NOVEL *IN PLANTA* TRANSFORMATION OF NIPPONBARE RICE (*ORYZA SATIVA*) TISSUES USING ELECTROPORATION TYPE PLASMA JET

A Thesis

by

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Submitted to the Office of Graduate and Professional Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

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December 2020

Major Subject: Plant Breeding

ABSTRACT

Common plant transformation techniques, such as *Agrobacterium*-mediated and biolistic transformation, still rely on time-consuming and intensive tissue culture steps, including callus induction and regeneration. Eliminating these steps using *in planta* transformation may service to further improve advances in plant molecular biology. Moreover, CRISPR-Cas delivery using ribonucleoprotein (RNP) transformations may further improve the efficiency of transformation using a transient approach that does not require integration into the genome. **Similar to electroporation-protoplast transformation, non-thermal atmospheric-pressure dielectric barrier discharge jet (DBDJ) can generate an electric field near cells, inducing pore openings in the cellular membrane and cell wall, and allowing the insertion of exogenous genetic material through the cell wall and plasma membrane to be expressed in living plant cells.** The ability of DBDJ to cause electrostatic disruptions of cell wall structures has already been reported in gram-negative bacteria. To utilize this technology for plant genome editing, improvements to the electroporation-like plasma jet transformation must include temporary disruption of both the plant cell wall and membrane, without irreparable damage to plant tissues or function. Here we demonstrate the transformation potential of DBDJ by insertion of an eYFP expression plasmid into mature rice embryos (ME), and a variety of GFP binary and non-binary vectors into root tissues of rice seedlings. By exposing excised MEs and seedlings to the DBDJ electric field after treatment in a liquid osmotic medium, we allow for the successful insertion of eYFP and GFP plasmids. The application of this technology may not be limited to genome editing with the potential to be utilized for many other molecular biological processes.

DEDICATION

I'd like to dedicate this to my parents Stephon E Warren Sr, Denise L Warren and GOD. Thank you for always supporting me and always believing in my potential no matter what trials and tribulations came my way. You were the only ones who ever pushed me to be greater than what I thought I could be and never doubted my drive no matter what direction I took; I wouldn't be here without your saving grace. There aren't words to describe how much I love you all.

ACKNOWLEDGMENTS

I would like to thank my committee chair, Dr. Thomson, for allowing me to be a member of the Crop Genome Editing Lab here at Texas A&M. Even from undergraduate, you supported me as a student worker, which sparked my interest in graduate research. I'd also like to thank you for allowing me the opportunity to investigate this new and exciting technology in relation to crop genome editing.

Thanks go out to my lab mates and employees at the Crop Genome Editing Lab in College Station, for supporting me and my development as a molecular plant biologist. I'd like to especially thank Karina Morales for sharing vital professional and technical information with me from undergrad until now. I'd like to thank Sudip Biswas for being a well of experience and his hands-on help with lab protocols. Special thanks go out to Dr. Nikolaos Tsakirpalogiou, for his pivotal advice in regards to developing a publication and for motivating me and keeping me accountable through this journey.

Thanks also go to the Office of Graduate and Professional School for awarding me the Diversity Fellowship without which I wouldn't even be here; many thanks.

Finally, I'd like to thank Dr. David Staack and my collaborator Min Huang of the Texas A&M Plasma Engineering and Diagnostics Lab. Without your technology and help, this project wouldn't be possible. Thank you for allowing me the opportunity to work with this amazing technology.

CONTRIBUTORS AND FUNDING SOURCES

This work was supported by a thesis committee consisting of Professor Michael Thomson (Advisor) and Associate Professor Endang Septiningsih of the Department of Soil and Crop Sciences and Professor David Staack of the Department of Mechanical Engineering.

Plasma Jet Characterization data was provided by Min Huang.

All other work conducted for the thesis was completed by the student independently.

Graduate study was supported by an Office of Graduate and Professional Studies Diversity Fellowship from Texas A&M University.

NOMENCLATURE

DBDJ	Dielectric Barrier Discharge Jet
FC	Foreign Constructs
GFP	Green Fluorescence Protein
ME	Mature Embryos
PDS	Phytoene Desaturase
RAM	Root Apical Meristem
RNP	Ribonucleoprotein
ROS	Reactive Oxygen Species
SAM	Shoot Apical Meristem
WS	Whole Seeds
YFP	Yellow Fluorescence Protein

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CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

I.1 Introduction

By 2050, the Earth's global urban population is predicted to increase from 7.6 billion to 9 billion people (Deelstra et al., 2000). This increase in population will lead to a higher percentage of poverty in developing regions and the demand for food will exponentially increase as well. The growing population will demand increased food and fiber security (predicted within the next 40 years), causing a rush in agricultural breeding and research. The rising population will cause competition for land, water, and energy use and negatively affect standard agricultural practices to produce food. Therein, the ability to quickly engineer new cultivars capable of adapting to changing terrain and climate will help crop production in the future. Climate change has led to many adverse effects in crop production; these effects include increased temperatures, drought and flooding (Parry et al., 2004). Many current research programs have already begun to focus on these issues, such as the development of heat tolerance and submergence tolerance crops (Fukao et al., 2011, Xu et al., 2006). This research has led to the development of crops capable of thriving in what would be detrimental abiotic stress conditions. This is exemplified by the development of salt tolerant rice via the upregulation of the chloroplastic glutamine synthetase (GS2) gene from rice, increasing the plant's photorespiratory capacity, thus conferring resistance to high salt conditions (Hoshida et al., 2000).

Once potential genes are identified, they must then be tested in relevant crop species using an appropriate plant transformation procedure. Plant transformation has become a crucial step in developing both transgenic and non-transgenic crops. It is a crucial practice wherein foreign constructs (FC) (DNA, RNA, Protein) can be introduced into viable plant cells. Current methods

of delivery include electroporation, callus induction, biolistics, micro-nano tubules, etc. Each of the mentioned delivery methods serves to either allow for the infiltration of FC, or creates an environment or surface that is readily accepting of FC, such as callus induction. We have outlined here a new method of delivery, "plasma jet". Similar to electroporation, it uses an electrical field to permeabilize plant tissue by dilating the pores in the cell wall to allow for infiltration. Practically, electroporation can be difficult to use; in many instances' samples are destroyed due to issues with cassettes or human error in applying samples to the cassette. Also, the use of electroporation as a method of crop transformation is limited to crops that are readily regenerable via callus induction. Using a Plasma jet may serve as a gentler method of delivery. Mature embryos and seedlings exposed to charged helium gas retain their totipotency and viability and are unaffected by the process. Post-exposure to the plasma jet, excised mature embryos and seedlings are readily accepting of FC. Using plasmids encoding for eYFP and GFP, we have shown that plasma jet can be used to transfect imbibed mature embryos and germinating seedlings.

I.1.1 Agrobacterium Transformation

The most common method used to produce a transgenic crop is *Agrobacterium*-mediated transformation. Typical techniques involving *Agrobacterium* require the isolation of a large tumor-inducing plasmid (Ti) from the pathogen, cloning a gene of interest into the T-region of the plasmid, and co-culturing of this new plasmid with protoplasts. One of the major issues with this transformation method is the time requirement. For methods utilizing protoplast, you must first isolate protoplasts, a technique that must be optimized for each species, then induce callus growth and regenerate. Callus induction to regeneration in rice takes about 3-5 months in a closely watched environment. This regeneration time is highly dependent on the species and

variety (Delporte et al., 2001). While Agrobacterium transformation using callus induction will remain useful for crops lacking a reference genome, a more promising experiment is to attempt the transformation of plants *in vivo*. Recently researchers Maher, Michael F., et al. 2019 at the University of Minnesota have demonstrated how to generate gene-edited dicotyledonous plants through *de novo* meristem induction. Plant meristems are the progenitor of almost all post-embryonic organs both above and below the soil surface. These meristems maintain a group of undifferentiated cells located in the central zone (CZ) that will proliferate new cells used for organ development. These totipotent undifferentiated cells have the potential to transdifferentiate into other cells; thus, the targeted transformation of these cells should affect the resulting organ primordia. As the meristem apex moves away from the undifferentiated cells via cell division, they enter different developmental pathways leading to cell differentiation resulting in roots, leaves and floral organs. This proves that meristematic tissues like those found in the shoot apical meristems (SAM) and root apical meristems (RAM) or in the case of Maher, et al. the nodes of dicots, contain cells that can be targeted for *de novo* transformations. However, contrasting the *de novo* experiment described above, plasma jet transformation on soil-grown dicots will only rely on topical treatment and involves minimal (microscopic) damage to the plant. In contrast, Maher et al. removed existing meristems to access delivery sites. For dicots, this could be as simple as pointing the system at a node, while meristematic tissues of monocots that do not have exposed nodes will have to be surgically exposed. Mature embryos isolated from germinating seedlings can be excised in such a way that both the SAM and RAM are exposed [Fig.1] while leaving the viability of the embryos unaffected. Transformation of mature embryos with a plasma jet allows us to skip the co-culturing and callus regeneration phase used with Agrobacterium and electroporation and quickly generates transformed plants.

I.1.2 Development of Plasma Jet

Dielectric barrier discharges (DBDs) are electrical discharges in electrode configurations that contain an insulating material in the discharge path in our case that is Alumina. This dielectric barrier is responsible for a pulsing plasma action and subsequently, the formation of a nonthermal plasma at normal pressure (Brandenburg, 2017). Initially, investigations by Siemens in 1857 were focused on ozone generation. This was done by exposing oxygen to a dielectric-barrier discharge (DBD) maintained in a narrow gap between two glass tubes by an alternating electric field of sufficient amplitude (Kogelschatz, 2003). When air is used as the carrier gas, DBD produces reactive oxygen species (ROS) like ozone, hydrogen peroxide, hydroxyl radicals, and reactive nitrogen species (RNS) such as nitric acids and peroxynitrites (Sakiyama et al., 2012). The technology has since been used for other applications such as surface treatment, degradation of pollutant molecules in gases, pumping of gas lasers, plasma displays, and generation of excimer radiation (Brandenburg, 2017). From an agricultural perspective, some recent applications include the sterilization of fungal pathogens in rice seeds and the improvement of the hydrophilicity of cotton seeds (Jo et al., 2014, Wang et al., 2017).

In terms of surface sterilization, a plasma jet has been postulated as a method for bacterial sterilization. Researchers aimed to use the technology for water purification, surface sterilization, and a wide variety of agricultural uses. Particularly DBDJ has been successful in inactivating both gram-negative and gram-positive bacteria (Estifaee et al., 2019). It was this finding that initially sparked the idea that DBDJ could be used to transfect plant cells, as both gram-negative cells and plants have a cell wall and an inner cell membrane. Although the bacterial cell wall is made of lipopolysaccharides (LPS) and plant cell walls are made up of a mix of cellulose, hemicellulose, and lignin. Research has shown that the primary cause of cell wall disruption in

gram-negative cells was due to the electric field generated by the DBDJ. These cell wall disruptions, when visualized, appear as holes or pores in the cell wall in bacteria suspended in solution. This would result in a leakage of genetic material. However, during these experiments, they noted that large amounts of reactive oxygen species (ROS) were released from the solution and may play a role in the degradation of the bacterial cell wall. This could be due to excited electrons interacting with aqueous solutions. It is also possible that the electrons were reacting to the oxygen in the LPS, potentially leading to cell disruption.

A study by Jun et al. demonstrated that DBD could be used to reduce the crystallinity of cellulose, the major structural component of plant cell walls (Jun et al., 2008). Argon gas was used to treat cotton fiber with high alpha-cellulose content, wherein both increased treatment time and power applied to the plasma increased the solubility of cellulose. This was represented by a decrease in hydrogen bonds, thus increasing the solubility of celluloses. This could suggest that the successful disruption of cellulose within the plant cell wall may be specific to the carrier gas used.

However, a more recent study by Wright, et al. 2018, suggests that the carrier gas had no significant effect on the treatment and the radical species generated by the plasma, such as hydroxyl radicals, hydrogen peroxide, and hydrated electrons, were mainly responsible for the disruption of hydrogen bonds. Wright, et al., designed a pretreatment reactor that produces a DBD plasma above a liquid surface to treat microcrystalline cellulose (MCC) solutions. They found that a 50min treatment of MCC with DBD plasma reduced the crystallinity index (CI) from 82.8% to 58.9%, proving that plasma treatment can be used to reduce intramolecular and intermolecular bonds. Even though this study demonstrated that radicals and ROS were crucial for reducing the crystallinity of MCC, the effect of reactive nitrogen species (RNS)

remains relevant (Wright et al., 2018). In a separate study, NO_2 was shown to oxidize cellulose to glucose effectively (Zhou et al., 2015). However, further investigation into the exact mechanism of cell wall disruption is needed. Using this information and the knowledge that cellulose rings are attached to one another by a single oxygen atom, it is possible that DBDJ could be used on plant tissues to disrupt the cell wall causing pore openings. There has been research into the effects of direct barrier discharge plasma on plant seeds. Treatment of coriander seeds with direct barrier discharge plasma resulted in the surface structure of the seed being partially destroyed or roughened by exposure (Ji et al., 2015).

1.1.3 Plasma Jet and Electroporation

A similar method of plant cell transfection is electroporation, wherein a plant cell is stripped of its cell wall via enzymatic digestion, suspended in an electroporation buffer and exposed to an electric field of varying parameters. Some common electroporation buffers include saline-based, phosphate-based, HEPES-based, or cell-culture-based, with electric conductivity and osmolarity tailored by additional salts and sugars, respectively (Sherba et al., 2020). The solution is then given an electric pulse of appropriate strength, duration, form, and number that causes a potential difference across the membrane and induces a temporary and reversible increase in permeability, allowing entry of large molecules such as DNA, RNA, proteins, and most often plasmid DNA (pDNA) into an otherwise impermeable cell membrane (Bolhassani et al., 2014). This is due to changes to the transmembrane voltage (TMV), which is the electric potential difference between the inner and outer plasma membrane, which is generated and regulated by a system of ion pumps and channels. Exposure of a cell to external electric field results in TMVs exceeding their resting stage causes structural changes to the membrane and its constituent molecules, such as membrane transport proteins as well as membrane lipids (Kotnik et al., 2019). The effect of this

is permeabilization of the plasma membrane is the allowance for passage of otherwise unpassable molecules (Kennedy et al., 2008). Following this, the cells are pelleted and re-suspended in culture media, then plated on selection media and regenerated. This technique implies that the plasma membrane can withstand an electric pulse and that pore openings are temporary, also that the cell wall can be regenerated rather expeditiously as well. The temporary nature of the pore dilation was verified by Tsong, Tian Yow, 1989. Tsong reasoned that if pores can be created in a cell membrane, and if small molecules can penetrate before cytosolic macromolecular contents leak out, it is likely that one can load molecules into pulse electric field (PEF) treated cells and then reseal the perforated membranes before these cells reach the lysis stage. They confirmed this by designing a protocol wherein they loaded exogenous molecules into lOO%-hemoglobin-retained erythrocytes (Kanehisa et al., 1978). Combined with the knowledge regarding bacterial sterilization and the increased solubility of cellulose by DBD, it seems possible to create a temporary pore opening that will allow the infiltration of genetic material such as plasmids and proteins.

I.1.4 Plasma Jet and Gene Gun

A rapidly improving technology in genome editing delivery systems is biolistics. Similar to electroporation and DBDJ, this system utilizes physical forces in order to insert FCs. Wherein electroporation and DBDJ provide electrostatic forces to permeabilize plant tissues; biolistics utilize what is called a Gene Gun to force FCs into tissue using high pressure, thus the term biolistics. High-velocity microparticles, typically gold, are attached to DNA or proteins and carried past cell walls and membranes. There are many benefits to biolistics; due to the high velocity of projectiles, biolistic protocols are shown to be nonspecific in terms of tissues affected; biolistics also shows limited host specificity, unlike *Agrobacterium*-mediated

transformation. This nonspecific technique has allowed for the development of previously limited germplasms, specifically those of previously recalcitrant cereals, legumes and woody species. Some of these major transgenic crops include soybean, peanut, corn, rice and wheat (Christou, 1995). It is also specifically applicable to organelle transformation (Sanford, 1990). Biolistics also allows for the potential of multiple gene transfer, which is crucial for complex genetic modifications, such as modifications to metabolic pathways that may require the production of both enzymes and transcription factors (Sanford, 1990).

However, like most techniques, it does have its disadvantages, mainly the use of very specific and expensive instrumentation and wide sample variation as compared to *Agrobacterium*. There is also a high frequency of integration of the vector backbone and subsequent loss of transgene integrity due to multicopy insertions (Yang et al., 2000, Taylor et al., 2002). Like most transformation techniques, a certain amount of optimization is required for successful transformation using biolistics as well. Electroporation may be proposed as a substitute to biolistics, as it is inexpensive and simple, however, the technology hasn't been very successful for a wide array of crops, as the requirement for tissue culture due to the thick cell wall remains a hindrance.

DBDJ may develop to be a crucial transformation technique as it has the potential to transform tissues in a non-specific manner once optimized, as well as directly permeabilize the cell wall of plants *in vivo*.

I.2 Approach and Rationale

I.2.1 Leading Evidence

In terms of genetic modification of plant cells retaining their cell wall, Hiromichi, Morikawa, et al. (1986) demonstrated the possibility of *ex vivo* plant modification by electro

injecting the Tobacco Mosaic Virus (TMV) RNA directly into intact isolated mesophyll cells, with the injected gene being successfully expressed. 40ul of a tobacco protoplast mixture in a homemade platinum electrofusion chamber received an electric impulse of 200 V (1 KV/cm) and 0.1 μ. F was given to the protoplast mixture. The electric resistance of the protoplast suspension in the fusion chamber was 1 to 2 KΩ, and the time constant of the decay of the electric pulse was I 00 to 200 μ.s (Morikawa et al., 1986). They argued their protocol would address the limiting host specificity inherent in protoplast protocols. After treatment with TMV RNA, they used a TMV fluorescent antibody to assay the electro injected cells, with some cells showing fluorescent specks due to aggregates of TMV, with inclusion body structures seen after 20 days (Hiromichi et al., 1986). Recently, another group has found similar success in plant transformation using a multi-gas plasma jet. They have demonstrated the potential by introducing cell-penetrating peptides (CPPs) as well as a fluorescent His-tagged sGFP-CyaA protein of about 75kDa into tobacco cuttings (Yanagawa et al., 2017). With this information, there is enough supporting evidence to assume that the insertion of Cas proteins may be possible.

I.2.2 Goals and Objectives

In order to optimize the system first, we are first using eYFP and GFP to visually verify that FC is taken up by the tissue, integrated into the plant genome and expressed. Once a consistent phenotype is noted from one set of experimental conditions, we will proceed with knockouts of Phytoene Desaturase (PDS). The strategy to mutate and knockout the phytoene PDS by CRISPR/Cas9 has been widely used to demonstrate the feasibility of CRISPR/Cas9 since its mutation results in early seedling death and chlorosis or photobleaching of early leaves (Miki et al., 2004, Su et al., 2012). If successful, the speed and flexibility of this technology could revolutionize the field of plant molecular biology as well as crop genome editing, possibly

leading to the industrial mechanization (assembly line) of crop transformation. With a successful precedent for transformation *in vivo,* DBDJ could be used in lieu of timely tissue culture steps and instant validation of FC function. For example, if you wanted to identify the exact location of *pin*7, a novel auxin efflux regulator, in plant roots, you could design an experiment wherein you use DBDJ to insert a plasmid encoding a protein specific to the promoter region of the *pin*7 gene tagged with GFP. In this experiment, you would target the root tissue with DBDJ system, expose the tissue to plasma and apply the plasmid. This would allow you to visualize *pin*7 expression in the root tissue without having to culture tissue until roots develop.

Our ultimate goal will be to use CRISPR/Cas in order to make knockouts, knock-ins, interfere and activate genes. The use of CRISPR will allow for permanent/integrated edits to the plant genome. The successful use of CRISPR FCs will prove the utility of the technology as a tool for molecular biology as well as a viable method for crop transformation.

I.2.3 Conditions for success

Notably, certain conditions are optimal for successful transfection. It seems that the cells may need to be primed in order to more efficiently receive FCs. Sabri et al. demonstrated in 1996 that cell plasmolysis before electropulsation (one square wave pulse of 15 ms at 750 V/cm) is an efficient approach to DNA delivery into intact plant cells (black Mexican sweet maize). They demonstrated that osmotic changes, which create a void between the cell wall and membrane, allowed macromolecules to pass through the cell wall, maximizing the contact of macromolecules with the cell membrane (Delsart, 2017). Similarly, the use of high osmotic media is used today in other genetic delivery systems such as electroporation and the Ribonucleoprotein complex (RNP) system. RNP, wherein gold particles coated with Cas protein and a guide RNA (gRNA) are shot in a high-pressure vacuum through plant tissue to cause

genetic modification. This method has been used successfully to genetically modify bread wheat (Liang et al., 2018). In this protocol prior to shooting plant tissue with RNPs, the tissue is soaked in an osmotic media (OM) in order to cause plasmolysis. Therein OMs as a source of plasmolysis were included in the experimental procedures here.

I.3 Determining Success

Ultimately the transformation of an intact seedling would serve as an indication of the powerful potential of this technology. Here we intend to insert eYFP and GFP vectors into mature embryos of an intact rice seedling, respectively. If successful, we will [1] verify that experimental treatment of mature embryos will not affect the viability of the tissue [2] demonstrate that meristematic tissues of mature embryos are susceptible to transformation using DBDJ [3] verify that experimental treatment of whole seeds will not affect seed viability [4] demonstrate that imbibed seeds are capable of transfection regardless of bran and aleurone layers. Future applications of this research are relevant to a fast-growing crop genome editing field. The ability to efficiently transfect plant tissues in a matter of minutes could possibly lead to the acceleration of genetic crop improvement and the release of subsequent cultivars. It is my intention to optimize the protocol for this method of transfection, as well as methods to minimize experimental costs.

CHAPTER II

DETERMINING MATURE EMBRYO VIABILITY AND TRANSFORMATION POTENTIAL OF MATURE EMBRYOS

II.1 Synopsis

Mature Embryos (MEs) are embryos excised from a mature fertilized seed. By carefully excising these tissues from mature seeds, we expose the apical meristems of the plant. The apical meristem of the plant possesses an area of meristematic totipotent cells that are akin to mammalian stem cells that can give rise to any cell type. Plant meristematic tissues can further differentiate into other meristematic tissues or vascular tissues such as leaves or roots. This area is responsible for growth, and by targeting this area for DBDJ transformation, we aim to induce changes that will be apparent in developing tissues. However, currently, we are using transient eYFP to verify the potential of this genetic delivery system. In order to target these areas, we must first know if excision and treatment of these tissues will have adverse effects on tissue development or germination.

II.2 Introduction

Many current crop transformation protocols require the use of embryogenic cultures or callus. These are plant embryos that have gone through somatic embryogenesis via preliminary culture in osmotic media and subsequent culture on a root inducing media. Most often, this rooting media contains the auxin analog 2,4-dichlorophenoxyacetate (2,4-D). 2,4-D is a synthetic small molecule that plants cannot degrade *in vivo*. Natural auxin indole-3-acetic-acid (IAA) plays a crucial role in plant growth development, specifically for embryogenesis. In contrast, 2,4-D kills plants and is a common ingredient in selective broadleaf herbicides like Roundup. Yet 2,4-D is an important ingredient in callus induction, causing a buildup of auxin in the plant and

inducing uncontrolled somatic embryogenesis, creating a callus. These new cells are akin to the meristematic cells of a plant embryo. As a mass of undifferentiated cells, the tissue of these calli are primed for coculture with *Agrobacterium* or transformation using particle bombardment.

As mentioned previously, transformation using callus can be difficult as it requires a certain level of expertise to grow and maintain these tissues to regeneration. There are many factors that affect the frequency of callus formation in tissue cultures, such as culture medium composition, explants source, genotype, and environment (Abbas et al., 2018). Once an acceptable protocol is identified, it still can take over three months to regenerate a healthy explant.

In order to expedite this process, we are testing the direct treatment of embryonic cells. By carefully excising mature embryos from germinating seeds, we can expose meristematic tissues that can be targeted for DBDJ transformation. To test this, we must know if an excised mature embryo can still germinate into a seedling, will treatment with osmotic solution impair the germination of the embryo, and can an excised embryo be transformed using DBDJ. Here we demonstrate the viability of mature embryos in an osmotic solution and the transformation of mature embryos using an eYFP vector.

II.3 Materials and Methods

II.3.1 Plant Materials

The rice variety used in this section is the *japonica* rice cv Nippobare. Nipponbare seeds were acquired from the GRIN U.S. National Plant Germplasm System. Seeds were dehusked using a hand-held rice husker, then soaked in double-distilled water (ddH_2O) at room temperature over the course of two days. In between each day, the seeds were washed in ddH_2O and re-submerged in ddH_2O. On the third day, germinated seeds were transferred to 5” pots containing a

1:1 mix of Turface MVP soil conditioning clay and SunGro Professional Mix soil, respectively. Pots were placed on flood trays, and water levels were kept consistent throughout the growing season. Plants were fertilized after 85 days using NPK 15-5-15 Peters-Water Soluble Fertilizer acquired from Scotts. Peters stock fertilizer was made using 10lbs/5 gallons and applied as a 1:100 dilution in the flood tray. Plants were harvested after 125 days and left in a 50C dryer over the course of 5 days.

II.3.2 Plasma Jet Characterization

In this experiment, a Dielectric Barrier Discharge Jet (DBDJ) is utilized to provide non-thermal atmospheric pressure plasma at high amplitude and short durations to create reversible pore opening in plant tissues for the insertion of FCs. The low power used prevents damage to the plant tissue. Grade 6 ultra-high purity helium was used as the carrier gas for this experiment. Helium gas was passed through an alumina tube at 10.6 standard liters per minute (SLPM). Alumina has high hardiness and a dielectric constant of 9.8, making it the optimal material for the DBDJ system. A BERGOZ current transformer was used to measure current, and a NorthStar voltage probe was utilized. Power for these experiments was set at 20kHz with a 5% duty cycle. Characterization of the system power shows an average power of ~0.72 W, average power at burst ~7.1W, and max instantaneous power at ~69W. The voltage was 7-8kV, and pulse power was at 14-16kV/cm. No streamers or filamentary discharge was allowed during transformation trials; if streamers were apparent, the experiment was stopped, and the gas flow rate was readjusted.

II.3.2 Genetic Material

Previously assembled 17kb eYFP plasmid containing a 720bp eYFP gene insert was obtained from the Texas A&M Institute for Plant Genomics and Biotechnology. [Appendix A] Colonies grown on selection plates were picked and cultured overnight in LB broth. Plasmids were isolated to manufacturer's instructions using Qiagen QIAprep Spin Miniprep Kit. The plasmid was eluted using the Elution Buffer supplied. Plasmid concentrations were measured using a spectrophotometer nanodrop from Thermo Scientific. All samples with >400ng/ul were used.

II.3.3 Mature Embryo Excision and Osmotic Solution Viability

Nipponbare seeds were dehusked using a hand-held rice husker, then soaked in double-distilled water (ddH_2O) at 29C over the course of two days. In between each day, the seeds were washed in ddH_2O and re-submerged in ddH_2O. On the third day, germinated seeds were collected and excised. Excisions were done using a scalpel under a LEICA EZ4 dissecting microscope. Care was taken to only cut along the scutellum, which separates the embryo from the starchy endosperm. Excised embryos were placed into Petri dishes containing either ddH_2O or 2.5M D-Mannitol acquired from Sigma-Aldrich and soaked for 2hrs. Mature embryos were then plated on 0.004% agar gel Petri dishes and stored in a 28C growth chamber. The growth chamber was set to 12hrs day and 12hrs night. Microscope images were taken using Olympus SZX10 stereoscope at 1.25X magnification and 50ms exposure over a period of 3 days.

II.3.4 Mature Embryo Transformation

Excised embryos were placed into Petri dishes containing either ddH_2O or 2.5M D-Mannitol acquired from Sigma-Aldrich and soaked for 2hrs. Mature embryos were then plated on 0.004% agar gel Petri dishes with excision side exposed. Plates were placed on the platform of the AZ1 DBDJ system. The height of the platform was adjusted so that the excised embryo was 5mm away from the jet nozzle. Embryos were exposed to plasma array for 30s at 20% power, immediately after which 5ul of plasmid was applied topically. Microscope images were taken using GFP filter on Olympus SZX10 stereoscope at 4X magnification and 50ms exposure.

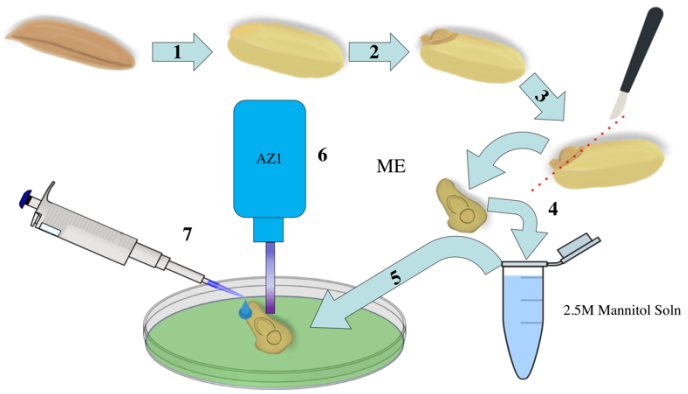

Figure 1. Work flow of electroporation-like plasma jet transformation on mature embryos. 1. Dehusk rice seed. 2. Imbibe with sterile water overnight at 29C. 3. Excise mature embryo (ME). 4. Incubate in 2.5M Mannitol soln. for 2-3 hrs at 29C. 5. Plate ME on 0.004% Agar gel. 6. Expose ME to DBDJ for 30s at 20% power (1.1601 J/s). 7. Immediately apply >400ng/µl (EB buffer) eYFP plasmid.

II.4 Results

III.4.1 Mature Embryo Excision Viability Results

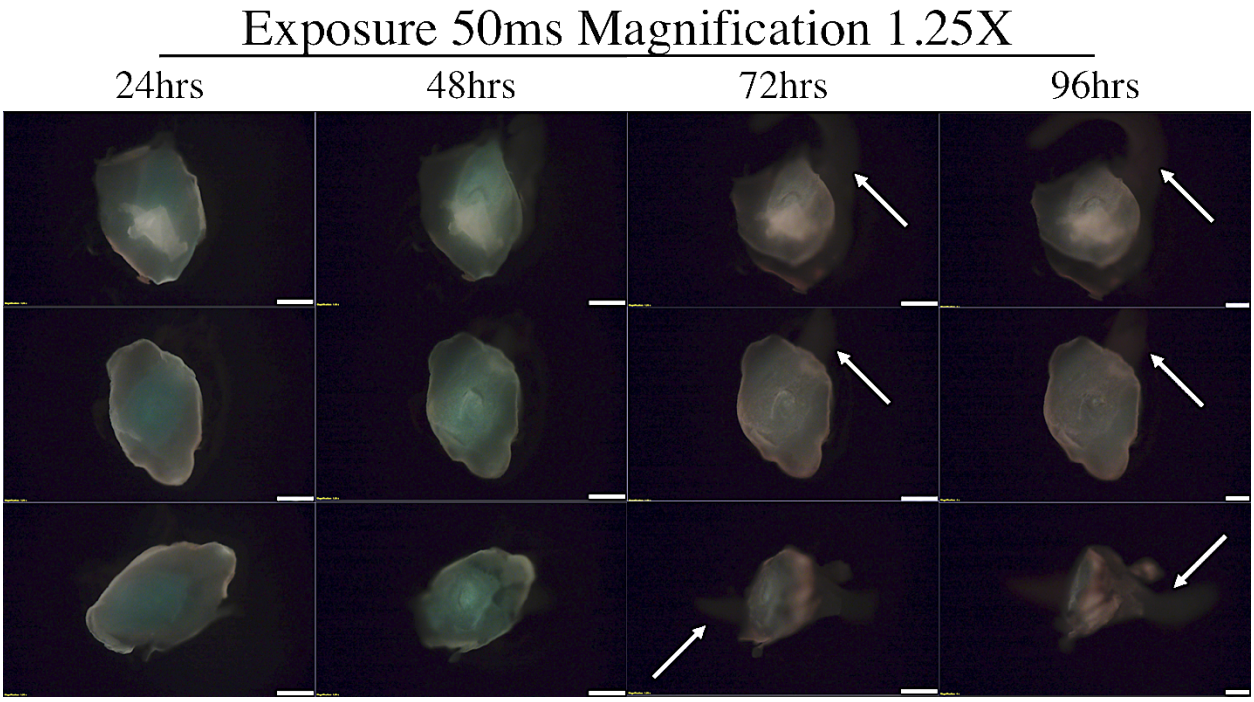

Figure 2. Here we see germinating mature embryos, white arrows indicate root emergence. The brightness of this figure was increased by 50% in order to visualize root growth.

III.4.2 Mature Embryo Osmotic Solution Viability Results

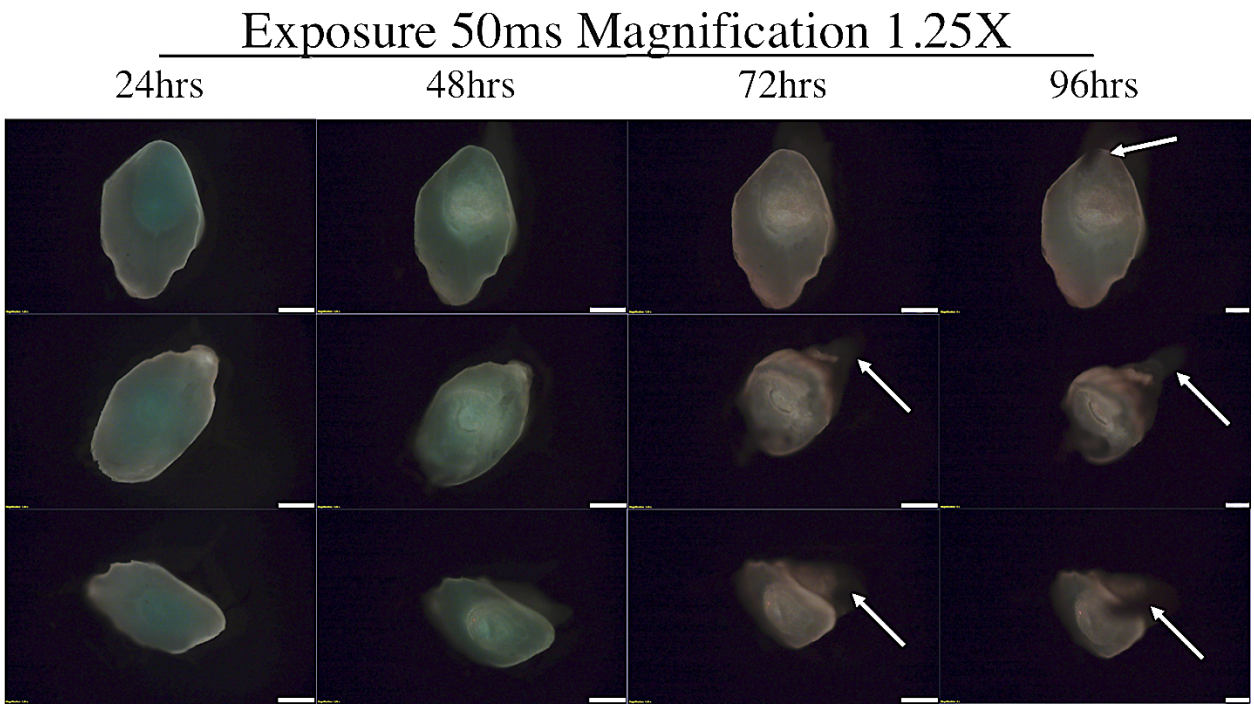

Figure 3. Here we see germinating mature embryo that has been treated with osmotic solution. The brightness of this figure was increased by 50% in order to visualize root growth.

1I1.4.3 Mature Embryo Transformation Results

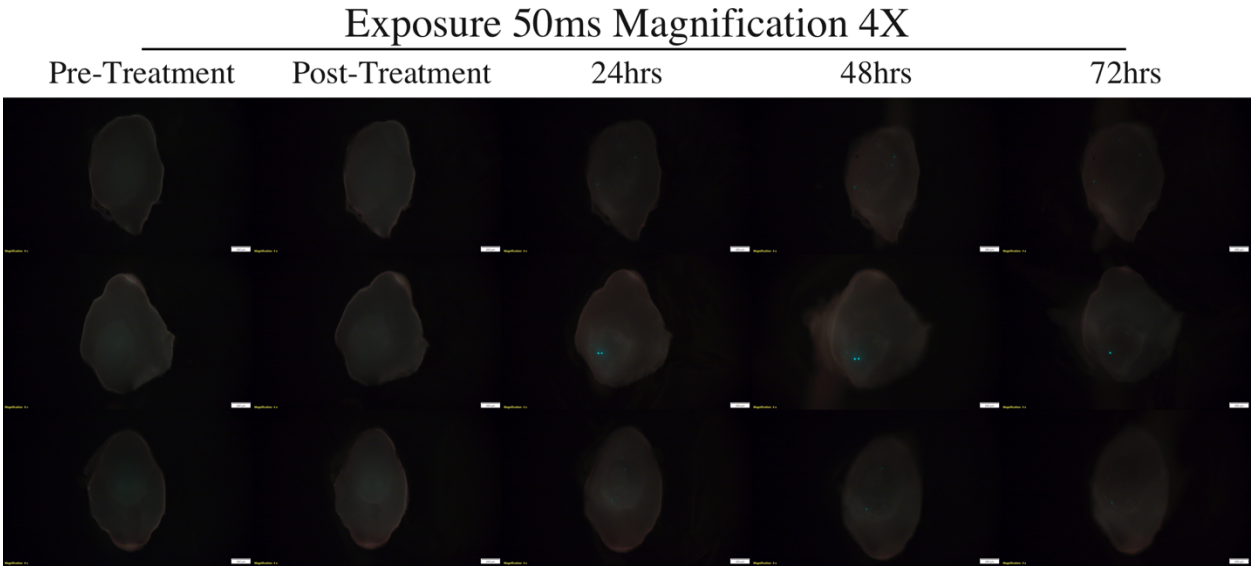

Figure 4. Here we see mature embryos expressing eYFP in clusters around the SAM. It should be noted that out of 20 samples, the fluorescent dot phenotype was observed in 10 samples.

II.5 Discussion

Mature embryos do not lose viability after excision or soaking in an osmotic medium. This can be seen by germinating root apical meristems (RAM). These findings indicate that the preparation of mature embryos for DBDJ transformation will not have adverse effects on the subsequent viability of the seedlings. It should be noted that senescence of seedlings could be seen after 7 days; this is most likely due to lack of nutrients in the pure 0.004% agar gel, therein replacing the media with an MS media supplemented with PPM (plant tissue culture preservation media) should work to grow full seedlings. A 0.004% gel was chosen initially to avoid complications associated with bacterial or fungal growth on nutrient media, as imaging would require exposing the embryos to non-sterile air over the span of three days; also we only wanted to check germination potential.

It is possible to insert FC into plant meristematic tissue using DBDJ. The resulting dot phenotype validates the potential of the technology. eYFP appears in small dot-like clusters around or inside of the meristematic tissue near the SAM, raising questions as to whether resulting outgrowth would possess the same phenotype if a constitutively active GFP plasmid was used. The dot-like phenotype was unexpected as it provokes the idea that the electric field caused by DBDJ may be imbalanced, electroporating the tissue unevenly. Other potential causes could be inaccurate excisions of the mature embryos, wherein certain portions of the scutellum remained and disproportionately absorbed FCs and expressed eYFP. Upon further investigation, experiments involving electroporation of mature embryos found that electroporation was tissue-specific, in that scutellum cells were found to be much more susceptible to gene transfer than other cell types of the embryo (Klöti et al., 1993). The two main roles of the scutellum include the production of gibberellins, which initiate the production of enzymes responsible for

breaking down starch and the transfer of sugars and amino acids into the embryo. Recent studies have found that after germination, the cells within the scutellum go through programmed cell death (PCD) (Domínguez et al., 2012). While dots found directly on the meristematic tissues in clean-cut embryos still lend to the potential success of a whole seed transformation, dots found on the peripheral may not provide the same confidence. Transformed cells found on the periphery of the meristematic tissues may not be incorporated into mature plants as the scutellum undergoes PCD.

CHAPTER III

DETERMINING WHOLE SEED VIABILITY AND TRANSFORMATION POTENTIAL OF WHOLE SEEDS

III.1 Synopsis

In order to appropriately utilize this technology for plant transformation, it must not only be quick but easy and efficient. Following the results from experiments involving excised embryos, we aim to test DBDJ on imbibed whole seeds. Unlike excised embryos, whole seeds have a bran and aleurone layer over the seed; therein, there may be some barriers to the infiltration of the plasmid. To industrialize this process, these barriers must be overcome. Here we target meristematic tissues associated with germinating meristematic tissue, either the RAM or SAM. As these tissues already have a designated cell fate, we are testing the potential for these different tissue types to be transformed using DBDJ. Positive transformations found in these tissues may suggest the potential for *in vivo* transformation of rice seeds. This may also give insight into future experiments involving long term genetic modifications from seed to mature plant.

III.2 Introduction

Plant molecular biology is a rapidly growing field and has many applications in both agriculture and pharmaceuticals. In agriculture, genetic engineering has led to crops capable of resisting herbicides, pathogens, abiotic stresses and insects (Demirer et al., 2017). Probably most notably was the development of crops capable of producing the same toxins as the bacterium *Bacillus thuringiensis*; these crops have since been called BT crops, such as cotton and corn. This engineered toxicity allows plants to be able to resist insect predators, reducing the use of pesticides. Plants can also be modified to increase their nutrient profile and density; these crops

are often called biofortified. One such example is the development of the biofortified rice, Golden Rice, rich in vitamin A; it is a transgenic crop combining genes from both daffodil and the bacterium *Erwinia uredovoia* (Hefferon, 2015). Golden Rice is proposed as a solution to the underlying nutritional deficiencies associated with night blindness, which affects 5.2 million preschool-age children and 9.8 million pregnant women (World Health Organization, 2009). Plant genetic engineering has become a crucial part of the pharmaceutical industry as plants can be used as bioreactors for the production of mammalian pharmaceuticals. Some benefits include that plant cells have no cross-species transmission of mammalian viral pathogens and vice versa (European Medicines Agency, 2008). An early indication that mammalian proteins could be produced in plants was the successful expression of a functional human antibody in tobacco (Hiatt et al., 1989). Hepatitis B virus-like particles (VLPs) have also been isolated from transgenic plants (Mason et al., 1992). From agricultural purposes to pharmaceutical plant genetic engineering has led to many innovations that we now come to rely on. Each of the above transformants must rely on some sort of genetic delivery system.

As protocols for crop genome editing continue to improve, so too must their delivery methods. The field of crop genome editing is rapidly growing to include new delivery systems, including Carbon Nano-Tubes (CNTs) and biolistic particle delivery. Each of which is being tested both *in vitro* and *in vivo*. Here we would like to propose DBDJ as another option, with the benefit of being easy to set up, involving minimal chemical preparation, potentially cost-effective and non-damaging. DBDJ allows for the infiltration of plasmids past the cell wall and plasma membrane to integrate into the plant genome while also maintaining plant viability.

III.3 Materials and Methods

III.3.1 Plant Materials

The rice variety used in this section is the *japonica* rice cv Nippobare. Nipponbare seeds were acquired from the GRIN U.S. National Plant Germplasm System. Seeds were dehusked using a hand-held rice husker, then soaked in double-distilled water (ddH_2O) at room temperature over the course of two days. In between each day, the seeds were washed in ddH_2O and re-submerged in ddH_2O. On the third day, germinated seeds were transferred to 5" pots containing a 1:1 mix of Turface MVP soil conditioning clay and SunGro Professional Mix soil, respectively. Pots were placed on flood trays, and water levels were kept consistent throughout the growing season. Plants were fertilized after 85 days using NPK 15-5-15 Peters-Water Soluble Fertilizer acquired from Scotts. Peters stock fertilizer was made using 10lbs/5 gallons and applied as a 1:100 dilution in the flood tray. Plants were harvested after 125 days and left in a 50C dryer over the course of 5 days.

III.3.2 Genetic Materials

Four plasmids containing constitutive promoters driving Green Fluorescence Protein (GFP) were constructed using golden gate cloning. Protocol from Čermák, Tomáš, et al. 2017 was used to prepare plasmids.

Glycerol stocks of the following plasmids were streaked on antibiotic selection media, and 2 colonies from each were isolated, cultured in LB at 37C overnight, and purified using the Qiagen QIAprep Spin Miniprep Kit. Module A plasmids (pMOD_A) were restriction enzyme digested using *Asc*1 and *Sbf*1 from NEB. Restriction digestion reaction solutions were run on a 5% agarose gel, and the appropriate fragment was isolated using QIAquick Gel Extraction Kit. Inserts and backbone were combined with T4 DNA Ligase Buffer (10X), nuclease-free water,

and T4 DNA Ligase according to Ligation Protocol with T4 DNA Ligase (M0202) from NEB website. The reaction was gently mixed by pipetting. The reaction was then incubated at 16C overnight in a PCR machine. The reaction was then heat-inactivated at 65C for 10min, then chilled on ice, and 5ul of the reaction was added into 50ul of thawed DH5a competent cells from Thermo Fischer Scientific. The mixture was placed on ice for 30min, then heat shocked at exactly 42C for 30s. The mixture was placed back on ice for 5min. 950ul of room temperature SOC was added to the mixture, then placed on a shaker at 250rpm at 37C for an hour. The mixture was plated on an Ampicillin selection plate warmed to 37C and kept in an incubator overnight at 37C. The resulting colonies were picked and cultured overnight in LB at 37C, then purified using the Qiagen QIAprep Spin Miniprep Kit. Golden Gate assembly reaction was set up according to Čermák, Tomáš, et al. 2017 up to step 5c. [Appendix A] Resulting colonies were picked and cultured overnight in LB at 37C, then purified using the Qiagen QIAprep Spin Miniprep Kit, making sure to leave some culture behind. Plasmid DNA was then verified using primers specific to each backbone NT-067and NT-065 for non-binary, NT-030, and NT-065 for binary. PCR reaction was run on a 5% gel, and correct transformants were plated on antibiotic selection plates overnight. Plates were then stored in 4C.

III.3.3 Whole Seed Osmotic Solution Viability

Nipponbare seeds were dehusked using a hand-held rice husker, then soaked in double-distilled water (ddH_2O) at 29C over the course of two days. In between each day the seeds were washed in ddH_2O and re-submerged in ddH_2O. On the third day, germinated seeds were collected. Germinated seeds were placed into Petri dishes containing either ddH_2O or 2.5M D-Mannitol acquired from Sigma-Aldrich and soaked for 2hrs. Seedlings were then plated on 0.004% agar gel Petri dishes and stored in a 28C growth chamber. The growth chamber was set

to 12hrs day and 12hrs night. Microscope images were taken using Olympus SZX10 stereoscope at 3.2X magnification and 32.4ms exposure over a period of 3 days.

III.3.4 Whole Seed Transformation

Germinated seeds were placed into Petri dishes containing either ddH_2O or 2.5M D-Mannitol acquired from Sigma-Aldrich and soaked for 2hrs. Seedlings were then plated on 0.004% agar gel Petri dishes. Microscope images were taken using the Olympus SZX10 stereoscope at 2.5X magnification and 50ms exposure before plasma transformation. Plates were then placed on the platform of the AZ1 DBDJ system. The height of the platform was adjusted so that the embryo of the germinating seedling was 5mm away from the jet nozzle. Seeds were exposed to plasma array for 30s at 20% power, immediately after witch 5ul of plasmid was applied topically to the embryo. Microscope images were taken again using the same parameters, and seeds were stored in a 28C growth chamber overnight. The growth chamber was set to 12hrs day and 12hrs night. Images were taken every 24hrs after the initial DBDJ trial over a period of 3 days. Microscope images were taken using GFP filter on Olympus SZX10 stereoscope and EchoRevolve Confocal Microscope.

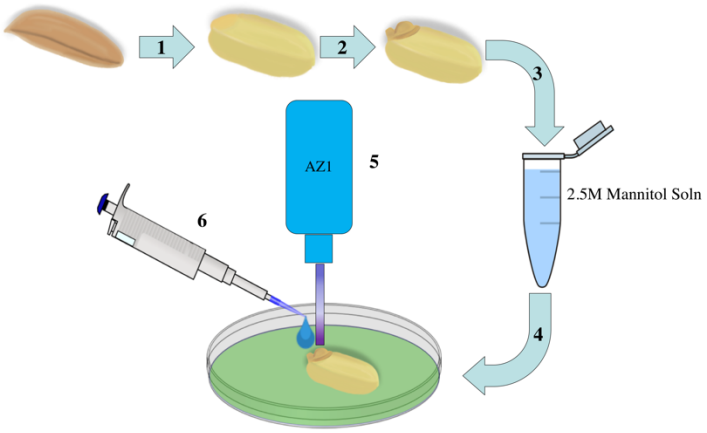

Figure 5. Work flow of electroporation-like plasma jet transformation on whole seeds. (1) Dehusk rice seed. (2) Imbibe with sterile water over 48 hrs at 29C. (3) Incubate in 2.5M Mannitol soln. (or H2O) for 2-3 hrs at 29C. (4) Plate seeds on 0.004% Agar gel. (5) Expose ME to DBDJ for 30s at 20% power (1.1601 J/s). (6) Immediately apply >400ng/μl (EB buffer or H2O) plasmid.

III.3.5 Whole Seed Transformation Image Analysis Validation

Initial validation of 11 suspected transformants across all plasmids was done by image analysis using ImageJ. TIFF images were taken on the Olympus stereoscope over three days (pre-treatment, post-treatment, 24hrs, 48hrs) were opened in the ImageJ platform, and ROIs were selected to match the germinating embryo. ROI parameters for area integrated density and mean grey value was measured for each seed ROI, as well as three points included from the black background. [Fig.7] Measurements were also taken for six control images that received no plasmid. Corrected Total Cell Fluorescence (CTCF) and Corrected Total Cell Autofluorescence (CTCAF) for experimental samples and controls were calculated using the formula below:

$$CTCF\ \&\ CTCAF = Integrated\ Density - (Area\ of\ selected\ cells \times Mean\ of\ background\ readings)$$

Once all points were acquired, t-tests assuming unequal variance were performed comparing data sets from CTCF and CTCAF, for each time period with a significance level of 0.05. It should be noted that using a P(T<=t) two-tail p-value, there was no statistical significance

between pre and post-treatment trials between the two groups. There was a statistical significance between both 24hrs and 48hrs, with p-values of 0.0113 and 0.0006, respectively. Data sets and calculations can be found in Appendix B.

Following statistical analysis Box and Whisker plots were created to visualize the difference between controls and experimental samples.

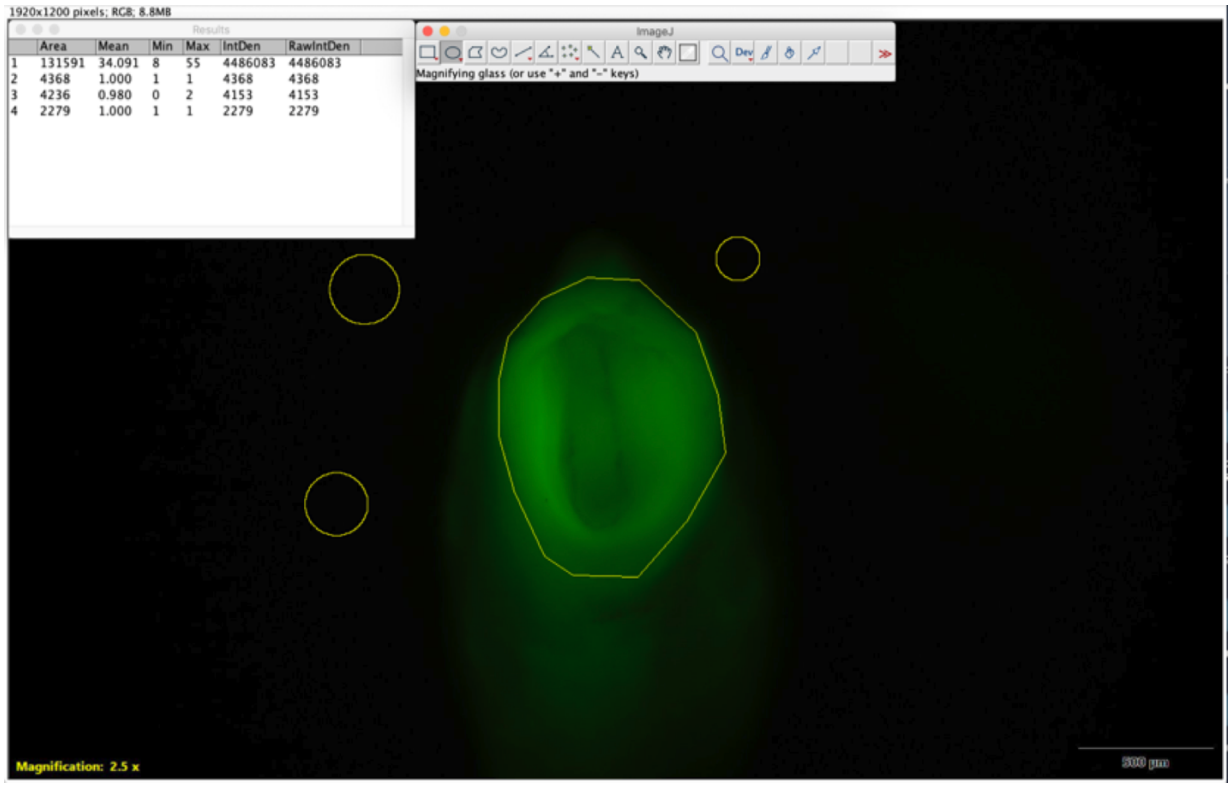

Figure 6. Selection of ROIs for ImageJ analysis of transformed whole seeds.

III.4 Results

III.4.1 Whole Seed Osmotic Solution Viability Results

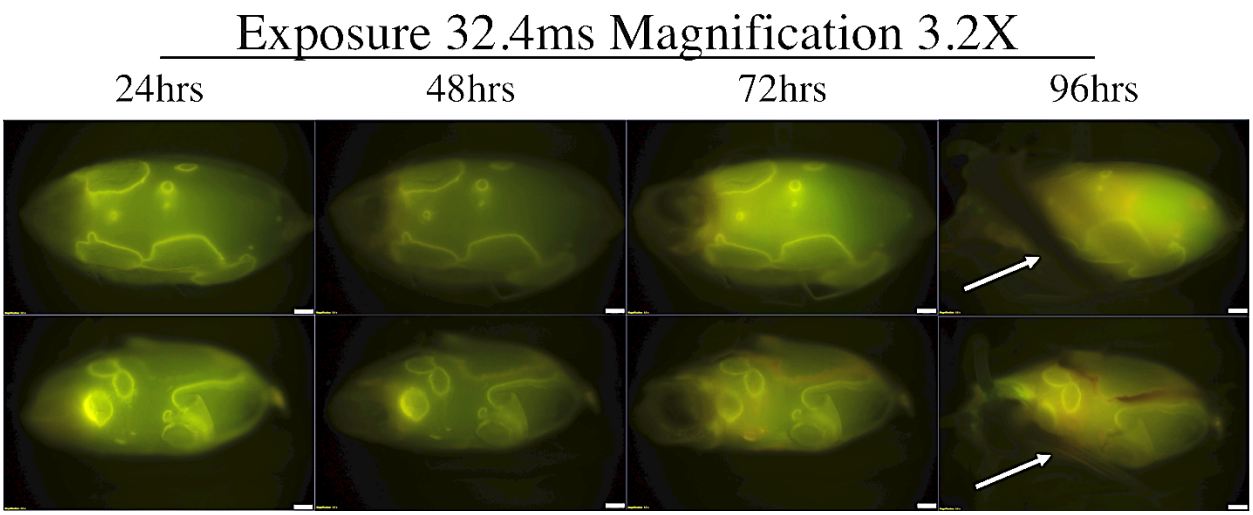

Figure 7. Here we see seeds continue to germinate after being soaked in 2.5M OM solution for 2hrs. The brightness of this figure was increased by 50% in order to visualize root growth.

III.4.2 Whole Seed Transformation Results

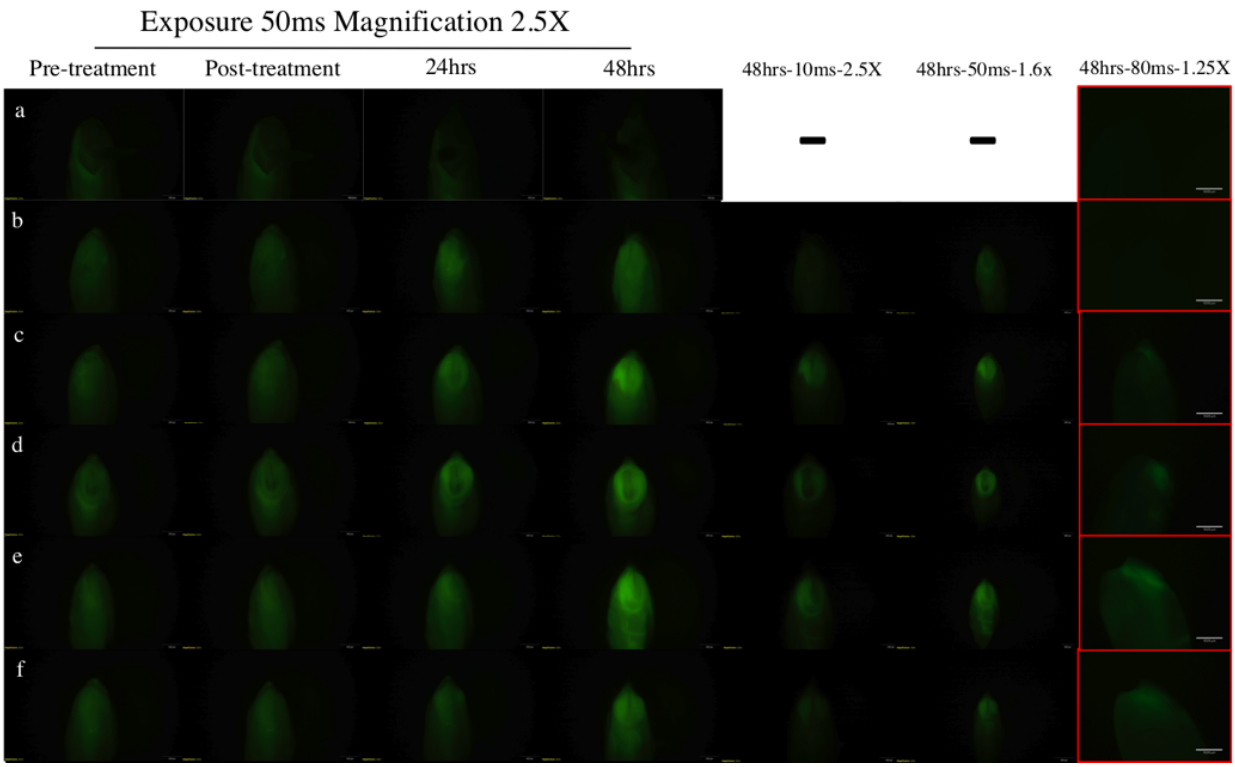

Figure 8. Whole seeds (japonica variety Nipponbare) expressing GFP. (a) WT seed imaged before group treatment at 50ms exposure and 2.5X magnification (50ms:2.5X), imaged after group treatment (without treatment), imaged at 24hrs and 48hrs. (b) WT seed imaged before group treatment at 50ms:2.5X, imaged after group treatment (without treatment), imaged at 24hrs and 48hrs. (c) Seed treated with DBDJ and 35S:GFP binary plasmid in EB buffer, imaged before group treatment at 50ms:2.5X, imaged after treatment, imaged at 24hrs and 48hrs. At 48hrs additionally imaged at 10ms:2.5X, 50ms:1.6X, 80ms:1.25X. (d) Seed treated with DBDJ and CmYLCV:GFP binary plasmid in EB, imaged before group treatment at 50ms:2.5X, imaged after treatment, imaged at 24hrs and 48hrs. At 48hrs additionally imaged at 10ms:2.5X, 50ms:1.6X, 80ms:1.25X. (e) Seed treated with DBDJ and CmYLCV:GFP binary plasmid in H_2O, imaged before group treatment at 50ms:2.5X, imaged after treatment, imaged at 24hrs and 48hrs. At 48hrs additionally imaged at 10ms:2.5X, 50ms:1.6X, 80ms:1.25X. (f) Seed treated with DBDJ and CmYLCV:GFP non-binary plasmid in EB, imaged before group treatment at 50ms:2.5X, imaged after treatment, imaged at 24hrs and 48hrs. At 48hrs additionally imaged at 10ms:2.5X, 50ms:1.6X, 80ms:1.25X. It should be noted that out of 51 samples across all promoter variations, only 11 successful transformations were noted.

III.4.3 Whole Seed Transformation Image Analysis Validation Results

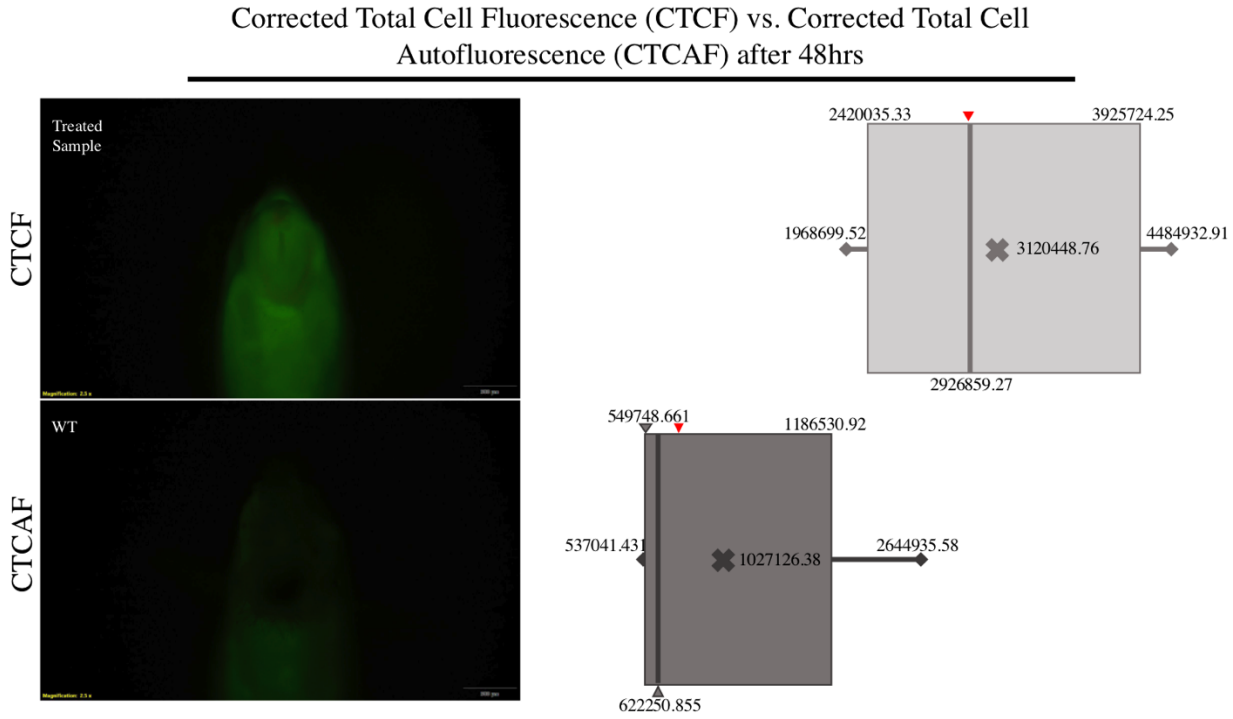

Figure 9. Image analysis results for whole seed transformation after 48hrs. Here we see a comparison of CTCF (above) and CTCAF (below). Red arrows indicate sample location on box and whisker plot. It should be noted that the max value for CTCAF sample was found in B of the previous figure.

III.5 Discussion

Whole seeds do not lose viability after soaking in the osmotic medium. This can be seen by germinating roots and shoots days after DBDJ transformation. These findings indicate that the preparation of whole seeds for DBDJ transformation will not have adverse effects on the subsequent viability of the seedlings. It should be noted that the senescence of seedlings could be seen after 14 days. This is most likely due to lack of nutrients in the pure 0.004% agar gel; therein, replacing the media with an MS media supplemented with PPM (plant tissue culture preservation media) should work to grow full seedlings.

It is possible to insert FCs into seedlings using DBDJ. Here we see a very different phenotype as compared to our previous mature embryo experiment. Instead of a dot phenotype, we see more of a gaussian spread, which makes it very difficult to distinguish from autofluorescence. Yet compared to a control image that appears almost black due to low exposure, we can tell there is some GFP expression when compared to the experimental samples. Loss of contrast may be due to the presence of a bran and aleurone layer over the embryo; however, if that is the case, then the potential for seed transformation *in vivo* may be more realistic than expected. The ability for FC to infiltrate past the bran and aleurone layer due to plasma array demonstrated the penetrating power of the system.

CHAPTER IV

CONCLUSIONS AND FUTURE WORK

Here we have demonstrated that neither excised mature embryos nor whole seeds lose viability due to treatment with an osmotic solution. We have also demonstrated that meristematic cells are accepting of FC after treatment with DBDJ. It does appear that some barriers to transformation are inherent in whole seeds compared to excised mature embryos, as we see a 50% (10:20) transformation efficiency with mature embryos vs. 22% (11:51) of whole seeds. This may be due to the bran and aleurone layer covering the meristematic tissues of whole seeds. To test this, a future experiment may focus on removing those layers surgically.

Some procedural updates could include the optimization of elution buffer vs. water as the pH of the plasmid elution could play a role in transformation efficiency. Another material update could be the use of differing osmotic solutions of varying osmolarity to more efficiently plasmolyze plant cells. The size of the plasmid may also play a role as pulsated pore diameter may be a limiting factor.

As previously mentioned, it's difficult to discern whether cell permeabilization is due to electric fields or due to the production of ROS or RNS. To test this, different carrier gasses should be trialed, including both Argon and O_2. If possible, this should be done in a closed system wherein the levels of ROS production can be measured and compared to resulting transformation efficiency.

For future validation of genomic integration, DNA should be isolated from transformed seeds and subsequent PCR with primers meant to amplify GFP DNA sequences. To validate expression RT-PCR can be done rather expeditiously without the need for the purchase of antibodies. Another form of validation should include the disruption of the Phytoene Desaturase

gene, which is a common knockout used to validate genetic delivery systems. The resulting phenotype should be albinism. Ultimately CRISP/Cas should be tested using this system to gain the full benefits of the technology, with the potential to make permanent genetic modifications *in vivo*.

There is not enough data to make statistical comparisons between the transformation efficiency of biolistics and DBDJ. Larger trial numbers should be used in the future in order to mitigate loss due to poor germination. Once fully optimized in monocots DBDJ should be tested on developed dicots such as cotton at their nodes to see if the resulting outgrowth will contain the desired modification.

In conclusion, the Dielectric Barrier Discharge Jet system could become a powerful tool in crop genome editing as it has the potential to be tissue non-specific and without the need for tissue culture, similar to biolistic methods. Moreover, it could be an inexpensive and easy way to perform genetic engineering.

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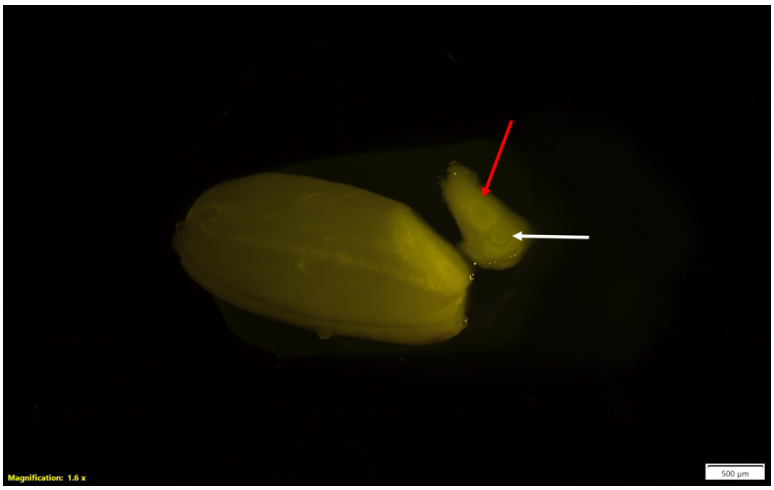
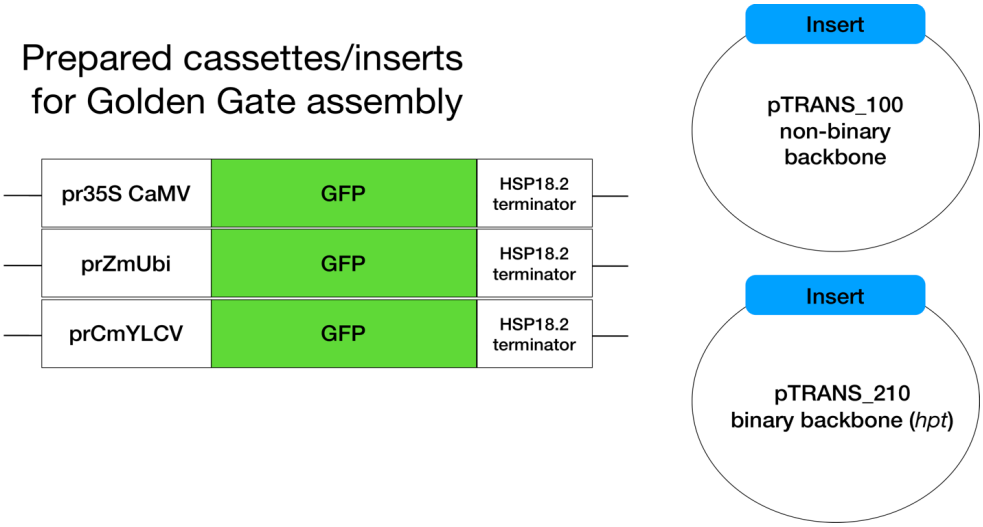
APPENDIX A

SUPPLEMENTAL FIGURES



Supplementary Figure 1. Stereoscope image of an imbibed seed with excised mature embryo. Red arrow indicates the root apical meristem (RAM), white arrow indicates the shoot apical meristem (SAM).

Supplementary Figure 2. eYFP vector used for transformation of mature embryos.

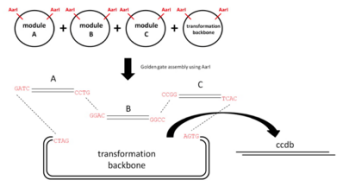


Supplementary Figure 3. Prepared cassettes for Golden Gate assembly

Plasmid preparation of constitutive promoters driving the Green Fluorescence Protein (GFP) gene using Golden Gate Assembly.

Reference: Čermák, T., Curtin, S. J., Gil-Humanes, J., Čegan, R., Kono, T. J. Y., Konečná, E., et al. (2017). A Multipurpose Toolkit to Enable Advanced Genome Engineering in Plants. Plant Cell 29, 1196–1217. doi:10.1105/tpc.16.00922.

Figure. Summary of the strategy to be followed.



Procedure

1. Streak glycerol stocks of the following plasmids and elute plasmid DNA from **2 colonies per plasmid**.

Plasmid ID	Plasmid Name	Antibiotic	Purpose
CGEL-004	pTRANS_100	Spectinomycin	Backbone, non-binary
CGEL-005	pTRANS_210	Kanamycin	Backbone, binary
CGEL-007	pMOD_A0101	Ampicillin	Promoter CaMV 35S
CGEL-008	pMOD_A1110	Ampicillin	Promoter ZmUbi1
CGEL-011	pMOD_A3003	Ampicillin	Promoter CmYLCV
CGEL-012	pMOD_B0000	Ampicillin	Empty module B
CGEL-020	pMOD_C0000	Ampicillin	Empty module C

2. Digest the following plasmids the *Asc*I and *Sbf*I enzymes and elute the appropriate fragment.

Plasmid ID	Plasmid Name	Size of expected fragment to be excised	Notes
CGEL-007	pMOD_A0101	854 bp	Insert
CGEL-008	pMOD_A1110	1,998 bp	Insert
CGEL-011	pMOD_A3003	3,008 bp	Backbone

3. Overnight ligation of the inserts to the backbone plasmid (see Step 2), using T4 Ligase[1].

4. Transformation of the new ligated products to DH5a competent cells (*E. coli*) and elute the plasmid DNA.

5. Golden Gate Assembly
   a. Set up a Golden Gate reaction:
      a. 75 ng transformation backbone (CGEL-004 or CGEL-005)
      b. 150 ng module A plasmid (CGEL-011, [CGEL-011 + Insert (CGEL-007)], [CGEL-011 + Insert (CGEL-008)])
      c. 150 ng module B plasmid (CGEL-012)
      d. 150 ng module C plasmid (CGEL-020)
      e. 0.4 µl AarI oligonucleotide (comes with the AarI enzyme)
      f. 0.5 µl AarI
      g. 1 µl T4 DNA ligase
      h. 2 µl 10x T4 DNA ligase buffer
      i. H2O up to 20 µl
   b. Place the reactions in the PCR machine and run cycle: 10x (37˚C/5min + 16˚C/10min) + 37°C/15min + 80°C/5min + 4°C hold.
   c. Transform 5 µl of the Golden Gate reaction into E. coli (DH5α cells) and plate on LB + 50mg/L of appropriate antibiotic - kanamycin for T-DNA vectors or spectinomycin for non-T-DNA vectors.
   d. Colony PCR (http://cshprotocols.cshlp.org/content/2006/1/pdb.prot4141) of 10 colonies.
      a. For cassettes in pTRANS_100 backbone, use the NT-065 & NT-067 set of primers (expected amplicon size: **340 bp**).
      b. For cassettes in pTRANS_210 backbone, use the NT-030 & NT-065 set of primers (expected amplicon size: **250 bp**).
   e. Isolate the plasmid DNA for one correct clone. The Golden Gate junctions can be sequenced, although the fidelity of cloning is usually high. Alternatively, the structure of the final plasmid can be verified by restriction digest analysis.

[1] Use the protocol for the T4 Ligase that we have in the lab.

Supplementary Figure 4. Golden Gate assembly protocol.

CTAB:

2% CTAB
1.42 M NaCl
20 mM EDTA
100 mM Tris pH 8
*Autoclave

(From "Rapid generation of a transgene-free powdery mildew resistant tomato by genome deletion." Nekrasov et al, 2017)

Supplementary Figure 5. Small scale CTAB DNA extraction recipe

APPENDIX B

IMAGE ANALYSIS TABLES AND CALCULATION

	SAMPLE ID	PRE TREATM	POST TREATI	24HRS	48HRS
Corrected Total Cell Fluorescence (CTCF)	35SB-OM-EB	1499059	1445485	2282842	3602345.13
	35SB-W-W-1	920573	1028827	1150915.55	1968699.52
	CmYLCVB-OM	2160320.69	2357385.08	3347125.97	4474870
	CmYLCVB-OM	1582329	1775752	2243396	4484932.91
	CmYLCVB-W-	1298176.81	1293892	1576468.49	2926859.27
	CmYLCVB-W-	1420747.16	1426883.86	1889261	2420035.33
	CmYLCVNB-C	1947076	1716357	1587732.58	2613061.88
	CmYLCVNB-V	1270676	1353157	1422130.1	1992294.46
	CmYLCVNB-V	1299539	1432930	2524778.73	3925724.25
	ZmUb1-W-EI	1662243.77	1653806	1194358.29	2456644
	ZmUb1-W-W	1959869	2009765	2940618.77	3459469.6
Corrected Total Cell Autofluorescence (CTCAF)	C-OM-1	954833.117	961542.64	475768.53	551249.377
	C-OM-2	1130796.54	1354564.02	601974.849	537041.431
	C-OM-3	1221440.73	1165470	483604.933	549748.661
	C-W-1	1584592	1410463	425654.775	693252.333
	C-W-2	1563778.26	1436974	447576.729	1186530.92
	C-W-3	1551612.12	1747008	2419614.58	2644935.58

Pre-Treatment: CTCF vs CTCAF

t-Test: Two-Sample Assuming Unequal Variances

	Variable 1	*Variable 2*
Mean	1547328.13	1334508.79
Variance	1.3314E+11	7.2135E+10
Observations	11	6
Hypothesizec	0	
df	13	
t Stat	1.37015439	
P(T<=t) one-t	0.09691862	
t Critical one	1.7709334	
P(T<=t) two-	0.19383724	
t Critical two	2.16036866	

24hrs: CTCF vs CTCAF

t-Test: Two-Sample Assuming Unequal Variances

	Variable 1	*Variable 2*
Mean	2014511.59	809032.399
Variance	5.1774E+11	6.2629E+11
Observations	11	6
Hypothesizec	0	
df	10	
t Stat	3.09761353	
P(T<=t) one-t	0.00564824	
t Critical one	1.81246112	
P(T<=t) two-	0.01129649	
t Critical two	2.22813885	

Post-Treatment: CTCF vs CTCAF

t-Test: Two-Sample Assuming Unequal Variances

	Variable 1	*Variable 2*
Mean	1590385.45	1346003.61
Variance	1.3505E+11	7.0742E+10
Observations	11	6
Hypothesizec	0	
df	14	
t Stat	1.57526742	
P(T<=t) one-t	0.06875823	
t Critical one	1.76131014	
P(T<=t) two-	0.13751646	
t Critical two	2.14478669	

48hrs: CTCF vs CTCAF

t-Test: Two-Sample Assuming Unequal Variances

	Variable 1	*Variable 2*
Mean	3120448.76	1027126.38
Variance	8.5173E+11	6.8974E+11
Observations	11	6
Hypothesizec	0	
df	11	
t Stat	4.77252199	
P(T<=t) one-t	0.00028916	
t Critical one	1.79588482	
P(T<=t) two-	0.00057833	
t Critical two	2.20098516	