Final Outcome Report

Constructing the Nitrogen Flux Maps (NFMs) of Plants

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Website: https://nfluxmap.github.io/

Nitrogen (N) is an essential element of organic molecules, such as amino acids, and hence is critical for overall metabolism and performance of all organisms. In plants, however, availability of N is highly variable in soil and often limited, unlike carbon (C) that can be captured from atmospheric CO_2 by photosynthesis. This limitation can be released by the application of N fertilizer, but this leads to a series of adverse environmental issues such as eutrophication and greenhouse gas emission. Therefore, it is critical to understand how different plants utilize N through **N metabolic networks**, so that we can identify key genetic components to optimize plant **N use efficiency**, especially for bioenergy crops grown on marginal lands. Yet, little is known how this fundamental N metabolic network evolved and function in plants and other organisms.

Aminotransferase (AT) family of enzymes play critical roles in distributing reduced N across different branches of the metabolic network for synthesis of various organonitrogen compounds. AT enzymes utilize two substrates—amino donor and keto acceptor—and often exhibit substrate promiscuity, which likely contributes to the complexity and diversity of N metabolic networks (Koper et al., 2022; Han and Yoshikuni, 2022). While many enzymes involved in specific AT reactions have been identified, the full spectra of AT multi-substrate specificity and their evolutionary divergence remain poorly understood. This limits our fundamental understanding of N metabolic networks that exist and operate in different organisms.

The **main objectives** of this project are to construct plant N flux maps (NFMs) from plant genomes and to determine functionality of AT enzymes and plant N metabolic network. To address this grand challenge, this project made use of rapidly growing numbers of plant genomes, high-throughput functional characterization platforms, and computational modeling to deduce both biochemical and systems level functionality of ATs and NFMs. The obtained NFMs will provide a novel framework to advance basic understanding of plant N metabolism and facilitate rational engineering of plants with high productivity even under limited N input.

To construct the framework of NFMs, we generated an N atomic map of *Arabidopsis thaliana* plant, based on metabolic models that include pathways in primary metabolism, including N compound-containing metabolic pathways (Huß et al., 2022). The utility of our automated workflow was further demonstrated by simulating ^{15}N-isotope enrichment and identifying metabolites which show enrichment patterns that are informative for estimation of fluxes for specific reactions in *A. thaliana* using metabolic flux analysis (MFA) (Huß & Nikoloski, 2023). To validate the NFM and estimate N reaction fluxes, ^{15}N-labeled precursor feeding has been set up using a hydroponic growth system and showed time-dependent incorporation of ^{15}N labeling in

major N-containing compounds (e.g., amino acids). After further optimization, the kinetic ^{15}N labeling data will be integrated into the N atomic map and be used to determine which reactions carry flux in specific conditions by using non-stationary ^{15}N-MFA at a genome-scale level. The resulting NFMs will serve as a novel framework to i) elucidate how N flows through the plant metabolic network in a quantitative manner, ii) simulate how plant metabolism responds to different N availability at a systems level, and iii) identify potential targets for improving N use efficiency.

The AT family enzymes likely evolved and already diverged before the last common universal common ancestor (LUCA). To determine evolutionary history of this ancient enzyme family, ATs and related sequences were collected from 90 representative species across five kingdoms (i.e., plant, animal, fungi, bacteria, and archaea) and protists and conducted deep phylogenetic analyses across the tree of life (ToL). The study revealed that the broad substrate promiscuity of ATs, which is unusual for core metabolic enzymes, allowed recruitment of distinct, non-orthologous ATs to carry out essential AT reactions in different taxa but without increasing their copy numbers (Koper and Han et al., 2024). To experimentally test multi-substrate specificity of AT enzymes, high-throughput AT enzyme assay platforms were established using nanostructure-initiator mass spectrometry (NIMS), which demonstrated broad substrate specificity of tyrosine and tryptophan ATs from *A. thaliana* (de Raad et al., 2023). The detailed kinetic analyses of these enzymes (Koper et al., 2023) further confirmed the findings from the NIMS assay. Additional analyses of class I aromatic amino acid ATs further revealed that some distantly related ATs exhibit a common signature of multi-substrate specificity by employing different non-conserved active site residues (Koper and Han et al., 2024). The established pipeline was further utilized to generate a global map of AT substrate specificity by generating ~forty AT enzymes and screening their enzyme substrates for over 100 amino donor and keto acid acceptor combinations in an unbiased manner (Koper et al., *in preparation*). The obtained map of AT substrate specificity revealed multi-substrate specificity of most AT enzymes and a number of new AT activities. Integration of the obtained biochemical data in the enzyme-constrained metabolic model of *A. thaliana* and *in silico* simulation further revealed that multi-substrate specificity of ATs contributes to growth phenotypes under different N environments. These results revealed that the versatile evolutionary trajectory of the promiscuous AT enzyme family likely led to biochemical diversity of the robust nitrogen metabolic networks that exist among various extant organisms. The findings provide a critical foundation to improve N use efficiency of crops.

References (Project publications to date):

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**Equal contribution.*