Comprehensive Characterization of Multi-Substrate Specificity of Aminotransferase Family Enzymes

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

Project goals: To comprehensively characterize multi-substrate specificities of all AT enzymes of *Arabidopsis* and *sorghum* through high-throughput mass spectrometry-based assays, and to integrate novel AT reactions to construct nitrogen flux map (NFM) of plants.

Abstract: Aminotransferases (ATs) are critical enzymes of plant **nitrogen (N)** metabolism that facilitate the transfer of amino N between biomolecules, namely amino acid donors and keto acid acceptors. Biochemical properties of many plant ATs have been tested with a certain substrate(s); however, the full spectra of **AT multi-substrate specificity** remain largely unexplored due to the tedious nature of AT characterization and poor sequence-function relationship. As a result, many cross-pathway N transfer reactions remain uncharacterized and unaccounted, making our understanding of plant N metabolic network far from complete.

To address these major knowledge gaps here we first generated a comprehensive phylogeny of AT enzyme families by utilizing fully-sequenced high-quality genomes of 15 species from each of six kingdoms, which identified a number of AT groups (i.e., well supported monophyletic clades, **Fig. 1A**). To further examine AT functionality, we successfully expressed and purified all 49 *Arabidopsis* AT candidates using *E. coli* and/or cell-free wheat germ expression systems (**Fig. 1B**) and validated previously-reported enzymatic activity for at least ~50% of them so far. To comprehensively examine their substrate specificity, we established high-throughput substrate screening methods using nanostructure-initiator mass spectrometry (NIMS) and matrix-assisted laser desorption/ionization MS (MALDI-MS) and determined substrate utilization profiles of eight ATs against 31 amino donors with 4 different amino acceptors (124 reactions for each). Even for ATs with well-established functions (e.g. AlaAT), we uncovered that many of them exhibit previously unknown reactions and multi-substrate specificities (**Fig. 1C**). Notably, we identified an unusual pattern of multi-substrate specificity among cytosolic aromatic ATs that allows them to utilize Phe, Tyr, Trp, Met, His, and Leu, which were further validated and quantified by LC-MS^2-based assays. These findings revealed the presence of a novel N network linking cytosolic pools of these six amino acids, which could be important for recycling N and rebalancing Phe and Met levels, such as during lignin biosynthesis (**Fig. 1D**). As we are completing the multi-substrate specificity screening for ATs from *Arabidopsis* and then *sorghum*, the obtained biochemical data are being integrated into the **nitrogen flux map (NFM)** that we are also developing in this project (see the other poster by Sebastian Huss *et al.*). The joint effort will discovery previously unknown AT-mediated N metabolic connections and thereby generate accurate NFMs of plants, which will guide us to identify engineering targets of N metabolic network to improve nitrogen use efficiency in crops.

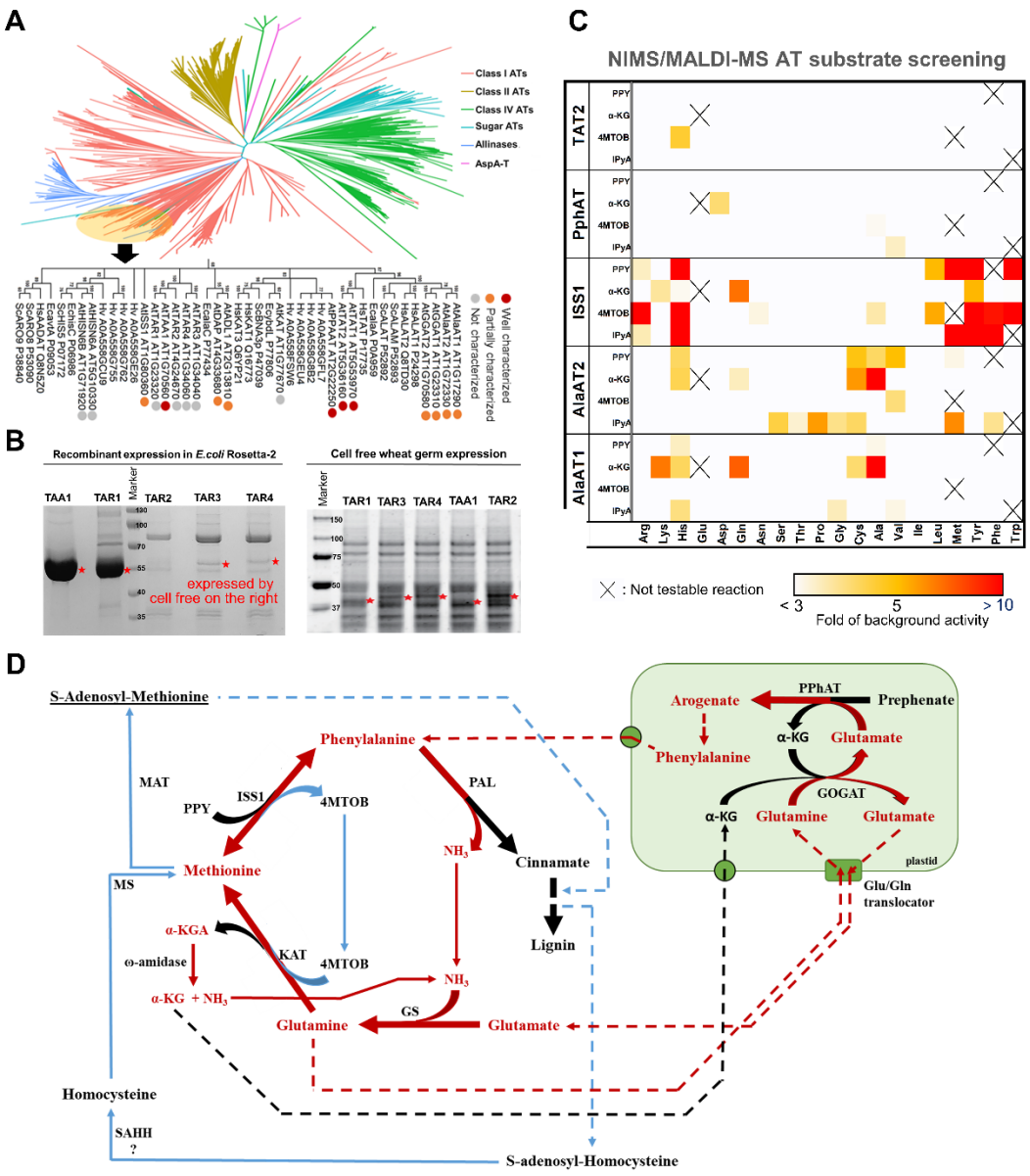

Figure 1: Functional characterization of Arabidopsis AT family enzymes. A. Phylogeny of ATs from 90 species of plant, animal, fungi, protist, eubacteria, and archaebacteria kingdoms, with the rectangular subtree highlighting a subset of AT clades from the representative model organisms – *Arabidopsis thaliana* (At), *Saccharomyces cerevisiae* (Sc), *Homo sapiens* (Hs), *Escherichia coli* (Ec) and *Halobacterium volcanii* (Hv). **B.** AT enzymes expressed recombinantly (left) and using the cell free wheat germ system (right). **C.** MALDI screening of multi-substrate specificities of five AT enzymes tested with phenylpyruvate (PPY), α-ketoglutarate (KG), 4-methyl-2-oxopentanoate (4MTOB) or indole-3-pyruvate (IPyA) as keto acid acceptors and twenty proteinogenic amino acids as amino donors. **D.** A new network of N rebalancing around phenylalanine biosynthesis and metabolism along with methionine and other amino acids (red). The *S*-adenosylmethionine (SAM) cycle is in light blue.

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