5-2023

The role of FTO, a human RNA demethylase in perennial grass development and abiotic stress responses

Andrew Fiorentino

Qian Hu

Xiaotong Chen

Zhaohui Chen

Honors Thesis in Biochemistry

The role of FTO, a human RNA demethylase in perennial grass development and abiotic stress responses

Andrew Fiorentino, Qian Hu, Xiaotong Chen, Zhaohui Chen, Dr. Hong Luo

Abstract

The integration of the human *fat mass and obesity associated (FTO*) gene into turfgrass is a novel approach at improving cell proliferation and abiotic stress resistance. The FTO protein is an RNA demethylase responsible for epigenetic regulation of the genome. In related rice, the gene is associated with increased crop yield, tiller number, and aerial biomass. It is proposed to work via demethylation of repeat RNA associated with chromatin remodeling, causing widespread transcriptional activation. In this study, the feasibility of using FTO for plant trait modification in perennial grasses is being investigated. Potentially transformed embryogenic calli of creeping bentgrass with *FTO* gene have been developed and are awaiting regeneration into plants, which can be assessed for *FTO* gene expression as well as abiotic stress resistance. Responsible application of transgenic FTO turfgrass is also being explored via a novel sterility mechanism that involves knockout of the gene responsible for flowering.

Introduction

Turf grass is an important crop across a wide variety of disciplines from infrastructure to agriculture. It is abundant in landscaping and environments like golf courses, which require extensive resources to maintain. On farms the crop can be used as food for animals or even buffer zones preventing toxic fertilizer runoff. Grass is an economically important crop, so ways to improve its viability are paramount. Genetic modifications using traditional breeding and biotechnology have been successful in trait modification for enhanced stress tolerance and plant proliferation of the species. However, epigenetics may offer novel avenues for plant improvement. Epigenetics differs from conventional modification of gene expression in that they do not directly change the DNA sequences located in the nucleus of cells. Instead, it targets proteins, DNA, and RNA to modify chromatin and RNA structures in a reversible manner, to achieve modification of gene expression. The epitranscriptome, being the modification of RNA, is a subcategory of epigenetic modification. It is one of the most powerful avenues to manipulate plant cell differentiation and development without altering DNA sequences [1]. The most abundant mRNA modification in higher eukaryotes is methylation of adenosine, specifically N6-methyladenosine (m6A). This methylation mark is essential whereas knockout of the gene coding for methylation (METTL3) is embryonic lethal in mice [2]. In plants, specifically *Arabidopsis,* similar disruption of m6A modification caused embryonic lethality [3] [4] [5].

Interestingly, mRNA methylation occurs in functionally critical positions rather than randomly placed in the RNA genome. M^6A maps preferentially bind to the transcriptional start site, the stop codon, and the 3' UTR [6] [7]. It is a versatile modification that can be subsequently written (methyltransferase), read (RNA binding protein), and erased (demethylase). Simply,

writers add methyl groups onto RNA, readers bind to methylated sequences, and erasers remove methyl groups from RNA. Writers are shown to be essential in plant development and erasers (ALKBH9B) are shown to be involved with floral transition [8] and viral infection [9]. Readers can have a wide diversity of effects; some being mRNA fate via nucleus-to-cytoplasm export [10], RNA turnover [11], and translation [12].

These processes occur in both animals and plants; however, the functions of these writers, readers, and erasers are just recently deciphered in plants. The identity of many of these proteins in plants are relatively unclear in comparison to animals. Many of the plant enzymes relevant in methylation are animal orthologs such as mta (ortholog of human METTL3). Yet, many of these human enzymes including FTO appear to not have any plant ortholog. The human FTO gene, the fat mass- and obesity- associated protein is an eraser, demethylating protein, that as the name implies correlates with obesity and diabetes in people.

Dr. Yu et al. found that when implemented into grain and potatoes the FTO gene increased yield [13]. They found that FTO improves root growth, tiller bud formation, photosynthetic efficiency and drought tolerance. In fact, it is primarily the increase in tiller number that increases grain yield and areal biomass. Fascinatingly, the increase in agronomically beneficial traits is not only observed in monocot rice but also in eudicot potatoes. Even though there was an increase in crop biomass, nutritional content remained identical. Root length was shown to increase both in length and number of lateral roots, which may confer better resistance to drought conditions. By staining longitudinal tissue sections of rice in roots, Yu et al. were able to confirm that cell size was not responsible for the root elongation, suggesting that root growth was likely caused by cell division. Via confocal microscopy the scientists observed propidium iodide-stained root tips. It

was found that there was an increase in cell number within the same amount of space as a normal meristem zone confirming their prediction.

The mechanism of action for the FTO gene in plants is due to its demethylase activity according to Yu et al. They found a 7% reduction of m6A methylation in poly(A) RNA in both shoots and roots of rice. With substantial decreases specifically in nuclear RNA, which aligns with the finding that FTO locates to the nucleus. The FTO demethylase was shown not to alter cap and internal m6A in U1 and U2 snRNA, m1A in tRNA, and DNA m6A; which is also observed in mammals. Hypo-m6A peaks were enriched within coding sequences as well as 3' untranslated regions, and were in genes regulating cellular homeostatic process, one-carbon and small molecule metabolic process, and gene expression.

RNA polyA methylation is shown to promote degradation of RNA sequences through nuclear exosome-targeting-mediated nuclear degradation. So decreasing methylation with a demethylase like FTO increases the lifetime for these transcripts. Subsequently, FTO induced poly(A) RNA demethylation was primarily present in two different types of RNAs particularly mRNA (responsible for protein synthesis) and repeat RNA (affect chromatin remodeling and gene transcription). Yu et al. found that it was the upstream effect of repeat RNA that caused widespread chromatin openness and increased mRNA expression. Significant overlap between the demethylated repeat RNA and the specific upregulated mRNA along with various experiments validated their conclusion.

Hypothesis

The Luo lab is interested in understanding how epitranscriptomic RNA methylation impacts plant development and stress responses, especially in perennial grasses, and exploring the

potential of epitranscriptomic RNA modification as new molecular tools for plant trait modification. We hypothesize that the human demethylase gene *FTO* would also function in perennial grass species, creeping bentgras, positively impacting plant development and plant responses to various abiotic stresses. The Luo lab is also concerned with the growing problem of transgene escape and has been exploring novel molecular strategy for gene containment using controlled sterility in combination with other beneficial genes. We hypothesize that *FTO* gene would an ideal candidate serving this purpose in developing environmentally safe transgenic products with enhanced agronomic traits.

Results

Chimeric gene construction

To generate transgenic creeping bentgrass plants overexpressing *FTO* gene, a binary vector pHL1067 was constructed. The *FTO* gene (GenBank accession no. NP _001073902.1) was chemically synthesized (ThermoFisher, USA) with two added restriction enzyme cutting sites, XbaI and SalI in the 5' end and 3' end, respectively. The synthesized gene was released from the cloning vector by XbaI and SalI digestions, gel purified and ligated to the corresponding sites of a binary vector pHL236 to replace a miRNA 169g gene, resulting in pHL1067 (Figure 1). The insertion of the *FTO* gene in the pHL1067 was confirmed by DNA digestions of the extracted plasmid DNA (Figure 2). Specifically, the pHL1067 plasmid DNA was digested with restriction enzymes SalI and XbaI, which are the restriction sites flanking the *FTO* gene in the plasmid. The digested DNA was run on an 1% agarose gel. The two expected DNA bands, the 1.5 kb *FTO* gene and the 10 kb DNA for the remining part of the vector were revealed (Lane 1, Figure 2). Lane 2 is a 1kb ladder. And lane 3 is the SalI and XbaI digested DNA of the plasmid pHL236, which is the

same plasmid as pHL1067 with miR169g gene in place of the *FTO* gene. The pHL1067 plasmid was then transformed into Agrobacterium strain pEHA105 for use in creeping bentgrass transformation.

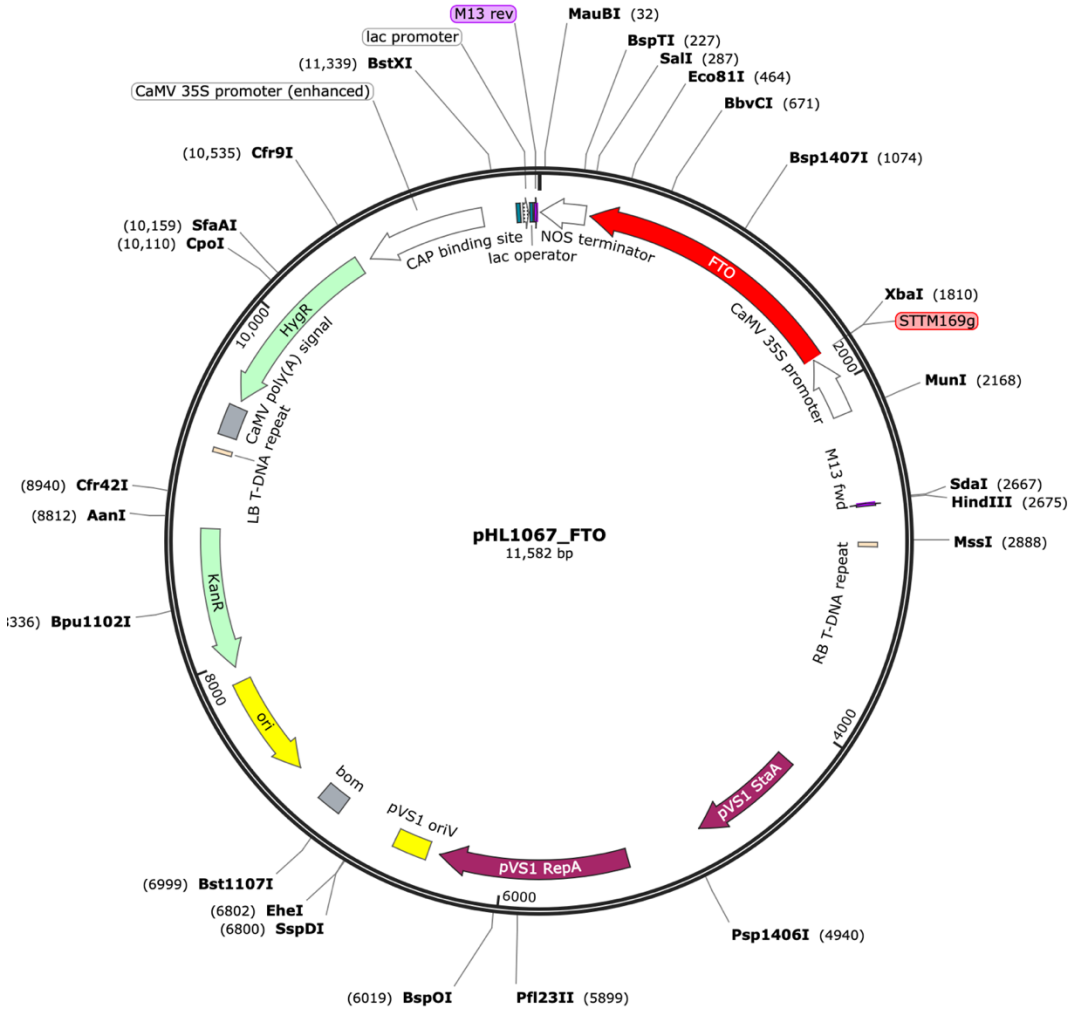

Figure 1. Plasmid map of the binary vector pHL1067, the *FTO* overexpression chimeric gene construct. the *FTO* gene is under the control of a constitutive CaMV35S (cauliflower mosaic virus 35S) promoter. The *FTO* expression cassette is linked to a hygromycin resistance gene *HygR* driven by CaMV35S promoter as a selectable marker for plant transformation. The "LB T-DNA repeat" and "RB T-DNA repeat" are the left and right borders of the transfer DNA (T-DNA), which will integrate into the host genome upon *Agrobacterium* infection of plant cell. The plasmid also contains an antibiotic kanamycin resistance gene *KanR* to facilitate *E. coli* culture for plasmid DNA extraction.

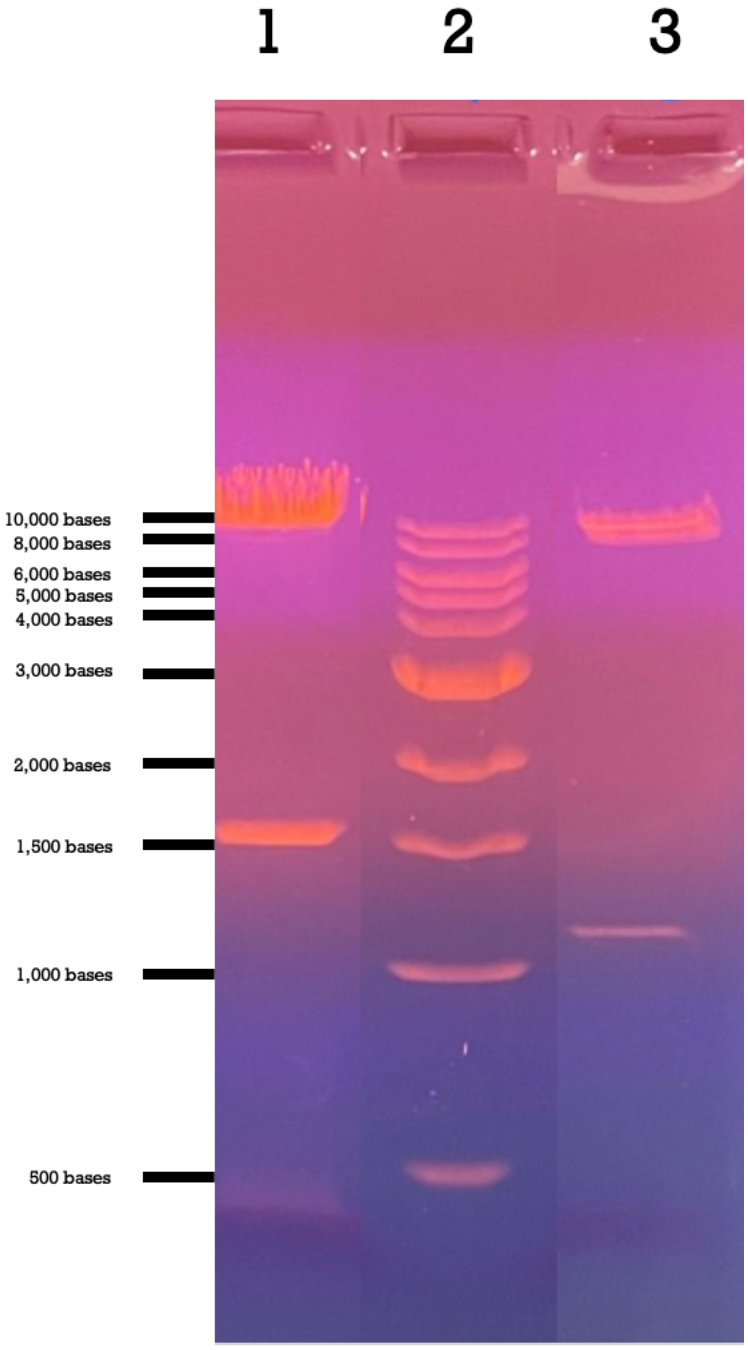

Figure 2. Plasmid DNA digestion to confirm the presence of *FTO* gene in pHL1067. Lane 1 contains pHL 1067 plasmid DNA digested with restriction enzymes Sal1 and Xba1. Lane 2 contains *Biolabs* 1 kb ladder. Lane 3 contains pHL 236 plasmid DNA digested with restriction enzymes Sal1 and Xba1. pHL 1067 has two separate bands: one near 10,000 bases as well as a band at approximately 1,500 bases corresponding to the *FTO* gene, which is 502 amino acids when translated. Lane 3 contains two bands one at 10,000 bases and another one close to 1,000, which corresponds with the gene that was replaced.

Embryogenic callus induction

Mature seeds were used to induce the embryogenic callus, which is critical to creating transgenic plant lines because the recombination that occurs on the cellular level of these stem cells can regenerate into adult plants. Creeping bentgrass seeds are dehusked with sandpaper and the surface sterilized with Clorox bleach (6% sodium hypochlorite) and rinsed with sterile distilled water. The calluses are then placed onto a callus-induction medium containing MS basal salts and vitamins[14], 30 g/l sucrose, 500 mg/l casein hydrolysate, 6.6 mg/l s,6 dichloro-o-anisic acid (dicamba), 0.5 mg/l 6-benzylaminopurine (BAP, and 2 g/l phytagel. The culture plates were kept in a dark room for 6 weeks. Callus induction was successful. The embryogenic calli were visually selected and subcultured on fresh callus-induction media as depicted in figure 3.

Figure 3. Turfgrass Callus

Creeping bentgrass transformation[17]

The process of creeping bentgrass transformation via *Agrobacterium*-mediated callus infection can be divided into five sequential steps: *Agrobacterium*-infection of callus, co-cultivation, antibiotic treatment, selection, and plant regeneration. The day before the plant calli are infected with *Agrobacterium* containing the pHL1067 plasmid, the callus is divided into 1- to 2-mm pieces and placed on callus-induction medium containing 100 µM acetosyringone, which is

a hormone that facilitates *Agrobacterium* infection via an ancient plant wound mechanism (*Agrobacterium* culture and callus maintenance are exemplified in Figure 4). The infection processes begin by adding 10 µL aliquots of *Agrobacterium* suspension to each individual callus. This is followed by 2 days of co-cultivation in the dark at 25 degrees Celsius. The calli are then transferred and cultured for 2 weeks on callus induction medium plus 125 mg/l cefotaxime and 250 mg/l carbenicillin to suppress bacterial growth. Next, for selection of the transgenic callus it is transferred onto selection media that contains 100mg/l of hygromycin antibiotic as well as cefotaxime. Several rounds of selection are conducted in which the calli are transferred onto fresh selection media every 3 weeks. For plant regeneration, the hygromycin resistant proliferating callus is moved to regeneration medium (MS basal medium, 30 g/l sucrose, 100 mg/l myo-inositol, 1 mg/l BAP and 2 g/l Phytagel) supplemented with cefotaxime and hygromycin. The calli are kept in the dark for 1 week before being moved under a light source for 2-3 weeks to develop shoots. The shoots are then separated and transferred to hormone-free regeneration medium containing hygromycin and cefotaxime to promote root growth and suppress any remaining *Agrobacterium*. Plantlets with well developing roots (3-5 weeks) are then transferred to soil and grown in the greenhouse. Schematic of the procedure is displayed in Figure 5.

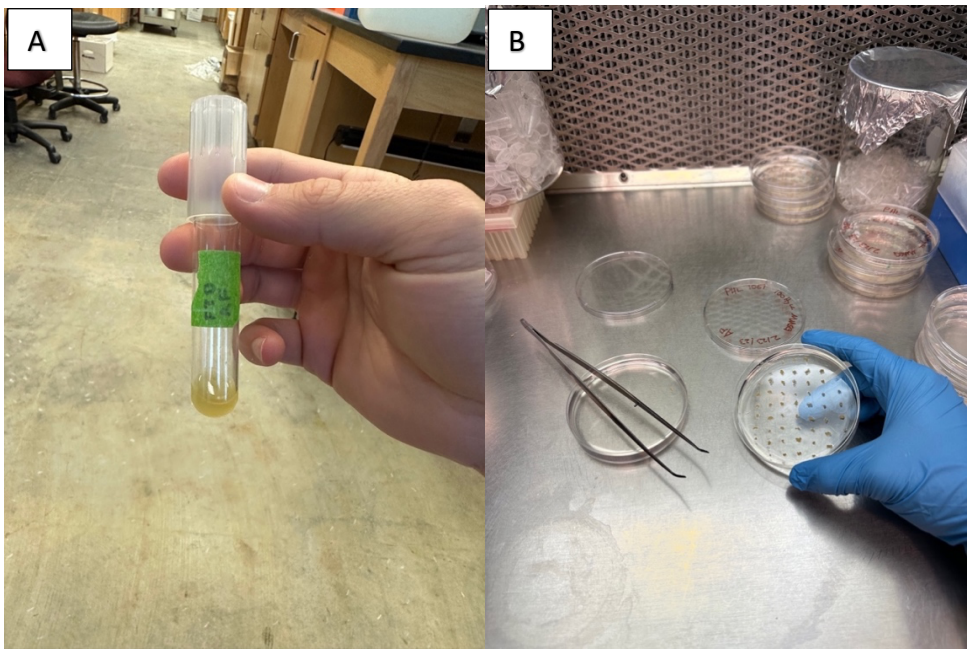

Figure 4. A) *Agrobacterium* preculture and B) Callus transfer

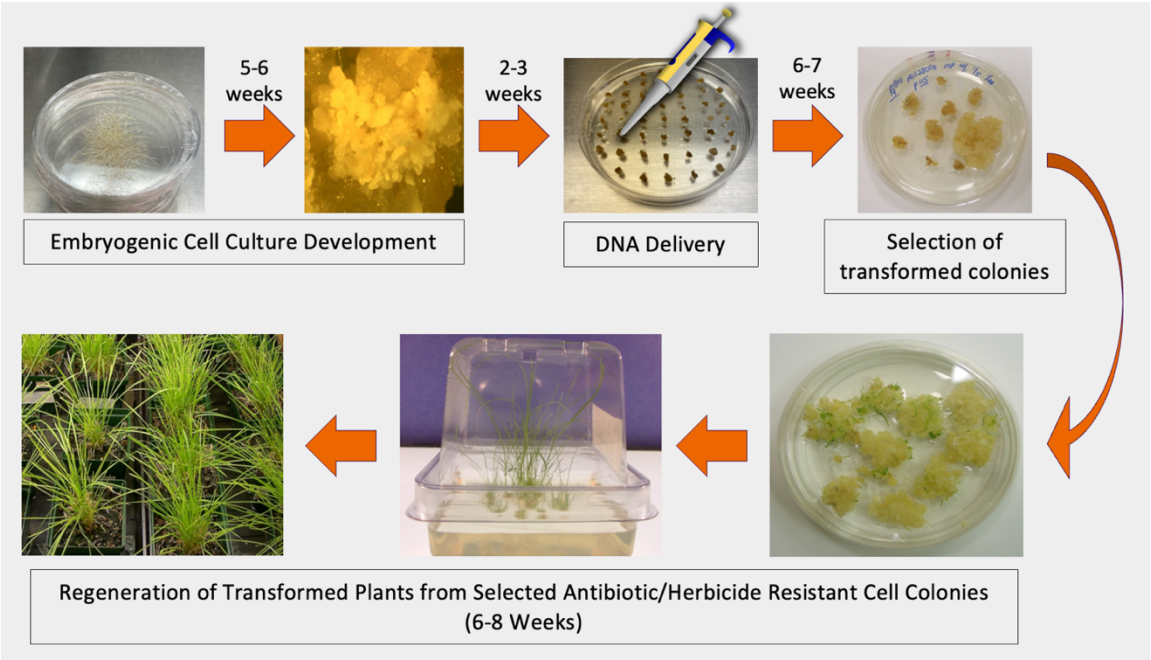

Figure 5. Turfgrass transformation procedure

Expectations

Currently, the *Agrobacterium*-infected calli are under the last round of selection. The next step would be to regenerate potentially transformed cells into plants for further analysis. I expect that FTO would function in transgenic creeping bentgrass, leading to an enhanced biomass production due to the increase in tiller bud formation. Likewise with an increase in biomass, it can also be expected that there will also be a corresponding increase in photosynthetic rate.

Abiotic stress resistance particularly drought resistance is expected to improve with increased root length. One of the biggest challenges with turfgrass is short root length; by lengthening roots the crop has better access to humid layers of soil and thus better accessibility to water.[15] This is particularly important under drought conditions. Yet, it is nonetheless hard to determine the impact that the *FTO* gene will have on drought tolerance. Although Yu et al. found that under two drought stress treatment conditions, FTO possessed enhanced drought resistance, they also found that the gene conferred ~34% increase in stomatal conductance and a 78% increase in transpiration rates which are generally correlated with drought hypersensitivity. Once transgenic FTO turfgrass is mature it will be compared to wildtype for resistance to abiotic stress conditions. Stress conditions can be measured by quantifying the proline content in the plant cell. Higher proline content indicated that the plant is experiencing stress to a great degree and has poor resistance to it. Proline is a way to maintain osmotic potential in a distressed state, and it can also act as a metal chelator, an antioxidative defense molecule, and a signaling molecule [16]. Electrolytic leakage is another tool that can be used to assess the status of a plant cell in abiotic stress conditions. It particularly is useful for looking a cell wall integrity. It is hard to predict the impact that the *FTO* gene will have on electrolytic leakage and proline content because Yu et al. did not

pursue those tests; however, it will undoubtedly offer novel information on FTO function in perennial grasses.

Trait modification using *FTO* gene in combination with a gene containment strategy

Because turfgrass is a highly-outcrossing, wind-pollinated, and a stoloniferous perennial species, it is highly at risk for introgression of alien genes into an adjacent population. There are serious ecological concerns about the possibility of *FTO* escape from transgenic plants to natural populations. In fact, a three-year field study on pollen-mediated gene movement from genetically modified creeping bentgrass found that pollen from transgenic crops could spread as far as 978 feet [19]. Therefore, it is paramount that effective reproductive confinement systems are established before the FTO transgenic line can be used practically.

There are two ways to achieve a confined system. One way which has been used in the Luo lab (Luo et al. 2004a; 2005) is male sterility. If no pollen grains are produced from the transgenic plants, it will effectively interrupt gene flow. Although it is an effective way to control transgenic plants, the development of new strategies that may confer better functionality in different situations is critical. The other way that plant sterility can be approached is via total sterility. The life cycle of flowering plants can be divided into three growth phases: vegetative, inflorescence, and floral. The switch from vegetative to floral needs a switch in the developmental program of the descendants of the stem cells in the shoot apical meristem (SAM). This transition is due to a set of genes called floral meristem identity genes such as *FLO* and *LFY*. If those genes can be knocked out, then the plant can remain stuck in the vegetative state. Flowering suppression prevents transgene escape through both pollen and seed dispersal. Knockout of the gene in turfgrass is

proposed via both RNA interference of the *FLO/LFY* genes as well as CRISPR/Cas9 genomic editing of the gene itself.

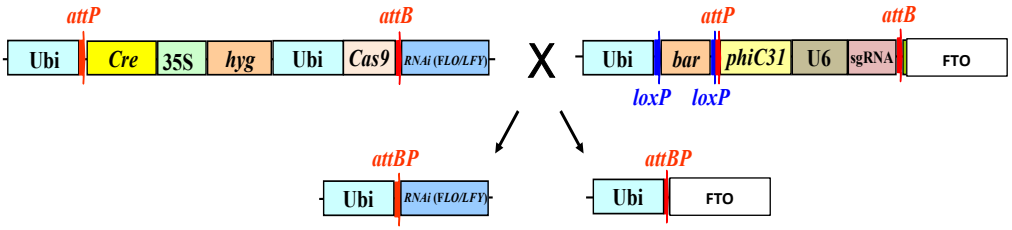

Figure 6: Total Sterility Construct

The strategy for producing environmentally clean transgenic plants involves crossing two transgenic lines to develop sterile offspring. The transgenic line on the top left (figure 6) contains the recombinase Cre linked to a CaMV35S promoter-driving the selectable marker gene hyg and a rice ubiquitin (Ubi) promoter-driving Cas9. Upstream of Cre and Downstream of Cas9 genes are recombinase target sites attP and attB. The fragment in between the target sites serve to separate the Ubi promoter from the RNAi expression cassette for FLO/LFY which causes plant sterility. This is important because the transgenic line needs to mate to produce offspring, so sterility needs to be inactive. The transgenic line on the right (figure 6) contains the loxP sites which are the target for Cre recombinase. In between the target sites loxP is the selectable marker gene Bar which is expressed via the Ubi promoter. Downstream of the gene is the recombinase phiC31 which targets the attBP in both transgenic lines. In fact, it this gene that is responsible for removing the majority of DNA (everything in between the red brackets). Linked to the phiC31 gene is the rice U6 promoter-driven FLO/LFY guide RNA (sgRNA), which needs the Cas9 protein to be effective. This plant line also has the same phiC31 target sites (attB) as the Ubi-attP-Cre-35S/hyg-Ubi/Cas9-

attB-RNAi-FLO/LFY (line to the left in figure 6). Lastly, the most downstream component is the FTO gene, which is separated from its Ubi promoter.

Upon cross-pollination of the two parental transgenic lines, the offspring will express both the Cas9 protein along with its guide RNA (sgRNA), which is specific for the FLO/LFY gene causing knockout of the flowering gene. This in turn will cause sterility in the offspring. Additionally, the Cre recombinase of the hybrid will cause excision of the loxP-flanked blocking fragment or the Bar gene. The importance of this is that it brings the Ubi promoter into proximity to express the phiC31 gene. The coding of the phiC31 gene causes self-excision of the phiC31 gene and linked U6 driven SgRNA resulting in expression of FTO via Ubi promoter. The expression of phiC31 also causes excision of the att-flanked Cre recombinase coding sequence with its linked CaMV35S-driven marker gene, hyg, and Cas9 gene. This brings the Ubi promoter in functional proximity to the downstream FLO/LFY RNAi cassette resulting in the sterility of hybrids. In other words, a fail-safe protocol for sterility is proposed where there are two mechanisms for induced plant sterility in the offspring: RNA interference of the FLO/LFY gene and direct knockout of the gene FLO/LFY gene. Additionally, all the superfluous DNA that is used as selectable markers is also removed creating a clean plant line.

Materials and Methods

Transformation of E. coli with plasmid

The first step requires a third-party company to synthesize the *FTO* gene (GenBank accession no. NP _001073902.1) from scratch flanked with the necessary restriction sites for SalI and XbaI restriction enzymes to integrate the gene into the plasmid. The company sends the gene

in the form of a powdered plasmid which must be excised to purify the gene of interest. First the plasmid is transformed into *E. coli* for culture to increase the copy number since the plasmid can replicate multiple times in a single cell. One microliter of plasmid DNA is added to 30 microliters of competent *E. coli* cells. It is then incubated at 37 degrees Celsius for one minute. Next it is added to 100 microliters of LB media (without antibiotic). Lastly the bacteria are spread on an LB plate with antibiotic and incubated overnight at 37 degrees Celsius.

E. coli amplification in liquid culture

The *E. coli* with plasmid are quadrant streaked on agar plate and incubate at 37 degrees Celsius overnight to isolate single colonies. A single colony is removed from the plate and inserted into 5 mL of liquid YEP media with antibiotics fitting plasmid resistance; this is referred to as preculture. This is incubated overnight at 37 degrees Celsius. This stock is then poured into 250 mL of YEP media with antibiotic overnight to amplify *E. coli* number.

Host Plasmid Isolation from E. coli

After the desired amount of incubation time the cells are centrifuged at 10,000 G for 1 minute to separate the media from the cells. Next the cells are lysed via solutions obtained from *Omega Bio-Tek E.Z.N.A Plasmid DNA Mini Kit I*. Following the kit, the suspension is centrifuged leaving a compact white pellet at the bottom of a tube, which is primarily the cellular debris. The supernatant contains the plasmid DNA as well as undesirable contaminants like cellular proteins, and it is passed through the HiBind DNA Mini Column in order to bind the plasmid DNA to the column and discard the proteins which will travel through as filtrate. This is followed by several wash steps with HBC Buffer in order to remove any proteins which may have been inadvertently

bound to the column. The column is then centrifuged by itself in order to removal all the trace amounts of HBC Buffer which contains alcohol in it. Lastly an elution buffer is added to the column and centrifuged at maximum speed leaving the plasmid DNA in the filtrate.

FTO gene isolation from host plasmid

Host plasmid, restriction enzymes Sal1 and Xba1, appropriate buffer, and dH2O are combined in a 1.5 mL tube. Two restriction enzymes are used so that the FTO gene will be inserted into the binary vector in the correct orientation. The solution is mixed by gently pipetting. The tube is then incubated at 37 degrees Celsius for 1 hour. Restriction enzymes are denatured by heating the solution at 70 degrees Celsius. In order to isolate the FTO gene from the rest of the plasmid, gel purification is conducted where the DNA is run on a gel to separate the heavy host plasmid from the relatively lighter FTO gene. The gene can then be cut out of the gel and purified using a gel extraction kit.

Subcloning: pHL 1067 Construction

The *FTO* gene is then integrated into a plasmid used in the Luo Lab as a binary vector for infection called pHL236. It is inserted into the polylinker region using corresponding restriction sites SalI and XbaI and DNA ligase. To achieve this the purified FTO gene, pHL236, ligase buffer, DNA ligase, and H2O are added to an Eppendorf tube. The tube is subsequently incubated overnight at 16 degrees Celsius. The newly created plasmid pHL1067 is then ran on agarose gel to see if the FTO gene successfully integrated.

Gel Electrophoresis of pHL1067

Gel is made using agarose powder with 100 mL of 1x TAE in a microwavable flask. The solution is then microwaved in pulses for 2 minutes. After a cooldown process ethidium bromide is added as an intercalating agent, which allows the DNA to be visualized under ultraviolet light. The solution is poured into to the gel tray with the comb in place. Next the gel is moved to the electrophoresis unit and covered with 1xTAE buffer until it is covered. Lane 1 of the gel contains the pHL 1067 plasmid with restriction enzymes Sal1 and Xba1. Lane 2 contains Biolab 1 kb ladder. And Lane 3 contains pHL 237 with restriction enzymes Sal1 and Xba1. The gel is run on 100 V and stopped when the dye reached about 70% of the way down the gel. The gel is lastly visualized under UV light to detect the location of the bands.

Agrobacterium tumefaciens Transformation

Once the pHL 1067 plasmid is completed and checked, it is transformed into *Agrobacterium*. Competent *Agrobacteria* are retrieved from the freezer and slowly thawed. pHL1067 and agrobacterium cells are transferred to electroporation cuvettes. The volts in the Gene Pulser are set to 2.5 kV and the CAP setting is set to 25 microFD. Resistance is set to 400 Ohms. The cuvette is placed in the cuvette holder and engage a pulse. 1mL of YEP media is added directly after pulse and mixed via pipetting. Tube contents are poured into a 1.5 mL microfuge tube and are spun for 5 minutes at 4000 rpm to pellet cells. The supernatant is removed, and the pellet is resuspended in 100 microliters of LB YEP media. The suspension is then plated on LB media with kanamycin resistance to select for bacteria that took up the plasmid. Colonies visible after 2 days are stored in the -80 degree Celsius freezer in glycerol stock.

Agrobacterium tumefaciens liquid culture

Glycerol stock of *Agrobacterium* containing pHL 1067 removed from -80 degree Celsius freezer and thawed. The *Agrobacteria* are quadrant streaked on kanamycin agar plates and incubated at 37 degrees overnight to isolate single colonies. A single colony is removed from the plate and inserted into 5 mL of liquid YEP media with kanamycin antibiotics; this is referred to as preculture. This is incubated overnight at 28-30 degrees Celsius. This stock is then poured into 250 mL of YEP media with kanamycin overnight to amplify *Agrobacterium* number. Approximately, 20 mL of agrobacterium plus media is poured into falcon tube and centrifuged leaving cellular pellet. The supernatant is removed and replaced by cocultivation media with acetosyringone. The falcon tube is placed on a shaker for 30 minutes then can be subsequently used.

Abiotic Stress Tests

Leaf relative water content (RWC) [18]: formula is RWC = [(FW-DW)/(TW-DW)] x 100% where FW is fresh weight, DW is dry weight and TW is turgid weight. The leaves from both the FTO recombinant and the wildtype were harvested and immediately weighed to record FW. They were then cut into small pieces and immersed in Millipore water at 4 degrees Celsius for 16 hours. After measuring the TW, the leaves were dehydrated in an oven at 37 degrees Celsius overnight and weighed (DW).

Leaf electrolytic leakage (EL) [18]: is measured to evaluate cell membrane stability. Fresh shoot segments (0.2-0.5g) from each sample are incubated in 20 mL of Millipore water at 4 degrees Celsius for 16 hours. The initial conductance (Ci) is measured using a conductance meter to estimate the quantity of ions released from the cell under normal and abiotic stress conditions. The leaf tissue is then autoclaved for 30 mins and followed by measuring the conductance

(Cmax). (Ci/Cmax) x 100: this measurement reflects the number of ions released from plant cells before and after heat treatment.

Proline content [18]: proline is extracted from 100 mg of plant tissue via mortar and pestle in 2 mL of 3% sulphosalicylic acid. 200 uL of extract is reacted with 200 uL of acid ninhydrin and 200 uL of glacial acetic acid for an hour at 100 degrees Celsius. An ice bath is used to terminate the reaction. The reaction mixture is extracted with 1mL of toluene, and then vortexed. Absorbance of the toluene layer will then be read at 520 nm in a Themo Spectronic BioMate 3. Proline concentration is determined from a standard curve and calculated as follows: [(ug proline/mL x mL extraction buffer)/115.5 ug ug/mol]/g sample = umol proline/g.

Acknowledgments

I would like to graciously thank the Honors College Grant and Luo Lab Members: Dr. Hong Luo, Qian Hu, Xiaotong Chen, Zhaohui Chen, Annslise Enger, Ryan Watts, Parth Patel, Morgan Kuess, Olivia Mathis, Lilli Hicks, and Emma Jenson.

References

1. Frye M, Harada BT, Behm M, He C. RNA modifications modulate gene expression during development. *Science*. 2018;361(6409):1346-1349. doi: 10.1126/science.aau1646.

2. Geula S, Moshitch-Moshkovitz S, Dominissini D, et al. m6A mRNA methylation facilitates resolution of naïve pluripotency toward differentiation. *Science (American Association for the Advancement of Science)*. 2015;347(6225):1002-1006. doi: 10.1126/science.1261417.

3. Zhong S, Li H, Bodi Z, et al. MTA is an arabidopsis messenger RNA adenosine methylase and interacts with a homolog of a sex-specific splicing factor. *Plant Cell*. 2008;20(5):1278-1288. doi: 10.1105/tpc.108.058883.

4. Bodi Z, Zhong S, Mehra S, et al. Adenosine methylation in arabidopsis mRNA is associated with the 3' end and reduced levels cause developmental defects. *Frontiers in plant science; Front Plant Sci*. 2012;3:48. doi: 10.3389/fpls.2012.00048.

5. Shen L, Liang Z, Gu X, et al. N6-methyladenosine RNA modification regulates shoot stem cell fate in arabidopsis. *Developmental cell*. 2016;38(2):186-200. doi: 10.1016/j.devcel.2016.06.008.

6. Dominissini D, Moshitch-Moshkovitz S, Schwartz S, et al. Topology of the human and mouse m^sup 6^A RNA methylomes revealed by m^sup 6^A-seq. *Nature (London)*. 2012;485(7397):201.

7. Luo S, Tong L. Molecular basis for the recognition of methylated adenines in RNA by the eukaryotic YTH domain. *Proc Natl Acad Sci U S A*. 2014;111(38):13834-13839. doi: 10.1073/pnas.1412742111.

8. Hong-Chao D, Lian-Huan W, Zhang C, et al. ALKBH10B is an RNA N6-methyladenosine demethylase affecting arabidopsis floral transition. *Plant Cell*. 2017;29(12):2995-3011. doi: 10.1105/tpc.16.00912.

9. Martínez-Pérez M, Aparicio F, López-Gresa MP, Bellés JM, Sánchez-Navarro JA, Pallás V. Arabidopsis m^{6}A demethylase activity modulates viral infection of a plant virus and the m^{6}A

abundance in its genomic RNAs. *Proceedings of the National Academy of Sciences - PNAS*. 2017;114(40):10755-10760. doi: 10.1073/pnas.1703139114.

10. Zheng G, Dahl JA, Niu Y, et al. ALKBH5 is a mammalian RNA demethylase that impacts RNA metabolism and mouse fertility. *Mol Cell*. 2013;49(1):18-29. doi: 10.1016/j.molcel.2012.10.015.

11. Du H, Zhao Y, He J, et al. YTHDF2 destabilizes m6A-containing RNA through direct recruitment of the CCR4–NOT deadenylase complex. *Nature communications*. 2016;7(1):12626-11. doi: 10.1038/ncomms12626.

12. Meyer KD, Patil DP, Zhou J, et al. 5' UTR m(6)A promotes cap-independent translation. *Cell*. 2015;163(4):999-1010. doi: 10.1016/j.cell.2015.10.012.

13. Yu Q, Liu S, Yu L, et al. RNA demethylation increases the yield and biomass of rice and potato plants in field trials. *Nat Biotechnol*. 2021;39(12):1581-1588. doi: 10.1038/s41587-021-00982-9.

14. Murashige T SF. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol Plant*. 1962.

15. Tajima R. Importance of individual root traits to understand crop root system in agronomic and environmental contexts. *Breed Sci*. 2021;71(1):13-19. doi: 10.1270/jsbbs.20095.

16. Hayat S, Hayat Q, Alyemeni MN, Wani AS, Pichtel J, Ahmad A. Role of proline under changing environments: A review. *Plant signaling & behavior; Plant Signal Behav*. 2012;7(11):1456-1466. doi: 10.4161/psb.21949.

17. Luo H, Hu Q, Nelson K, Longo C, Kausch AP, Chandlee JM, Wipff JK, Fricker CR. Agrobacterium tumefaciens-mediated creeping bentgrass (agrostis stolonifera L.) transformation using phosphinothricin selection results in a high frequency of single-copy transgene integration. *Plant Cell Rep.* 2004.

18. Yuan S, Zhao J, Li Z, et al. MicroRNA396-mediated alteration in plant development and salinity stress response in creeping bentgrass. *Horticulture research; Hortic Res*. 2019;6(1):48. doi: 10.1038/s41438-019-0130-x.

19. Wipff JK, Fricker C. Gene flow from transgenic creeping bentgrass (agrostis stolonifera L.) in the willamette valley, oregon. *International Turfgrass Society Research Journal*. 2001;9:224-242.