Call for Proposals 2023

Zimin Institute for Engineering Solutions Advancing Better Lives

Title:

Next-generation multi-targeted CRISPR genetic toolbox to reveal hidden traits and accelerate crop breeding

PIs:

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Abstract:

The need to accelerate crop breeding programs has never been greater, as the world population is exponentially increasing, the climate is changing, and resources are limited. Breeding relies on genetic variation. However, it is impossible to alter many phenotypes by introducing genetic variation in a single gene due to large gene families with high functional redundancy. For example, in tomato and rice, ~80% of coding genes belong to multi-gene families. Therefore, in many cases, mutating multiple gene family members is required to uncover "hidden" traits that are important for plant resilience and food security. Currently, there is no approach or technology that can dig into the hidden genetic redundancy at a genome-scale level (unbiased forward genetics) and reveal masked agricultural traits. To address these challenges, we developed the Multi-Crop technology - the first genome-scale multi-targeted CRISPR libraries in crops. We have constructed, validated, and patented the approach in Arabidopsis, tomato, and rice, demonstrating that the Multi-Crop technology can uncover hidden genetic traits and is the future of plant breeding programs. Not only is this the first demonstration of a large-scale, multi-targeted CRISPR technology in plants, but the unique approach also overcomes functional gene redundancy under any given conditions, such as the response to drought, pathogen, fruit size, and more. Multi-Crop can be applied to most crops and all breeding traits. Therefore, we expect the new toolbox we develop here to transform how scientists and breeders perform genetics.

Scientific Background and Innovation Potential

The problem: Feeding the World. The need to accelerate crop breeding programs has never been greater as the world population is exponentially increasing, climate change is altering growth conditions, and resources are limited [1,2,3]. Hence, novel methods for generating high-yielding and nutritious cultivars resilient to biotic and abiotic stresses and growing with limited water and nutrient resources are desperately required. Importantly, successful plant breeding relies on variation, be it natural, induced, or introduced. Genetic variation has been expanded over the years by introducing natural variation and by creating random mutagenized lines (e.g., using radiation). During the past two decades, these approaches have led to the identification of novel genes underlying agriculturally-important traits and their integration into breeding programs. Notwithstanding, comprehensive genetic studies and large-scale genome sequencing projects failed to alter many phenotypes via the introduction of genetic variation. This is due to numerous local and large-scale chromosomal duplications that have occurred over the course of plant evolution, resulting in large gene families of similar sequences and partially overlapping functions [4]. In tomato, for example, 80% of all protein-coding genes belong to families with at least two members, and these numbers are similar in other crops [5]. The high sequence similarity among plant gene families often results in complete, partial, or conditional functional redundancy, leading to substantial phenotypic buffering, such that single null mutants do not present an evident phenotype. Therefore, in many cases, mutating multiple gene family members is required to uncover "hidden" traits. Accordingly, it is estimated that ***the majority (~70%) of agricultural traits are currently genetically inaccessible*** [6-9]. Such traits must be identified to fuel and accelerate breeding programs.

Given these limitations, broadly applicable and innovative plant genomic technologies are greatly needed. Whereas the creation of random mutations (i.e., forward genetics) does not allow the modification of more than one gene in a family at once, reverse genetics does [9,10]. Several studies have applied classic methods and genome editing to target multiple family members simultaneously, creating new opportunities for modern plant breeding. However, these reverse genetics approaches require advanced knowledge of which genes to target in order to drive the needed phenotype. This is a significant bottleneck since, in most crops, the putative function of less than 5% of the genes is annotated.

We propose a novel molecular engineering technology based on genome editing that will overcome functional redundancy and will uncover hidden phenotypes critical to crop improvement. **We will demonstrate that the strategy can be efficiently applied in tomato and rice, representing two major, evolutionary-distant crops.** The developed interdisciplinary technology, illustrated schematically in Fig. 1, utilizes CRISPR/Cas9 genome editing tool to generate a large number of independent non-GMO mutants, each potentially containing mutations at functionally-redundant genes. First, we develop and apply a dedicated bioinformatics algorithm to design a library of a large number of different CRISPR-CAS constructs, each containing a single guide RNA (sgRNA) directed towards a different subgroup (typically 2-8 genes) of a gene family in the genome. To increase the flexibility and specificity of the genetic screen, the library is further partitioned into functional sub-libraries (e.g.,

all genes putatively involved in drought tolerance). The library is then synthesized, cloned into a genome-editing Cas9 vector[23], and introduced into plants, such that each plant contains mutations at multiple homologous genes. A phenotypic screen is then applied to detect the desired phenotype, and potential candidates are genotypes to identify the underlying genes.

To date, we have constructed, validated, and patented the approach in the model plant *Arabidopsis* and demonstrated its capability to overcome genetic redundancy at a genome-scale level (*Nature Plants, in press*). Specifically, we computationally designed a total of 59,129 sgRNAs targeting all gene families in *Arabidopsis*. The library was engineered molecularly into 10 functional sub-libraries, and the sub-library targeting over 800 transporter genes, encompassing 5,635 sgRNAs was synthesized, cloned into a genome-editing efficient intronized Cas9 vector [11], and transformed into *Arabidopsis*. A forward-genetic screen using over 3,500 CRISPR lines recovered many novel phenotypes whose function has been hidden due to genetic redundancy. For example, we identified a homologous subfamily of three previously unstudied genes with partially overlapping functions, *PUP7*, *PUP21*, and *PUP8*. We demonstrated that all three proteins biochemically function as cytokinin transporters, and regulate meristem function and plant development through a complex redundant activity, thus illustrating the power of the approach to discover new biological functions regulated by multiple genes with overlapping functions. *To our knowledge, this was the first genome-scale multi-targeted CRISPR library introduced into an eukaryote*. While the approach is general, a major challenge is its applicability in a genome-wide manner since it relies on the efficient large-scale transformation of the genome-editing vectors into plants, a procedure that must be carefully tuned and calibrated. Through this proposal, we aim to further develop the technology and apply it to two major food crops that span the two major plant lineages, tomato (eudicots) and rice (monocots), demonstrating that it can be integrated and accelerate an array of breeding programs. *We expect that the new technology we develop here will provide a platform for performing next-generation genetics enabling the agricultural field to develop more resilient and high-yielding crop varieties*.

Objectives and expected significance

Through this interdisciplinary proposal, we will develop the next generation of genetics in agriculture using a multi-targeted genome-wide CRISPR editing approach. Our strategy applies large-scale forward genetics to target the most beneficial genomic regions and can overcome functional redundancy and linkage among related genes. Here we will further develop the strategy allowing more flexible and efficient genome editing and employ it in two important model crops, thus demonstrating its general applicability.

Specific Aims:

1. Develop advanced computational design for multi-targeted genome editing.

2. Construct the multi-targeted gene-editing library and transform it into rice and tomato.

3. Apply the developed libraries to reveal drought tolerance mechanisms in tomato and rice.

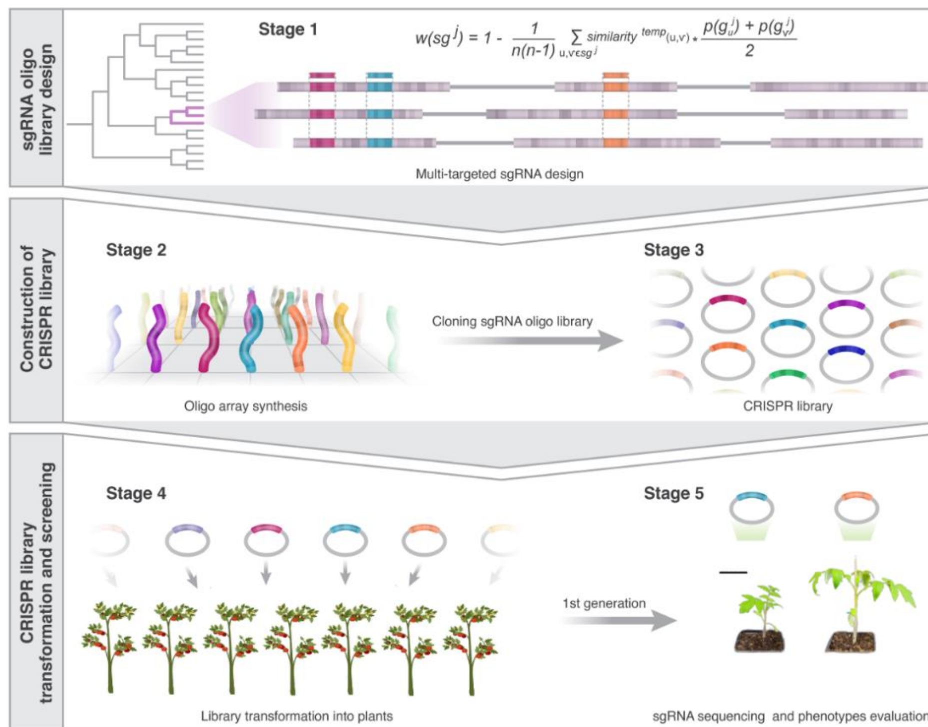

Fig. 1: Genome-scale multi-targeted CRISPR platform in crops. ***Stage 1:*** The genome of a crop, tomato in this illustration, is clustered into gene families, represented as phylogenetic trees. The trees are then used to guide the optimal computational design of multiple sgRNAs targeting each subfamily using the CRISPys [12] algorithm. ***Stages 2 & 3:*** An sgRNA sub-library is synthesized, amplified, and cloned into CRISPR/Cas9 vectors. ***Stage 4:*** The library is introduced into plants; each plant expresses a single sgRNA, targeting a subfamily of 2-8 genes. ***Stage 5:*** Candidates identified in a phenotypic forward genetic screen are genotyped for sgRNA sequence.

Detailed description

1. Develop advanced computational design for multi-targeted genome editing.

Although successful, our previous computational design and its application to the Arabidopsis genome suffered from several shortcomings. These will be addressed here, and we will further provide a novel algorithm that allows for multiple sgRNAs at each CRISPR/Cas9 vector, thus enhancing the probability of editing multiple genes simultaneously. First, our previous design harnessed the low specificity of the Cas9 nuclease to design an sgRNA that would simultaneously target multiple genes. The design was based on *off-target* specificity scoring functions (e.g., [13,14]) that provide the editing potential of an sgRNA to a given DNA target. These functions mainly account for the effect of different DNA::RNA mismatches and practically ignore the editing potential of different sgRNAs. Increasing evidence, however, demonstrates that some sgRNA provide more efficient genome editing than others, and this has been incorporated into *on-target* scoring functions [15]. We will develop a novel probabilistic scoring function based on function composition that accounts for both aspects of genome editing (efficiency and specificity), thus increasing the editing potential of our screen. The development will be general, allowing the incorporation of any of the widely-used off-target and on-target scoring functions, as well as those that will be developed during the course of this research. Second, our current algorithm is aimed at targeting multiple genes within a given gene family. Often, however, it is of interest to specifically edit some family members while avoiding others (e.g., due to lethality). We will thus allow the specification of a subgroup of genes whose editing is undesired. Treating our combined scoring function as the probability of success in a Bernoulli experiment, this option will be incorporated into our algorithm by maximizing the probability of successfully editing the given gene family, multiplied by the probability of not editing the specified undesired gene set. This enhancement should make our screen more versatile and could allow the design of larger combinations of subgroups of genes within each family (e.g., by iteratively choosing different genes in the

undesired gene set). Lastly, for each Cas9 vector, our current algorithm designs a single sgRNA for the editing of multiple homologous genes. Using multiplex genome editing, multiple sgRNAs can be integrated within a single Cas9 vector. We will thus extend our algorithm to allow the design of multiple sgRNAs that would collectively target the input gene set, such that each sgRNA could be more specific but the vector would target a larger fraction of the input gene set. Applying this algorithm in a large-scale manner will enable us to construct the first genome-scale multiplex library that is non-random and directed into specified gene members.

2. Construct the multi-targeted gene-editing library and transformation into rice and tomato.

We will provide the first evidence that our large-scale genome editing approach for targeting multiple functionally redundant genes can be successfully applied in two of the most important crops. Specifically, the tomato M82 (*sp-*, determinate tomato mutated in *SELF-PRUNING* [16] cultivar) and rice Zhonghua 11 background will be used in our experiments. We assessed and optimized aspects of the CRISPR library transformations, confirming: i) accuracy and coverage of the CRISPR library in bacteria, ii) number of plants to transform to achieve 1X coverage of all genotypes, and iii) ability to obtain and verify phenotypes. From our experience, generating 500,000 colonies of DNA is sufficient to fully represent the deep-sequenced sgRNA library.

Although our libraries can be designed to cover the entire genome, we will focus *in planta* on the transcriptome-enriched drought-mediated sub-library, designed to target genes that are differentially expressed under drought conditions. The library design is based on published data [17,18] and our tomato RNA-seq drought and ABA response data. Each library targets 500 genes with 1,000 sgRNAs. Each sgRNA targets 2-8 genes from the same family. Thus, the genes are targeted by multiple sgRNAs, and each sgRNA targets multiple genes. sgRNAs likely to have off-target effects are removed during the design.

<u>**Preliminary achievements:**</u> We have designed, synthesized, cloned, and transformed the transportome multi-targeted CRISPR sub-library as a case study to validate the developed tools. This transformation was carried out by *Agro*-based tissue culture approach. This sub-library targeted 250 transporters from the ABC, MFS, and MATEs families using 450 sgRNAs. sgRNAs were amplified and cloned into the *UBQ4:CAS9* library. The library was sequenced to evaluate sgRNA coverage (100%) and frequency and transformed into the M82 tomato cultivar (not shown, space limit). We sequenced DNA from 25 independent lines. In parallel, we have synthesized, cloned, and transformed a library of 800 sgRNAs into rice (Zhonghua 11 background) and generated 1,000 independent lines (Fig. 3).

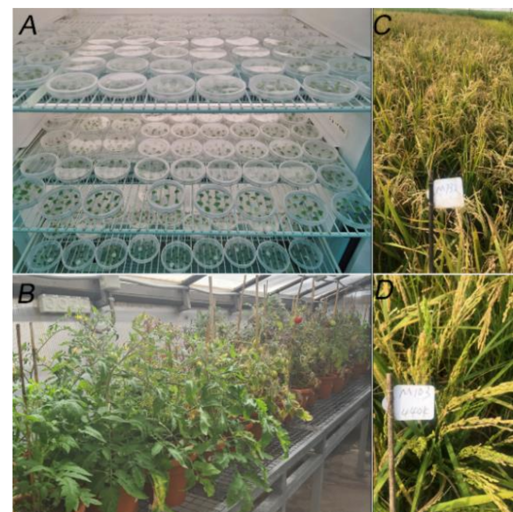

Fig. 3: Multi-Crop transformation into rice and tomato generating over 1,000 plants. A-B) High-throughput tomato tissue culture Multi-Crop transformation yielded hundreds of plants. **C-D**) One thousand independent rice Multi-Crop plants propagated in the field.

<u>**Deliverables:**</u> **Our unique technology allows overcoming functional redundancy for the first time, in an unbiased forward-genetic fashion, enabling targeted genetics for any agricultural trait. We expect that**

these new toolboxes will transform the way scientists and breeders carry out plant genetics.

3. Apply the developed libraries to reveal drought tolerance mechanisms in tomato and rice

This multi-targeted CRISPR approach overcomes redundancy at a defined genome-scale level to reveal hidden traits. Our validated pipeline will be used to construct the tool in rice and tomato. As a proof of concept, we will focus on drought tolerance. We will screen the library at T1 generation with eight plants per line planted in four pots. To identify mutant lines involved in drought tolerance, we will screen the library using powerful independent tools: 1) the Porometer (LI-190R Quantum Sensor), 2) thermal imaging, and 3) phenomics. We have gained extensive experience using these instruments [19,20]. Physiological data will be collected in week 3. At week 3, we will withhold irrigation for two weeks and monitor the plants for an additional two weeks. We do not have the capacity to apply weighted pots and lysimeter assays to over 500 pots at once. These quantitative tools will be applied to selected lines in the T2 generation.

Achievements: Our preliminary data in tomato identified several lines, among them *sgRNA-217,* which expresses an sgRNA that putatively targets three genes *Solyc05g054890*, *Solyc04g00102200*, and *Solyc04g00102210*. Expression of this sgRNA results in partial drought tolerance, reduced daily transpiration, and altered ABA levels (Fig. 4). This demonstrates the strength of our multi-targeted CRISPR approach. **Importantly, the methods developed here will enable the plant community to apply a multi-targeted genome-scale forward genetics approach to uncover hidden breeding traits in an unbiased fashion.**

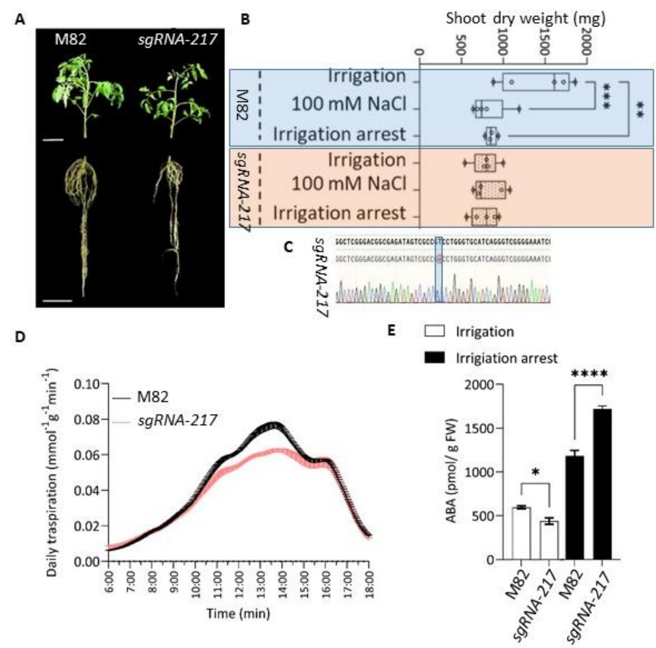

Fig. 4: sgRNA-217 mutants are partially resistant to salinity and water shortage. A) Six-week-old homozygous *sgRNA-217* plants grown under optimal growth conditions. **B)** *sgRNA-217* mutants show growth inhibition and drought resistance. **C)** Chromatogram mutations map illustrating the sgRNA cleavage site in one of the *sgRNA-217* target genes. **D)** Daily transpiration is reduced in *sgRNA-217* plants under optimal growth conditions. **E)** ABA levels are modified in *sgRNA-217* leaves, before and after stress. Unpublished.

Summary: We believe the proposed interdisciplinary research provides a game-changing technology to the breeding community. Unlike any other available technology, it enables uncovering hidden phenotypic variations due to functional redundancy in an unbiased forward-genetics fashion, enabling targeted genetics for any agricultural trait. Our labs have already exposed hundreds of phenotypes in *Arabidopsis* that the plant community could not find using the available genetic tools. By generating over 1,000 Multi-Crop tomato and rice lines, we will show that the technology is general and broadly applicable to many crops. Moreover, its high throughput nature of maximizing genetic diversity will enable a tailored fit for specific farmers, markets and growth conditions, enhancing productivity and farmer profitability. From seed producers' perspective, this technology has the potential to break the breeding glass ceiling and significantly reduce the high R&D expenses on quality seed development.

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Budget and justification

Cost Category	Budget (USD)
Bioinformatician (Computational Ph.D. student) Will devote 100% of its time to developing algorithmic solutions for the optimal choice of sgRNA and to designing CRISPR libraries directed toward specific crops.	35,000
Research genetics expert (Ph.D. student) Will devote 100% of its time to constructing the CRISPR-based libraries targeting gene families and leading plant genetics. This will include the development of the first CRISPR multi-targeted library in crops, tissue culture and genetic screens, plant propagation, physiology, and genotyping.	30,000
Consumables The necessary budget will cover disposables, cloning kits, tissue culture materials, genetics screening materials, hormones, enzymes, genotyping kits, and chemicals.	25,000
CRISPR library synthesis Service cost: CRISPR libraries will be synthesized by TWIST bio-sciences (Seattle) $3,000 per library.	6,000
Fieldwork, growth rooms, greenhouses, plant maintenance costs	4,000
Total personal, consumables, molecular material, and plants growth needs	$100,000

Justification

The central part of the research money will be dedicated to supporting research students (PhDs): one will be conducting the computational aspects and the other the genetics and experimental procedures. The Mayrose lab is already equipped with all the necessary computing infrastructure and no additional budget is required. Additional costs are requested to carry out the experimental procedures. The expected expenses should exceed the allocated sum, so the researchers will provide additional support from internal funds.

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Education

2008-2011 Ph.D. (with distinction), Cell Research and Immunology, Tel-Aviv University

2002-2004 M.Sc., Zoology, Tel-Aviv University

1997-2001 B.Sc., Multidisciplinary program in Computer Science and Biology, Tel Aviv University

Employment

2021-to date Professor, School of Plant Sciences and Food Security, Tel Aviv University

2016-2021 Associate Professor, School of Plant Sciences and Food Security, Tel Aviv University

2011-2016 Senior lecturer, Department of Molecular Biology & Ecology of Plants, Tel Aviv University

2008-2011 Post-doctoral fellow, Biodiversity Research Institute, University of British Columbia

Additional positions

2012– Head of the M.Sc. track in Theoretical and Mathematical Biology

2018– Board member of the Israeli Society of Bioinformatics

2021– Head of the B.Sc. track for excellent undergraduate students in the Life Sciences Faculty

Editorial Boards

New Phytologist, Methods in Ecology and Evolution, Genome Biology and Evolution

Supervision of graduate students and postdoctoral fellows

2011–Present 5 post-docs, 7 Ph.D., and 12 M.Sc. studetns all at Tel-Aviv University

Selected Grants (total of 8,500,000 NIS for own lab)

ISF (2012-1016, 2017-2021, 2021-2026), Marie Curie Reintegration Grants (2011-2015), NSF-BSF (2017-2020, 2020-2024), BSF (2014-2016), Ministry of Agriculture (2016-2019, 2020-2023), BARD (2018-2021), Israel Center of Innovation (2020-2023).

Selected publications (Google Scholar H index 40. 15,252 citations)

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Education

2003-2005	B.Sc.	Hebrew University of Jerusalem
2006-2010	Ph.D.	Hebrew University of Jerusalem

Employment

2018-present	Associate Professor, School of Plant Sciences and Food Security, Tel Aviv University
2013-2018	Senior lecturer, Department of Molecular Biology & Ecology of Plants, Tel Aviv University
2008-2011	Post-doctoral fellow, University of California San Diego

Selected academic and professional awards

Year	Name of Institution	Occasion
2011	Vaadia BARD Postdoctoral Fellowship	Binational Agricultural Research and Development Fund (BARD)
2011	Machiah Postdoctoral Fellowship	Postdoc excellence fellowship
2016	The Marker Magazine, Israel	[40 under 40], Most promising people in Israel under the age of 40
2017	Krill Prize – Wolf foundation	Excellence in research
2017	Tel Aviv University	Rector award for excellence in teaching.
2018	Member of the Israeli Young Academy	Contribution to science

Selected Grants

Year	Name of Agency (and collaborators)	Total	My use	Role
2014 - 2019	**ISF**	$520,000	$520,000	PI
2014 - 2016	**ISF** equipment	$240,000	$240,000	PI
2015 - 2018	**HSFP**	$1,050,000	$350,000	PI
2016 - 2018	**GIF**	€180,000	€84,000	PI
2016 - 2018	**MOAG**	$500,000	$100,000	PI
2017 - 2019	**F.I.R.S.T (BIKURA)**	$150,000	$75,000	PI
2017 - 2023	**ERC-SyG**	€1,500,000	€1,500,000	PI
2019 - 2024	**ISF**	$440,000	$440,000	PI
2020 - 2023	**ISF – NSFC**	$600,000	$300,000	PI
2020 - 2024	**HSFP**	$1,400,000	$350,000	PI
2023	**ERC-PoC**	€150,000	€150,000	PI

Editorial Boards

2020 - present, Associated Editor for the New Phytologist, IF 10.2. Handling over 30 manuscripts per year.

Supervision of graduate students and postdoctoral fellows 2013–Present 3 post-docs, 5 Ph.D., and 12 M.Sc. students, and 6 lab managers/technicians.

Selected publications (Google Scholar H index 21. 2,993 citations)

Tal I, Zhang Y, … and **Shani E**. (2016). The Arabidopsis NPF3 protein is a GA transporter. *Nature Communication*. 7: 11486

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30-1-23

To: The Zimin Institute for Engineering Solutions Advancing Better Lives.

I am happy to support the Proposal "Next-generation multi-targeted CRISPR genetic toolbox to reveal hidden traits and accelerate crop breeding" by Prof. Eilon Shani, and Prof. Itay Mayrose, School of Plant Sciences and Food Security, Tel Aviv University. I am confident that this multidisciplinary high throughput technology of maximizing genetic diversity, combining state-of-the-art bioinformatics and genome engineering tools, will enable plant scientists and breeders to enhance crop productivity.

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