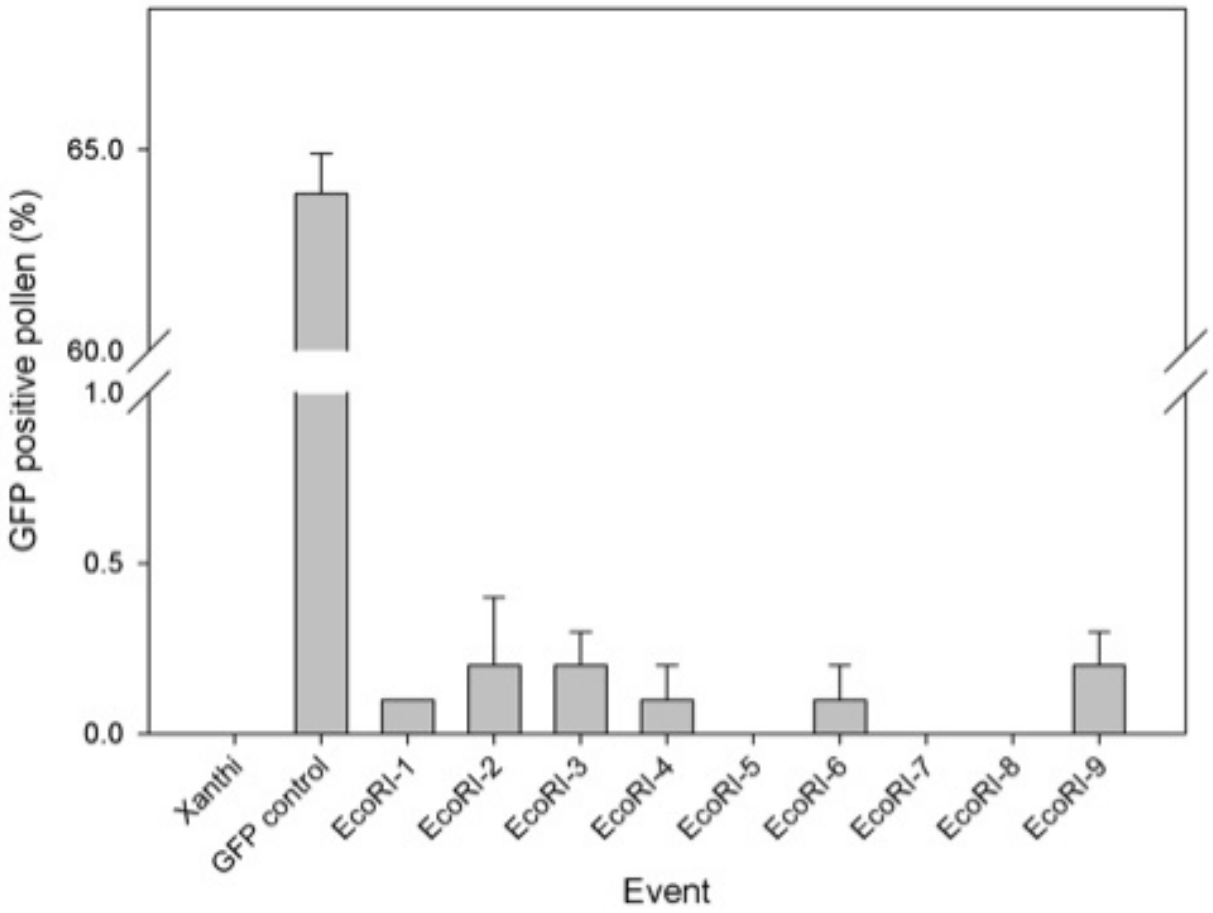

Figure 5-6 Percentage of GFP positive pollen in transgenic EcoRI events.

GFP positive pollen grains were detected and counted via the flow cytometry (FCM)-based transgenic pollen screening method. Percentage of GFP positive pollen ranged from 0 to 0.2 among nine different transgenic events, while the GFP control had 64 % and non-transgenic Xanthi had no GFP positives. GFP control pollen samples were collected from a mixture of hemizygous and homozygous plants. GFP positive pollen percentage was acquired based on 3,000 pollen counting for each measurement with 3 replications. Error bars represent standard deviation of the means.

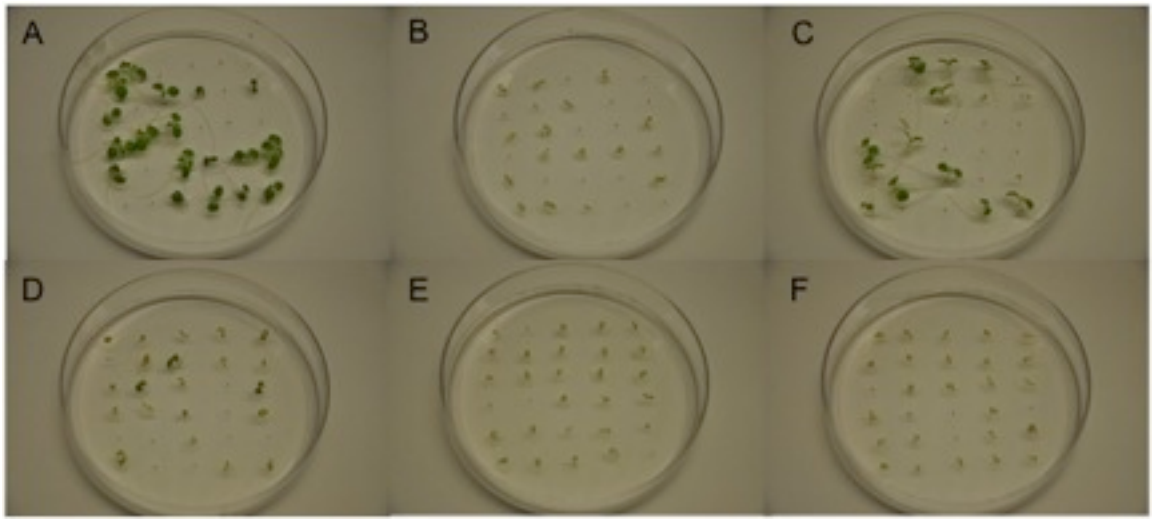

Figure 5-7 Segregation of test-crossed progeny.

(A) Non-transgenic Xanthi on MSO medium without selective agent. (B) Non-transgenic Xanthi on MSO medium containing hygromycin 50 mg l^{-1}. (C) GFP control tobacco on MSO medium containing glufosinate as a selective agent. (D-F) EcoRI-4, 5, and 8 events, respectively, on MSO media containing hygromycin 50 mg l^{-1}.

Chapter 6: Conclusions

Rapid detection and counting of relatively rare transgenic pollen in a mixture of predominantly non-transgenic pollen is necessary for efficient and reliable transgenic pollen screening and detection. FCM-based transgenic pollen screening is not only less laborious than other methods, but also extremely fast and accurate for high GFP-synthesis events. Transgene containment strategies and other transgene flow studies can be accurately and efficiently analyzed with this FCM-based method. Since high levels of expression of fluorescent marker genes would be expected in transgenic pollen containing a transgene removal system in the fields, the efficiency of the transgene removal system could be determined in a fast and accurate manner by analyzing large numbers of pollen using FCM. Also, if floating pollen grains in the air need to be analyzed for transgenic pollen tracking, collected pollen could be effectively analyzed for transgenic pollen detection by the FCM-based method. Even though transgenic plants do not have a built-in fluorescent marker for pollen, fluorescently labeled surface markers such as an antibody or quantum dot could possibly be employed and utilized for transgenic pollen detection.

Transgene excision via site-specific recombination could be an effective transgene biocontainment strategy when gene flow from pollen is the main concern for transgenes leaving the field of interest (Luo et al., 2007; Moon et al., 2010). A codon optimized serine resolvase recombinase CinH recognizes specific 119 base sequence recombination sites and excised embedded functional transgenes including the *eGFP* gene from the pollen genome. An absence of GFP expression served as an indicator of transgene excision. Three independent CinH tobacco T_1 events exhibited less than 1% GFP positive pollen, based on FCM analysis of 30,000 pollen grains. As compared to a control CinH_Drec event, which had GFP expression in 70% of pollen, CinH events had a significant decrease in percentage of GFP positive pollen. This indicated that transgenic events containing CinH recombinase efficiently excised transgenes from the pollen

genome. CinH recombinase has superior specificity by recognizing a 119 nucleic acid base sequence known as *RS2* (NCBI ref# AF213017). As compared to Cre and FLP recombinases, which have 34 bp recombination sites, CinH has recombination sites of 119 bp that should provide high specificity to the site-specific recombination, this negating the risk of illicit recombination within plant genomes. One *RS2* recombination site would remain in the pollen genome after transgene excision occurs. However, this single recombination site in the absence of a recombinase and another *RS2* sites should not lead to recombination in pollen genome.

Transgenic pollen was successfully ablated and/or rendered infertile by overexpression of the EcoRI restriction endonuclease in pollen. The type II restriction endonuclease EcoRI was used to destruct nuclear genomic DNA in pollen. Pollen specific LAT52 promoter drove a translational fusion of the EcoRI and GFP gene. Based on the FCM-based transgenic pollen screening result, three events, EcoRI-5, 7, and 8 had 100% efficiency on selective male sterility. However, further confirmation with the test-cross showed that most of the EcoRI events had higher efficiency on selective male sterility compared to the ones from the FCM-based pollen analysis. Seven independent EcoRI events had 100% efficiency on selective male sterility. This suggests that this selective male sterility approach could result in a perfect prevention of transgenic pollen-mediated transgene escape. This approach could be used as a highly efficient biocontainment strategy.

Vita

Hong Seok Moon was born on Feburary 12, 1976 in Seoul, Korea to Dr. Yang-Soo Moon and Mae-Ja Kang. He graduated from Yang Jae high school and started his college education at Korea University in Seoul, Korea. After sophomore year, he joined a mandatory army service and served as a riot police for 26 months. He returned to the campus and graduated in 2002 with a Bachelors of Science in Crop Science. He obtained a Masters of Science degree in Plant Sciences with a concentration of Plant Biotechnology at the University of Tennessee, Knoxville. During his Masters studies, his research focused on the consequences of transgene escape and introgression. In 2007, He started his Ph.D studies in the Stewart laboratory in Department of Plant Sciences at the University of Tennessee, Knoxville. His research focused on developing biocontainment strategies for genetically engineered crop cultivation. He plans to graduate with a Doctor of Philosophy degree in Plants, Soils, and Insects with a concentration of Plant Molecular Genetics in August 2011. He plans to do a postdoctoral training at USDA-ARS Southern Regional Research Center in New Orleans, Louisiana from August 2011.