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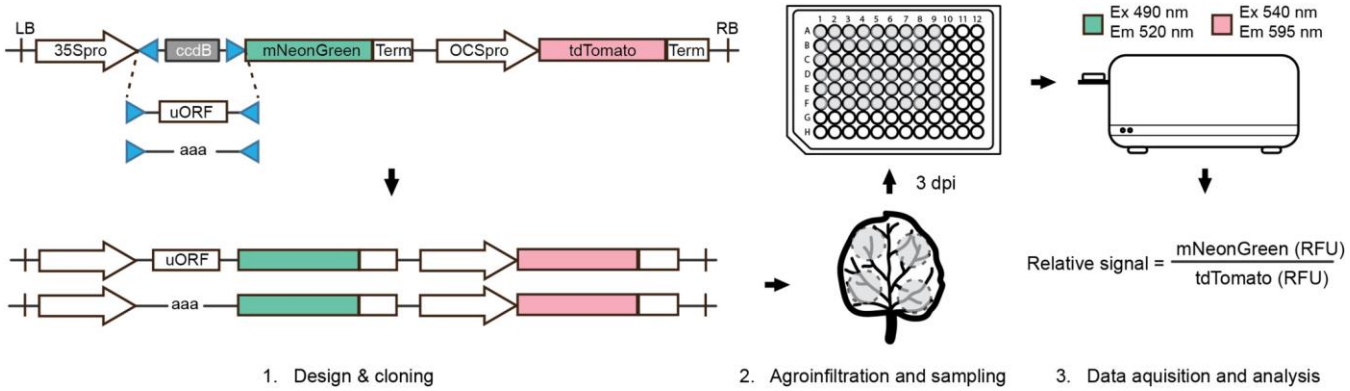
A Fluorescence-Based Transient Expression Assay for the Analysis of Upstream Open Reading Frames in Plants

Background/Objective

Upstream open reading frames (uORFs) are critical regulatory elements that control gene expression, offering a powerful tool for crop engineering via precise translational tuning. However, validation of predicted uORFs remains a bottleneck for engineering. Traditional validation methods, like luciferase assays, require reagent preparation and sample manipulation. Therefore, this study aimed to develop a high-throughput, fluorescence-based transient assay using *Nicotiana benthamiana* to rapidly characterize uORF function, facilitating the identification of targets for CRISPR-mediated genome editing to optimize traits in bioenergy crops.



A dual-fluorescence based assay for the evaluation of transcript leaders.

Approach

- Developed a modular dual-reporter system (mNeonGreen and tdTomato) using standardized Loop assembly for rapid, normalized vector construction.
- Employed *N. benthamiana* agroinfiltration for high-throughput transient expression and quantitative fluorescence imaging.
- Validated the system's sensitivity using known uORFs from *Arabidopsis* and *Lactuca sativa* to ensure accurate functional characterization.

Results

- Demonstrated that co-expression of mNeonGreen and tdTomato in *N. benthamiana* enabled selective quantification from excised leaf-discs in a monochromator-based plate reader. Dual-fluorescence normalization effectively accounted for biological variation and transformation efficiency across samples.
- Identified uORFs in photosynthesis genes from soybean and cowpea, facilitating the development of tunable gene expression.

Significance/Impacts

This research provides a streamlined and scalable platform for the functional validation of uORFs, effectively bridging the gap between genomic discovery and crop trait improvement. By enabling the rapid screening of regulatory elements, this tool accelerates the development of bioenergy crops with optimized metabolic pathways and enhanced resilience. Ultimately, this approach empowers researchers to use CRISPR-based editing to fine-tune native gene expression, providing a precise, non-transgenic path toward enhancing plant performance and cropping systems.

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