Understanding the Cell Type-Type Specific Response of Medicago Roots to Flood Stress

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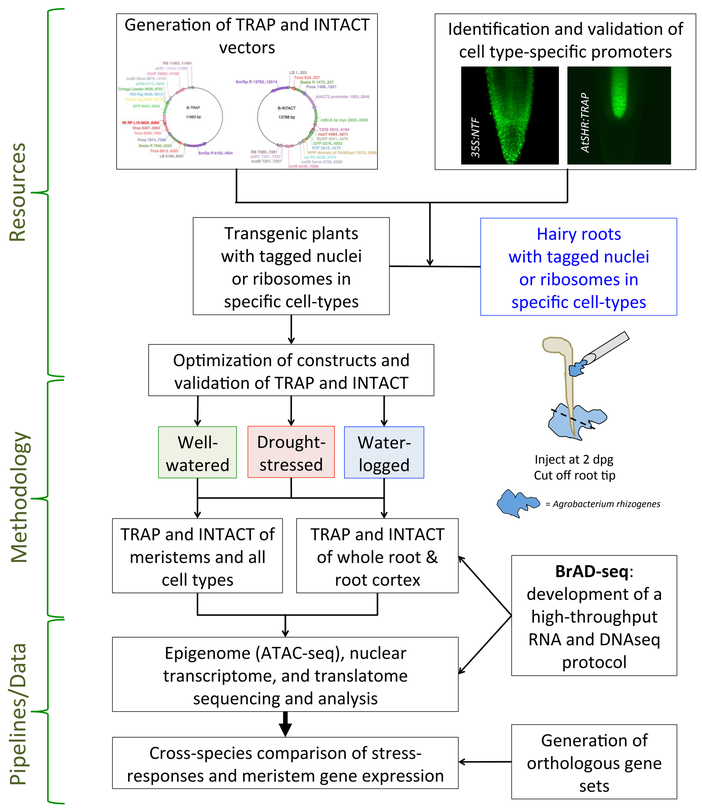
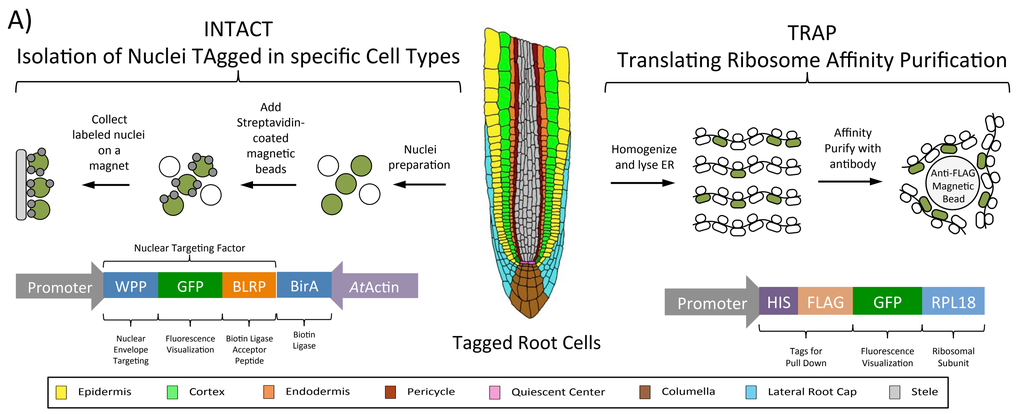
Abstract

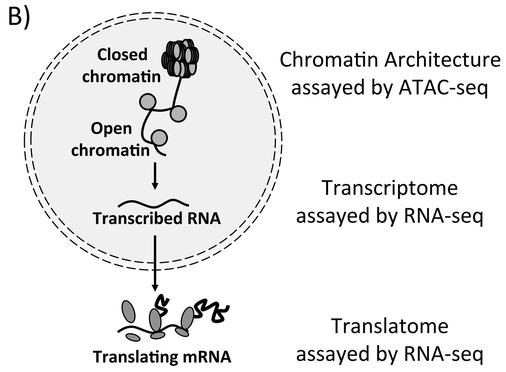
Plants are stationary organisms that must constantly respond to a changing environment in order to survive. Primary detection and response to flood stress occurs in the roots. However, how the specific cell types of the roots respond to these stresses is not completely understood. We have utilized two techniques, INTACT (Isolation of Nuclei TAgged in specific Cell Types) and TRAP (Tagged Ribosome Affinity Purification), to characterize the transcriptional and translational response of Medicago truncatula, alfalfa, roots to submergence stress. The nuclei and translating ribosomes are expressed in specific cell types of the root using *Arabidopsis thaliana* promoters. Our results show that all of the Arabidopsis promoters, with the exception of AtWOX5, had the same localized expression in *Medicago truncatula* as they do in *Arabidopsis*. We also show that INTACT and TRAP can be performed using *Medicago* tissue. We are currently characterizing the response of alfalfa root cells to 2 hours of submergence stress. Our results will be compared to parallel experiments performed in tomato and rice in order to identify conserved genes involved in flood stress response in crops. The long-term goal of this research is to establish a comprehensive understanding of drought and flood stress response in crops and to use this information to develop hardier crops.

Long Term Project Goals



Establishment of INTACT and TRAP lines in *M. truncatula* for isolation of nuclei and ribosomes from specific cell types





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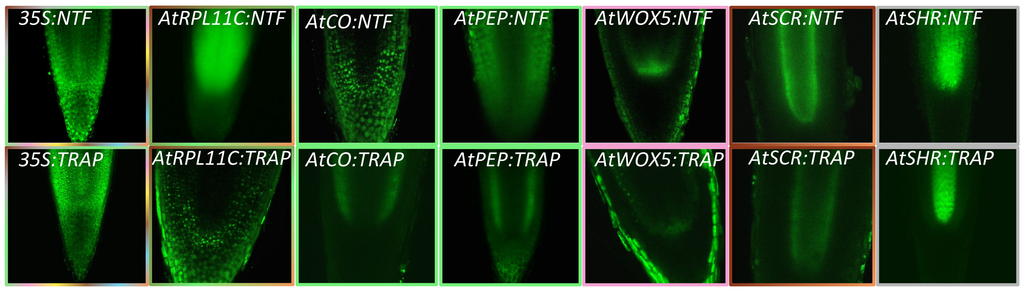

D)

Target Cell Type	Promoter
Near Constitutive	Cauliflower mosaic virus 35S (35S)
Root proliferating cells	Ribosomal Protein L11C (RPL11C)
Root endodermis, quiescent center	SCARECROW (SCR)
Root vasculature	SHORTROOT (SHR)
Root cortex meristematic zone	Cortex specific transcript (CO)
Root cortex elongation and maturation zone	Endopeptidase (PEP)
Quiescent center	WUSHEL-related homeobox 5 (WOX5)

A) The different cell types of the root are shown in the root diagram and labeled in the box below. The cell types that make up the root that are being targeted in this project are: Epidermis (yellow), Cortex (green), Endodermis (orange), Pericycle (red), Quiescent Center (pink), Columella (brown), Lateral Root Cap (light blue), and Stele (grey). A cartoon depicting the protocol for performing INTACT is shown on the left side of the root diagram and a cartoon depicting the TRAP protocol is shown on the right side of the root diagram. Under each cartoon depiction is a schematic depicting the transformed T-DNA that allows for each construct to be expressed in specific cell types.

B) From chromatin to translation, the molecular response of specific cells to stimuli can be studied in great detail by isolating nuclei and ribosomes using INTACT and TRAP, respectively. We are utilizing ATAC-seq (Assay for Transposase-Accessible Chromatin) to determine chromatin accessibility, and RNA sequencing for RNA isolated from nuclei and ribosomes to determine the transcriptional and translational response in the specific cell types.

C) Fluorescence visualization of GFAP was carried out using a fluorescent microscope and/or the Leica SP8 Scanning Laser Confocal Microscope. . Images shown are at different magnifications. All of the *A. thaliana* promoters show the expected localization and expression in *M. truncatula*, except for p*At*WOX5. The *At*WOX5 promoter should only express in the 2 cells of the Quiescent Center. However, in *M. truncatula* this promoter has ectopic expression throughout the Endodermis, Cortex, and Stele. Two lines imaged (p*At*RPL11C:TRAP and p*At*CO2:NTF) appear to be untransformed roots.

D) The *Arabidopsis thaliana* promoters listed were used to target expression of NTF or TRAP in the target cell types. The promoters listed were either already isolated or were amplified using primers reported in prior publications.

Figure 1: Nuclei Isolation Using *M. truncatula* Root Tissue Yielded High Purity

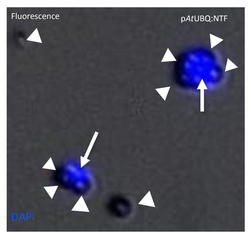

Figure 1: Nuclei isolation was performed using p*At*UBQ:NTF hairy root transformed tissue. a total of 100,000 nuclei were isolated with 9302% purity. Purity was calculated as the ratio of magnetic bead bound nuclei over the total number of observed nuclei. Arrowhead = magnetic bead, arrow = nucleus. DAPI was used to stain the DNA.

Figure 2: *Agrobacterium tumefaciens* Transformed Root Explants Have Yielded Adult Plants

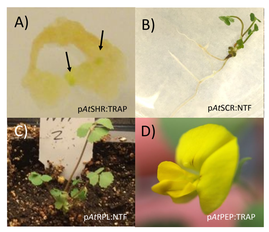

Figure 2: A) Callus obtained from *A. tumefaciens* transformed root explants. Arrows show green spots that will develop into seedlings. **B)** A seedling that has differentiated from a callus, ready to be planted. **C)** A planted seedling adjusting to soil. **D)** A flowering adult ready to form pods and produce seeds. T1 seeds have been collected from 6 lines. We are still working to establish the 10 remaining lines.

Figure 3: Validation of Common Submergence Conditions in *M. truncatula*

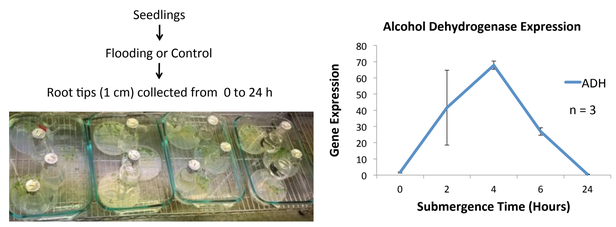

Figure 3: One-month-old *M. truncatula* seedlings were submerged under 3 centimeters of water for 0 to 24 hours. At each time point, 1 cm root tips were collected from 3 different plants. Alcohol dehydrogenase (*ADH*) mRNA levels, encoding an enzyme required for anaerobic metabolism, were quantified by qRT-PCR. Values were normalized to Ribosomal Protein Subunit L2 (*RPL2*). **The 2 hour time point was selected for inter-species comparison** of nuclear transcriptome, translatome and epigenetic regulation affected by the stress.

Figure 4: Chromatin accessibility in *M. truncatula* roots in response to submergence as determined by ATAC-seq

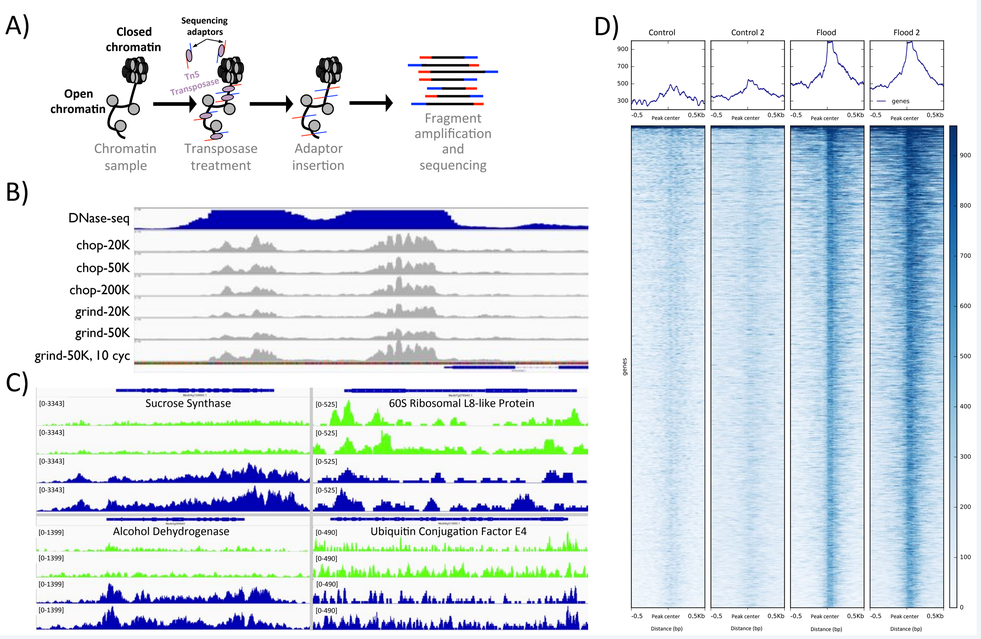

Figure 4: A) The hyperactive Tn5 transposase (violet), loaded with sequencing adapters (blue and red), inserts only in open chromatin regions (between gray nucleosomes) and generates sequencing library fragments that can be PCR-amplified. **B)** In *Arabidopsis thaliana*, 20,000 nuclei isolated from root epidermal cells are sufficient to make sequencing libraries with deep coverage. Our results show little difference between nuclei isolated from freshly chopped tissue and tissue that was frozen and then ground to a powder. Additionally, the sequencing reads obtained through ATAC-seq overlap with previously reported DNaseI hypersensitivity sequencing done in the nuclei of the same root cell type. Nuclei used to make the comparison between ATAC-seq and DHS were isolated from root non-hair epidermal cells. **C)** Libraries were prepared from 35S:NTF transformed hairy roots by collecting 1 cm root tips from plants that were either submerged for 2 hours (submergence stress) or grown under standard conditions for 2 (control). All four libraries shown had an average of 30 million reads. Chromatin accessibility is shown for four different genes. Open chromatin is expected for genes that are upregulated in response to submergence stress, such as Sucrose Synthase and Alcohol Dehydrogenase). Chromatin accessibility between the two sets of libraries should be the same in genes that do not respond to submergence stress, such as 60S Ribosomal L8-like Protein and Ubiquitin Conjugation Factor E4. Control libraries are shown in green and libraries from submerged samples are shown in blue. **D)** MACS2 was used to call peaks for the four different sequencing libraries shown. For all four samples a total of 40,000 unique peaks were called. 6,000 (15%) of these peaks show up only in libraries from submerged samples and are not present in control libraries. Deeptools was used to generate a heat map centered at the middle of each peak. The heat map is clustered with the most significantly different peaks between submergence and control samples are located at the top and less distinct peaks are found at the bottom.

Figure 5: Isolation of RNA from Purified Nuclei and Removal of rRNA

A)

Experiment #	Seedlings Injected	Survived Selection	Transformation Efficiency	Nuclei Isolated	RNA Yield (ng)
1	129	60	44 %	130,000	52
2	160	82	79 %	122,500	52
3	150	50	92%	57,000	37
4	150	54	70%	290,000	89
5	200	125	85%	160,000	64

B)

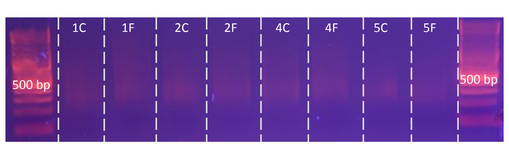

Figure 5: A) The submergence experiment was repeated at least 5 times. The injection protocol allows for efficient hairy root transformation that produces lots of transformed hairy roots in *Medicago* in 2-3 weeks. However, around 50% of the injected plants do not survive selection. Out of the survivors, around 80% of their hairy roots are transformed. The transformation efficiency is measured by collecting root tips and measuring how many of the have GFP signal. RNA was quantified using a spectrophotometer. On average, 0.44 picograms of RNA can be isolated from one nucleus. **B)** Ribosomal RNA was removed from the total RNA isolated from nuclei by treating the total RNA with rRNA DNA probes and then treating with DNase. The resulting libraries were amplified and 60% mapped to coding regions of the genome. Additionally, Duplex Specific Nuclease (DSN) was used to further remove rRNA from the mRNA and the resulting libraries mapped 75% to coding regions of the genome.

Current and Future Work

1. Isolate total and translating RNA from 35S:TRAP plants that have been stressed with 2 hours of submergence.
2. Sequence nuclear RNA and total and translating RNA from TRAP and compare.
3. Identify the locations and percentage breakdown of chromatin regions that “open” in response to submergence stress.
4. Compare ATAC and RNA-seq data to determine which open regions are transcribed, and which of these transcribed regions are translated.
5. Compare results to rice and tomato.

Conclusions

1. Except for p*At*WOX5, all the other *Arabidopsis thaliana* promoters showed similar expression in *Medicago truncatula* roots, compared to *Arabidopsis thaliana* roots.
2. Nuclei isolation using INTACT was successfully performed in *M. truncatula*.
3. Two hours of flood stress is sufficient to elicit a stress response in *M. truncatula* roots.
4. ATAC-seq can be performed with 20,000 nuclei from either freshly chopped or frozen and ground root tissue.
5. Fifteen percent of called peaks are unique to submergence stress.