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Pilot Project Proposal
(Not to exceed two pages)

Name of Project: Engineering and regulating plant genomes: build a map, editing and design of functional eukaryotic-based integrases and synthetic chromosomes

Proposer and Contact Information:

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Technical Idea:

The project aims are to: a) develop plant functional synthetic chromosomes in *Arabidopsis thaliana* and provide protocols to design, built, isolate and purify these synthetic chromosomes; b) utilize recombinases, such as serine integrases and novel designed recombinases to determine endogenous nonessential genes and develop genetic circuits to control endogenous and exogenous gene regulation. These developments will form the foundation for genetic systems capable of switching specific traits “on” and “off” in model and cultivated plant species through external chemical, physical and/or biologicals inducers; c) utilize soybean, as a cultivated model plant for engineering; d) generate a genetic map of AT and Soybean advanced lines directed for educational and engineering purposes.

- a) Develop plant functional synthetic chromosomes and systems to deliver foreign DNA into such chromosomes. Recently the international community has concentrated major efforts on building more precise and comprehensive genomes to be utilized as templates for editing and high precision engineering. Two examples to be cited are the development of a minimal prokaryotic genome (Craig Venter) and the Sc2.0 project. Those studies will ultimately make it easier to

find specific DNA involved in traits important for an organism's survival or for genetic manipulation. In this proposal our first short-term goal will be to complete a minimal semi synthetic plant chromosome of *Arabidopsis thaliana* (AT). Current limitations of existing gene delivery technologies, demand improvement and development of systems suitable for transferring large (up to Mb size or larger) into plant genomes. Recent studies have shown that 2.6 Kb of AT telomeric repeats was efficient to integrate a construct formed by a reporter gene and a selective marker gene at a distal chromosome location in maize, generating a minichromosome in the maize B chromosome. These results opened the possibility for the use of the telomere-mediated chromosome truncation technology as a platform for the production of artificial minichromosomes in maize plants. Later, it was shown that this technology could be also applied for AT and can be expanded to other plant species. For the utilization of this technology for the development of a synthetic plant chromosome, one should consider three important aspects. The first one is that the insertion of the artificial minichromosome vectors is currently random. Another aspect to be considered is that the occurrence of homologous recombination in plants is very low. Thus, one major problem becomes how the original chromosome will be replaced by the synthetic one. Here we propose utilizing telomere-mediated chromosome truncation to initiate replacement of the original chromosome by the synthetic. To initiate the process the telomere-mediated technology will be coupled to a modified CRISPR/Cas9 system that will allow replacement of the telomere at a specific sequence. Currently, we have been conducting preliminary experiments to evaluate whether this modified CRISPR/Cas9 could boost the rate of homologous recombination in AT, allowing the replacement of an original gene sequence with a mutated gene sequence. If the modified CRISPR/Cas9 system shows to be efficient to increase the homologous recombination in AT we intend to continue using this system to promote the replacement of the original DNA sequences to the synthetic ones, though out the chromosome. Except for the centromeric region, because the third important aspect to be considered for the development of the synthetic plant chromosome will be the centromeric region. Several previous studies had shown that the identity of the centromeric region is not determined by its sequence, but by its epigenetic markers. Therefore we intend to preserve the endogenous centromeric region to develop a semi-synthetic plant chromosome. It is also important to mention that previous studies had shown that the construction of a minichromosome line in AT required a tetraploid line.

- b) Utilize recombinases, such as serine integrases to determine the endogenous nonessential genes and also to design and provide systems to control endogenous and exogenous gene regulation, allowing in the

future the use of genetic circuits, which are capable of switching specific traits “on” and “off” in model plants and in cultivated crops, through external chemical, physical and/or biological inducers. Recombinases such as serine integrases are capable of recombination at a specific sites to integrate and excise bacteriophage genomes into and out of their host’s genome. These enzymes have the capacity to induce a remarkable directionality of DNA sequences. Studies in prokaryotic organisms have demonstrated the capacity to generate genetic circuits utilizing different recombinases to control gene regulation. However, knowledge about the functionality of integrases in eukaryotic cells is still limited. Our recent results demonstrated the functional capability of serine integrases in plant cells. A co-transformation plasmid system was utilized to evaluate two different integrases in *A. thaliana* protoplasts. We are currently expanding the number of integrases to be tested and we anticipate our results to be an initial point for development of more complex models of gene regulation in plants using integrases. This is important because another short goal of this proposal is to determine specific DNA involved in traits important for an organism's survival or for genetic manipulation. Although AT knockouts lines has been available for the scientific community for some time, the assembly of the minimal prokaryotic genome, by Craig Venter’s group and the SCRaMble analysis performed on the yeast chromosome III by Jeff Boeke’s group has shown us that the knockouts of unique genes is a limited method to determine essential genes probably due to unpredictable functional gene redundancy. Therefore, to obtain the knockouts of every major class of gene the next target will be to generate AT plants carrying inducible promoters attached to integrases-sensors, which, in turn, will respond for external inducers (physical, chemical and/or biologicals) under field conditions with a inducer to switch desirable gene “on” and/or “off”. In this case we intend to perform the SCRaMble analysis by flanking different groups of endogenous essential genes with different integrase target sites. We think it would increase the probability of eliminating the knockout of genes that function at the same pathway. Once the nonessential genes had been identified we could replace them by desirable genes/metabolic pathways. Another approach that one can use would be to develop a semi-synthetic chromosome AT with the Cas9 under an inducible promoter and loci specific for the introduction of DNA that would transcribe guided RNAs under an U6 promoter for each one of the desired genes.

- c) Utilize soybean, as a cultivated model plant: Soybean will also be a case study to engineer and regulate gene expression using built maps, synthetic chromosomes and recombinases aiming to provide a stably inherited plant artificial chromosome. Protein levels in soybean seeds continue to decline. As a consequence, there is current demand from

soybean growers to raise protein levels aiming to regulate proteins in soybean seeds to improve quality and quantity. An approach to be evaluated will explore the transcriptional regulatory system based on two plasmids with RNA scaffolds for the CRISPR-Cas9 system. Then, aiming to increase protein content by using VP64 to enhance transcription of target storage proteins and using SET domain to repress the oil content. Development of a system to increase the homologous recombination rates of CRISPR-Cas9 system in soybean plants will also be studied. Aiming to perform specific nucleotide substitutions and direct transgenes to specific genome sites (such as high transcription genome sites). In addition, we will introduce the orphan gene *Qua-Quine Starch* gene from AT in a location with high expression within the soy genome.

- d) Generate a map of AT and Soybean advanced lines as a “biochassis” for educational purposes. Education is probably the most important strategy for building sustainable progress and reduce inequity. Intensifying and updating the current format applied in most of schools is urgently needed. Students ought to be stimulated at the early age with novel thoughts having scientific and technological experiments as foundation.