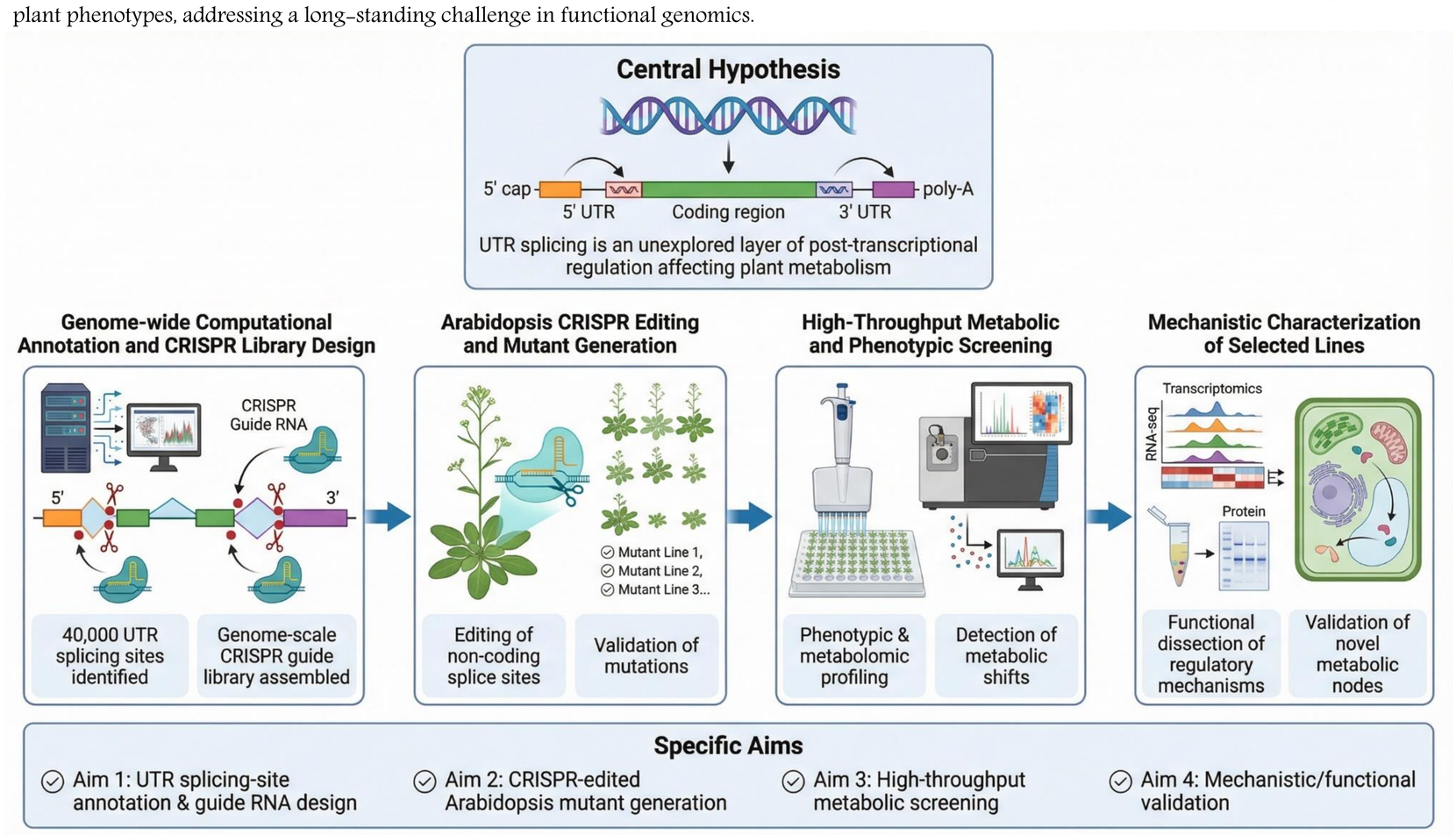
Genome-Scale CRISPR Interrogation of Untranslated Regions Reveals Regulatory Networks Shaping Plant Metabolism

E Aviv-Sharon[1], Y Hu[2], San Martin BD[1], Shachaf N[1], J Cai[1], E Shani[2], A Aharoni[1]

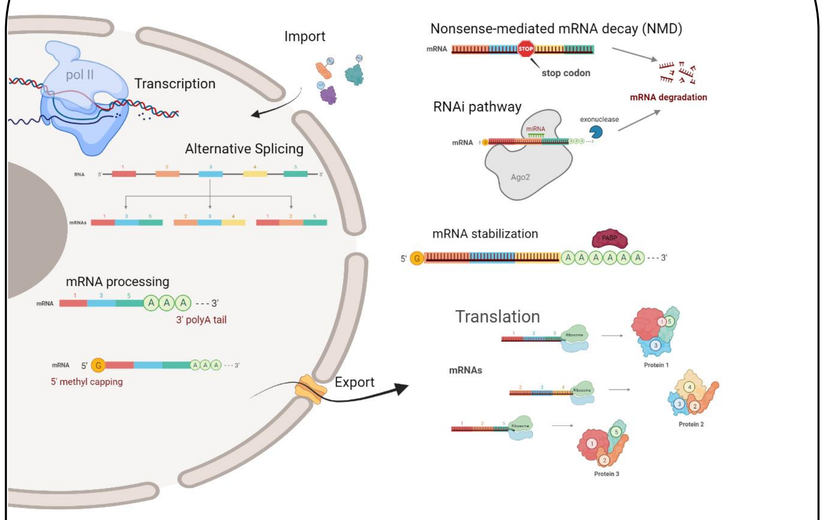
[1]Department of Plant and Environmental Sciences, Weizmann Institute of Science, Rehovot, 7610001, Israel;
[2]School of Plant Sciences and Food security, Tel Aviv University, Tel Aviv, Israel

Abstract

Plants continuously adapt to developmental and environmental cues through extensive transcriptional and post-transcriptional reprogramming. Untranslated regions (UTRs) of mRNAs serve as key regulatory hubs controlling transcript processing, stability, localization, and translation efficiency. Despite their central role in gene regulation, functional characterization of plant UTRs has largely remained limited to low-throughput, gene-by-gene studies. To systematically dissect UTR-mediated regulation, we generated a whole-genome CRISPR-Cas mutagenesis library targeting splice-related regulatory elements within 5′ and 3′ UTRs in *Arabidopsis thaliana*. We developed a computational framework, termed "UTRSplicer", for genome-wide identification and annotation of UTR splicing elements across the Arabidopsis transcriptome, identifying approximately 5,500 UTR-associated introns. Based on this annotation, we designed over 40,000 single-guide RNAs and generated more than 10,000 independent transgenic lines. To determine the functional consequences of UTR perturbations, we conducted large-scale high-throughput metabolomic profiling of approximately 900 edited lines, comprising over 6,000 metabolomic samples. Metabolic screening has been completed, and downstream computational and statistical analyses are currently underway to uncover novel metabolic rearrangements and regulatory networks associated with UTR-dependent regulation. To complement this unbiased discovery platform, we established targeted mutagenesis workflows for selected candidate loci and performed high-resolution amplicon sequencing of approximately 2,000 library-derived T-DNA lines to characterize editing outcomes. These datasets were further integrated to develop machine-learning models predictive of CRISPR editing efficiency and mutational outcomes in plant systems. Together, this work establishes a scalable platform for systematic functional interrogation of untranslated regions in plants and provides a unique resource for uncovering previously unknown links between post-transcriptional regulation and plant metabolism. More broadly, it enables genome-scale dissection of how untranslated regions shape plant phenotypes, addressing a long-standing challenge in functional genomics.

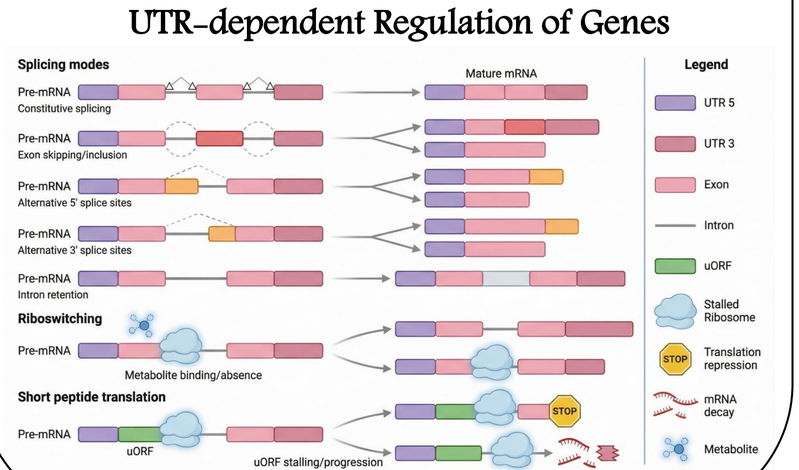


Background and Motivation



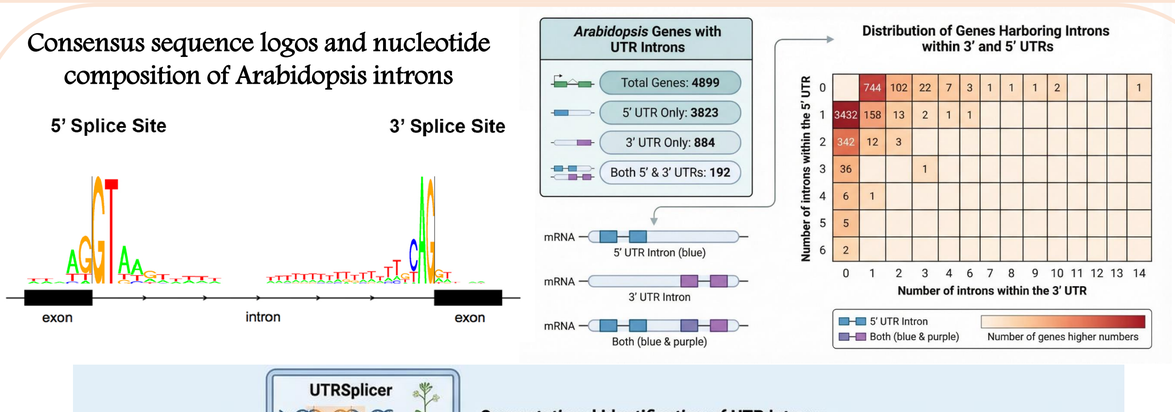
The 5′ and 3′ untranslated regions (UTRs) are key regulators of gene expression, influencing multiple stages of the mRNA life cycle. Despite their importance, many UTR-mediated regulatory mechanisms remain undiscovered..

UTR-dependent Regulation of Genes



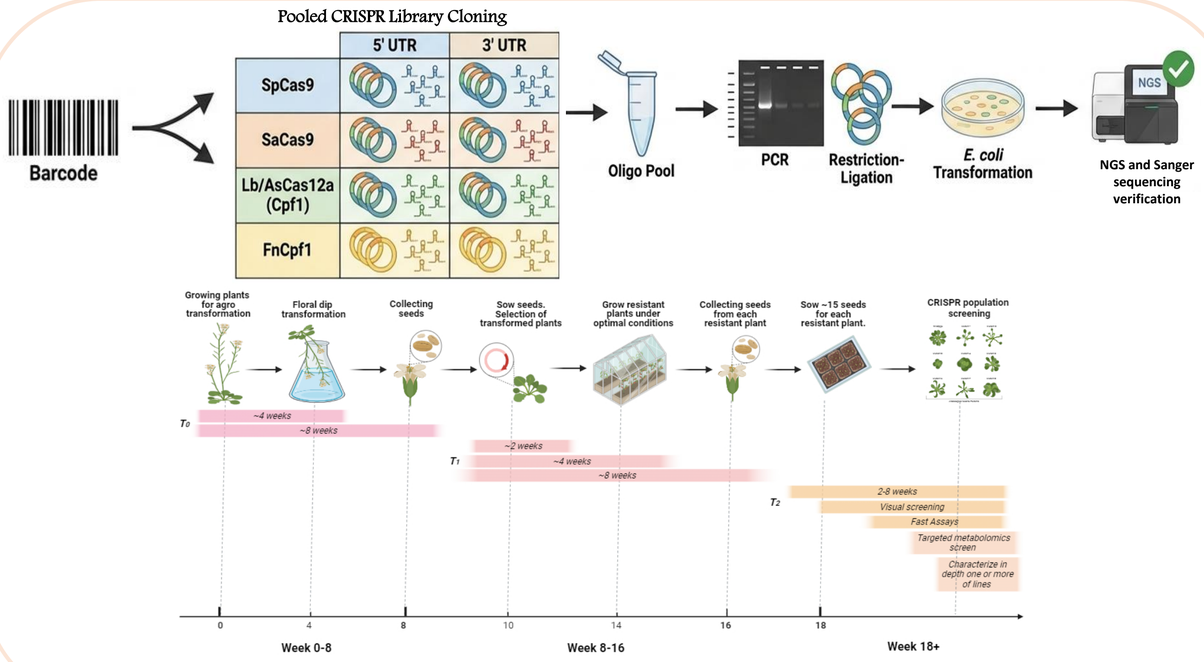
Results

1. Database construction, computational tools development and in silico sgRNAs design



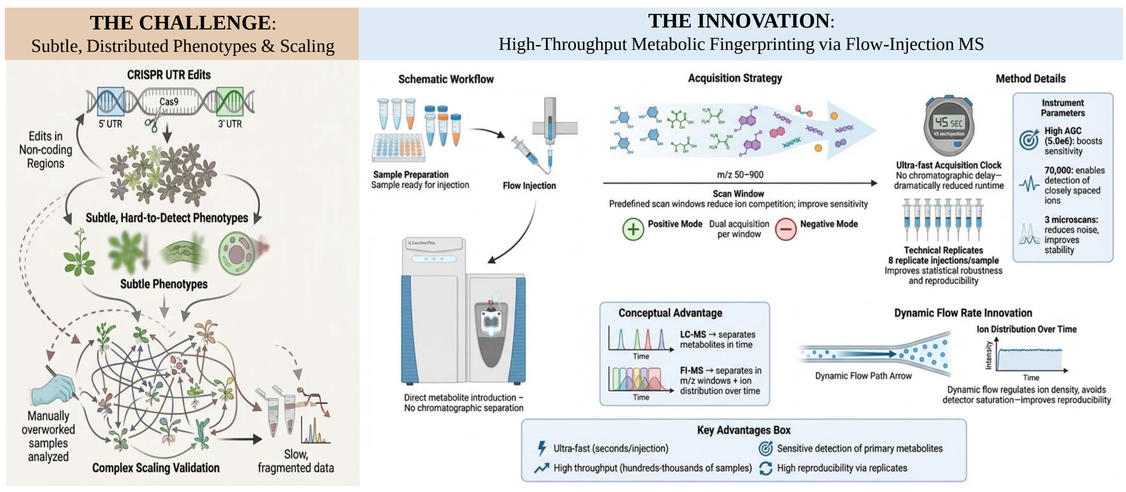
Cas enzyme	PAM	Number of guides
SpCas9	5'-NGG-3'	6,874
SaCas9	5'-NNGRRT-3' (R=A, G)	2,975
LbCas12a(Cpf1) AsCas12a(Cpf1)	5'-TTTV-3' (V=A, C, G)	5,448
FnCpf1	5'-TTN-3'	23,994
Total sgRNAs: 40,291		

2. Generation of an Arabidopsis Plant Gene Editing Population



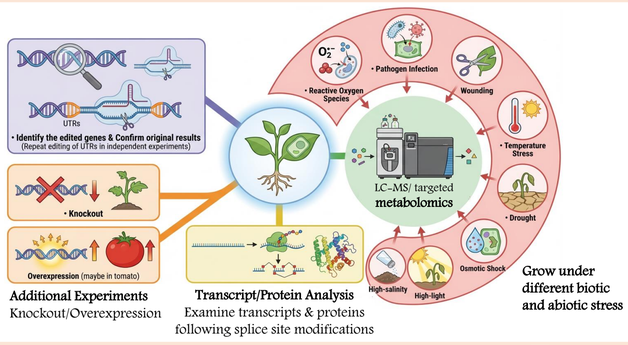
Construction, NGS validation, and Arabidopsis transformation of a pooled barcoded CRISPR library

3. Linking CRISPR Editing to Metabolic Function: Integrating HRFIMS-Based Screening

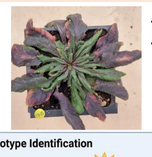


Regulatory edits, including UTR modifications, often generate subtle phenotypes that are difficult to validate at scale. Flow-injection metabolomics enables rapid, high-throughput detection of metabolic signatures linking genome edits to functional outcomes.

4. Characterize in Depth One or More Lines Showing Altered Metabolism/Phenotype

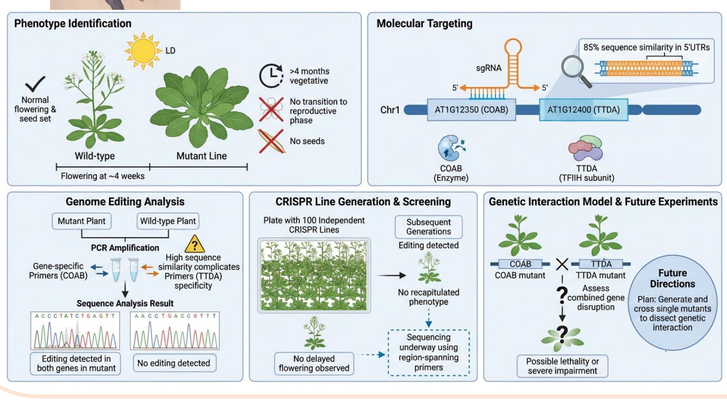


Candidate lines will be validated and characterized using targeted metabolomics, transcript/protein profiling, and stress-response assays to determine the functional impact of UTR editing.



Line SS2-1705: Unexpected Dual-Target Mutant Phenotype

CRISPR/Cas9 Dual-Gene Targeting Results in Severe Delayed Flowering Phenotype



A single sgRNA unexpectedly targeted two closely linked genes on chromosome 1: AT1G12350 (COAB) and AT1G12400 (TFIIH subunit), located only 23 kb apart. The original mutant showed delayed flowering and sterility, but the phenotype was not reproduced in independent CRISPR lines despite confirmed editing. Ongoing work includes long-range sequencing, generation of single mutants, genetic crosses, and targeting additional regulatory regions within the UTRs.

Toward Functional Decoding of Plant Regulatory Landscapes

By combining large-scale CRISPR editing of untranslated regions with high-throughput metabolomic profiling, this work establishes a powerful framework for uncovering hidden layers of gene regulation in plants. Beyond identifying novel regulatory elements, this approach enables functional exploration of non-coding genomic regions at unprecedented scale, opening new opportunities for understanding plant adaptation, metabolism, and crop improvement.