Exploring the structural assembly of rice ADP-glucose pyrophosphorylase subunits using MD simulation

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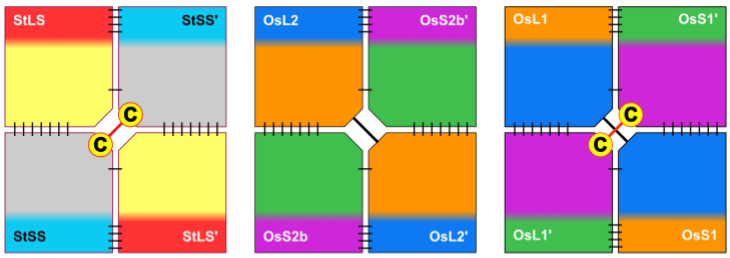
ABSTRACT

ADP-glucose pyrophosphorylase plays a pivotal role as an allosteric enzyme essential for starch biosynthesis in plants. The higher plant AGPase comparises of a pair of large and a pair of small subunits to form a heterotetrameric complex. Growing evidence indicates that each subunit plays a distinct role in regulating the underlying mechanism of starch biosynthesis. In the rice genome, there are four large subunit genes (OsL1-L4) and three small subunit genes (OsS1, OsS2a, and OsS2b). While the structural assembly of cytosolic rice AGPase subunits (OsL2:OsS2b) is known, no such study has been thus far reported for plastidial rice AGPase (OsL1:OsS1). In this study, we employed protein modeling and MD simulation approaches to gain insights into the structural association of plastidial AGPase subunits. The results demonstrate that the heterotetramic association of OsL1:OsS1 is very similar to that of cytosolic OsL2:OsS2b and potato plastidial AGPase heterotetramers. Moreover, the yeast-two-hybrid results, which resemble potato AGPase L1:S1, suggest a difference in protein conformation between OsL1:OsS1 and OsL2:OsS2b. Thus, the regulatory and catalytic mechanisms for plastidial AGPase (OsL1:OsS1) could be different in rice culm and developing endosperm compared to those of OsL2:OsS2b, which is found predominantly in rice endosperm.

Keywords: AGPase; rice plastid; starch biosynthesis; OsL1:OsS1 interaction; MD Simulation

Graphical Abstract



StLS:StSS OsL2:OsS2b OsL1:OsS1

Highlights

- Stronger ATP-binding with OsS1 than that of OsL1, evidenced from H-bond analysis.
- MD simulation predicted two stable OsL1:OsS1 AGPase complexes.
- Yeast-2-hybrid study suggest analogous pattern of OsL1:OsS1 association similar to potato AGPase heterotetramer.

1. Introduction

Plants rely on starch as their main source of energy and primary component. In photosynthetic tissues like leaves and stems, starch undergoes temporary synthesis and breakdown, whereas in storage organs such as grains, tubers, roots, and bulbs, it is stored in a more stable form [1-3]. Starch biosynthesis occurs via the coordinated actions of large number of enzymes including ADP-glucose pyrophosphorylases (AGPase; EC 2.7.7.27), starch synthases (EC 2.4.1.21), and starch branching enzymes (EC 2.4.1.18) as well as debranching enzymes. AGPase catalyzes the first rate-limiting step in starch biosynthesis where Glc-1-P and ATP are utilized for the synthesis of ADP-glucose (and inorganic pyrophosphate; PPi) [4]. Plant AGPases are regulated by a diverse range of metabolites, with 3-PGA serving as a main activator and Pi (inorganic phosphate) acting as an inhibitor of the enzyme [5]. The AGPase activity is additionally controlled by redox potential [6], light intensity and sugar levels [7], as well as concentrations of nitrate [8] and phosphate [9].

In higher plants, the AGPases function in a heterotetrameric form comprised of two large subunits (LS) and two small subunits (SS) [10-13]. It was previously believed that the LS and SS AGPase subunits served as regulatory and catalytic components of the enzyme, respectively [14]. However, recent comprehensive research suggests that both subunits contribute to both catalytic and regulatory functions, albeit to varying extents. The SSs were found to actively participate in both catalytic and regulatory functions, whereas LSs primarily contributing to controlling the regulatory properties of SSs, displaying lower catalytic properties [14-17]. The LS subunits play a crucial role in binding to the substrate (glucose-1 phosphate) and ATP, facilitating their subsequent interaction with the SS subunits [16, 18-20]. The collaborative interplay promotes and enhances the catalytic activity of the SS subunit. In the case of the potato tuber AGPase, heterologous expression of the SS subunits alone in bacteria forms a catalytically active homotetrameric enzyme ($StSS_4$). However, achieving maximal catalytic activity requires a considerably higher level of 3-PGA than required by the heterotetrameric enzyme [21]. In the Arabidopsis TL46 mutant line, which lacks LS, a homotetrameric small subunit AGPase is also formed. In contrast, the LS subunit is unable to form a homotetramer due to its insolubility. After introducing a mutation (S302N), the solubility of potato tuber LSs was greatly improved, enabling the formation of a homotetramer ($StLS_4$) but exhibiting marginal catalytic activity [20].

Plants contain two types of AGPase, one localized in the plastids and the other in the cytosol. In rice, the genome encodes four large AGPase subunits (OsL1-OsL4) and two small subunits (OsS1 and OsS2; where OsS2 further encodes two different isoforms through alternative mRNA splicing; OsS2a and OsS2b). The OsL2 and OsS2b isoforms are cytosolic while OsL1, OsL3, OsL4, OsS1 and OsS2a are plastidial in location [22-25]. The plastidial AGPase subunits, OsS1 and OsL1, are expressed during the early stages of endosperm development and plays a role in regulating phosphorus homeostasis [23]. On the other hand, the cytosolic isoforms, OsS2b, associates with OsL2 during endosperm maturation and are primarily responsible for the majority of starch synthesis during seed development [26].

In a previous study, we documented the interaction between cytosolic isoforms OsL2 and OsS2b and proposed the catalytic activities of OsS2b variants under heat stress [27]. However, to the best of our knowledge, no investigation has been conducted to date to explore the atomic-scale association of the OsL1:OsS1 heterotetrameric complex. Therefore, we employed structural bioinformatics approaches to explore the possible interfaces and crucial residues responsible for the assembly of the OsL1:OsS1 heterotetrameric complex. Through this analysis, we noticed similar mode of association between OsL1 and OsS1, which were corroborated by protein-protein interaction analysis. These findings not only contribute to a better understanding of AGPase mechanisms in rice but also present an opportunity to manipulate genes to enhance their activity, ultimately leading to the advancement of desirable agricultural traits in rice.

2. Materials and Methods

2.1. Homology modeling of rice AGPase sub-units:

The amino acid sequences of OsAGPase-L1 (OsL1; XP_015640472/ Os05g0580000 LOC_Os05g50380) and OsAGPase-S1 (OsS1; XP_015612224/ LOC_Os09g12660/ Os09g0298200) were retrieved from NCBI-protein database. Using MODELLER 9.17 [28], we generated three-dimensional models of the domain regions of OsL1 and OsS1. These models were created by employing the crystal structure of the potato AGPase SS (1YP3 [29]) as a template. In total, fifty 3D models were generated, and the representative models for each subunit were selected based on the lowest DOPE score. The random loop regions were subsequently remodeled using GalaxyLoop [30], and final refinement of the models was carried out using the GalaxyRefine

server [31]. Following the model refinement, the stereochemical properties and overall quality of the OsAGPase-L1 and OsS1 models were evaluated using PROCHECK [32], ProSA [33], ProQ [34], and Verify3D [35].

2.2. Molecular docking of ATP and modeling of OsL1:OsS1 heterotetrametric complexes

To investigate the binding of ATP, we followed the methods outlined in recent publications [18, 19, 27, 29] for conducting molecular docking. The ATP coordinates were integrated into the OsL1:OsS1 heterotetramer models derived from the potato AGPase SS structure (1YP3 [29]). To deduce the potential interfaces in forming the OsL1:OsS1 heterotetramer, we generated three OsL1:OsL1 heterotetrameric models (Complex-I, II, and III) using the crystal structure of potato AGPase SS homotetramer (1YP3 [29]) as a reference. Following the established procedures [11, 12, 27], we superimposed individual subunits of rice AGPase subunits (OsL1/OsS1) on to refecence structure to generate the heterotetramer complexes. The molecular docking of ATP and the modeling of heterotetrameric complexes were carried out using PyMOL, following the procedures described in our previous studies [27, 36-38].

2.3. Molecular Dynamics Simulation

In order to explore the structural and dynamic characteristics of the modeled complexes (OsL1/OsS1-ATP and OsL1:OsS1 heterotetramer), molecular dynamics (MD) simulations were conducted using GROMACS 5.1 [39] with CHARMM36 force field [40] and periodic boundary conditions. The topology parameters for ATP were generated using the SwissParam server [41]. The simulation systems were solvated using TIP3P water models and neutralized by adding Na^+ and Cl^- ions at a physiological concentration of 0.15 M to achieve electro-neutrality (Table. S1). Energy minimization of the systems were conducted using the steepest-descent method to avoid steric clashes and high energy interactions [42]. Particle-mesh Ewald (PME) method was employed to handle long-range electrostatic interactions [43]. Following energy minimization, the systems underwent position-restrained in two different phases; NVT (constant volume and temperature) and NPT (constant pressure and temperature) for 0.1 and 1 ns, respectively. Subsequently, the position-restrained monomeric systems (OsL1, OsS1, OsL1-ATP, and OsS1-ATP) were simulated up to 70 ns, and the heterotetrameric systems were subjected to 100 ns production run (in triplicate). In the C-III, we used the '-ss' flag in the gmx pdb2gmx command to

ensure that the disulfide bond between two OsS1 subunits remained intact during the simulation. Throughout, the temperature and pressure were maintained at 300 K and 1 bar, respectively. Trajectory analysis employed built-in GROMACS tools, utilizing gmx rms and gmx gyrate for backbone RMSD and Rg, and gmx hbond was used for computing intermolecular H-bonds as a function of simulation time. RMSD-based clustering analysis was conducted using gmx cluster. PDBe-PISA web interface was used to analyze protein-protein interactions [44]. PyMOL (https://pymol.org/) and ChimeraX [45] were used for structural visualization. 2D graphs were plotted in Grace 5.1.2 (http://plasma-gate.weiz-mann.ac.il/Grace/).

2.4. Yeast-two-hybrid Experiment

The cDNA sequences of the large (StLS) and small (StSS) subunits of potato tuber AGPase [46] and the large (OsL2) and small (OsS2b) subunits of rice endosperm AGPase [47] were amplified through PCR using gene specific primers (Table. S3) and cloned into yeast-two-hybrid vectors, specifically pGBKT7 and pGADT7 (Fig. S5). Following the preparation of cDNAs from immature rice seeds, the rice AGPase L1 (OsL1) and S1 (OsS1) subunits were amplified through PCR and subsequently cloned into the plasmid vectors. To perform protein-protein interaction experiments, a pair of plasmids carrying bait and prey sequences were co-transformed into *Saccharomyces cerevisiae* AH109 cells, according to the method described in [48]. Subsequently, the cells, which were grown on SD medium lacking leucine and tryptophane (SD/-Leu-Trp), were transferred to SD/-Leu-Trp-Ade-His media for testing the protein-protein interaction.

3. Results and Discussion

3.1. Three dimensional OsAGPaseL1 and OsS1 models

Domain analysis of OsL1 and OsS1 revealed an N-terminal loop-region followed by two structurally folded functional domains; catalytic domain and β-helix domain (Fig. 1A). Prior to 3D modeling, the N-terminal loop regions, OsL1 (1-73) and OsS1 (1-55), were removed and structurally folded regions were considered for model building. As displayed (Fig. 1B), both 3D models (OsL1 and OsS1) showed similar structural features with those of the potato AGPase SS (StSS) crystal structure (1YP3; [29]) with a minimum structural deviation (RSMD: 0.409 for OsL1 vs. StS1, 0.327 for OsS1 vs. StSS). The predicted models were energy optimized and validated

with their stereo-chemical parameters using several model validation servers (Table S1; Fig. S1) and considered for further structural study.

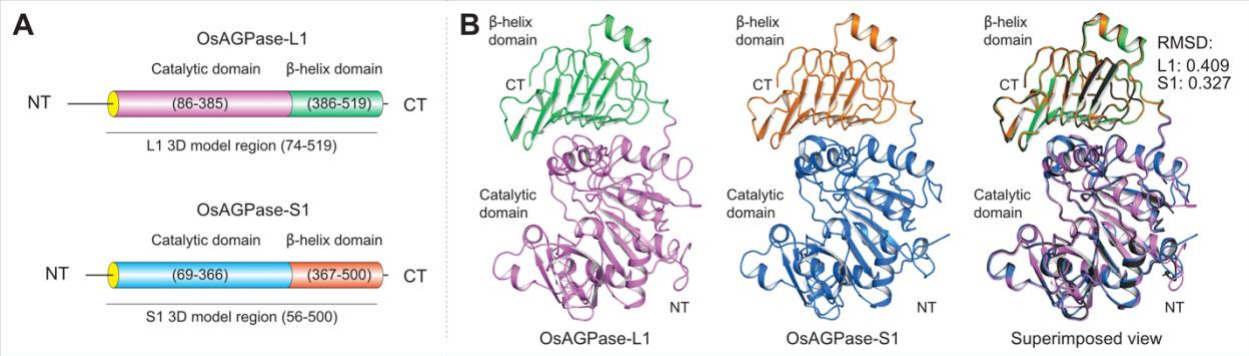


Figure 1. (A) Schematic overview of functional domains of rice AGPase-L1 and S1. The domains are displayed in colored cylinders [OsL1: catalytic domain (magenta), and β-helix domain (green); OsS1: catalytic domain (blue); β-helix domain (orange)]. N and C-terminal ends are represented by NT and CT, respectively. (B) Predicted 3D models of OsL1 and OsS1 and their superimposed view with potato AGPase SS structure with annotated functional domains.

3.2. Dynamic insights into interaction between rice AGPase-L1/S1 and ATP

To access the critical residues of OsL1 and OsS1 participating in ATP-binding, MD simulations of the ATP-docked complexes (OsL1-ATP and OsS1-ATP) were carried out. As shown in Fig. 2A, the AGPase subunits were found to be conversed (with backbone RMSD of ~3.5-3.7Å) just after the production run. The computed RMSD profiles of ATP (Fig. 2A) and conformational ensembles of the ATP-bound complexes (Fig. 2B) suggested a stable ATP-AGPase interaction during the course of simulation time. Furthermore, the nature of ATP-AGPase interaction was evaluated by computing intermolecular H-bonds and in-detail interaction analysis. The H-bond analysis revealed a higher number of H-bonds (7.42) in the trajectory of OsS1-ATP compared to OsL1-ATP (4.94) (Fig. 2C), suggesting a stronger binding of ATP to OsS1 than OsL1. This observation indicates a more stable interaction between OsS1 and ATP. To identify the residues essential for ATP-binding, we carried out RMSD-based cluster analysis and used best-clustered OsL1/S1-ATP coordinates for interaction analysis. As shown in Fig. 2D, both models showed 9-10 intermolecular polar contacts with several hydrophobic/electrostatic contacts. In particular, one aspartic acid residue in both subunits (D215; OsL1 and D194; OsS1) was found to form strong polar contacts with the sugar backbone; where the adenine moiety surrounded by several conserved residues (Fig. 2D). To assess the precision of ATP binding, we compared our findings with the

ATP-binding residues of potato AGPase subunits (Fig. 2E). The comparison revealed a high degree of conservation between the predicted ATP-binding residues of OsL1/OsS1 and their counterparts in potato [18], indicating the reliability and accuracy of our predictions.

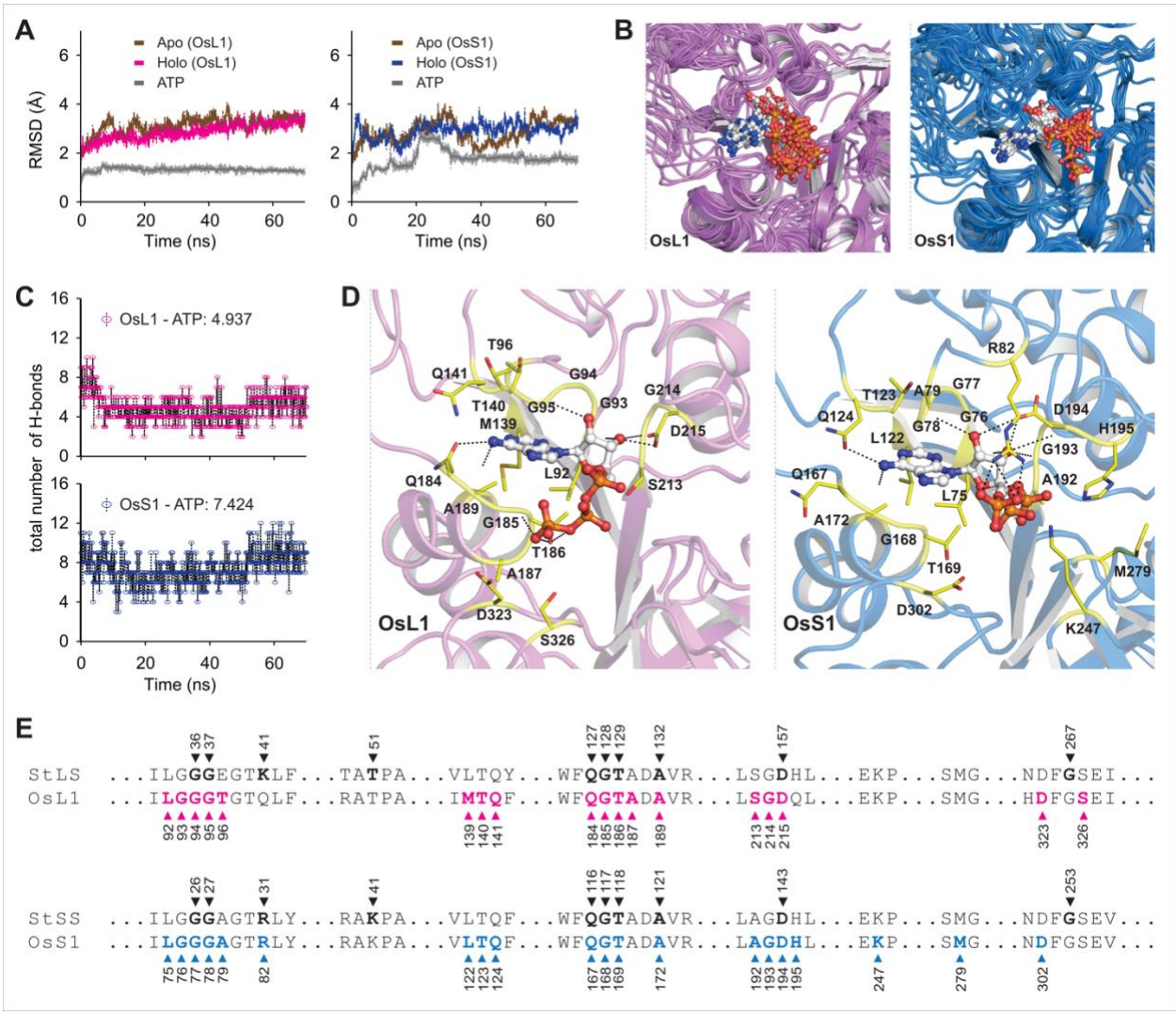


Figure 2. MD simulation of ATP-bound rice AGPase (L1 and S1) subunits. (A) Graphs represent RMSD profiles of rice AGPase backbone atoms and ATP. (B) Superimposed view of ATP-bound L1 (left panel) and S1 (right panel) snapshots (collected at 10 ns interval from respective trajectories), indicating dynamic stability of ATP in the binding cavity. (C) Total number of H-bonds formed between the AGPase subunits and ATP (OsL1-ATP in magenta and OsS1-ATP in deep blue) as a function of simulation time. (D) Comparative overview of in-detail molecular interactions observed. (E) Comparative overview of ATP-binding residues observed in potato (bold black fonts) and rice (colored-bold fonts) AGPase subunits.

3.3. Dynamic stability of designed OsL1:OsS1 heterotetramers

To gain a better understanding of the crucial interfaces and residues implicated in the interaction between OsL1 and OsS1, we initially constructed three hypothetical models of OsL1:OsS1 heterotetramers (C-I, C-II, and C-III; Fig. 3A). Subsequently, we performed molecular simulations for a cumulative duration of 100 ns (in triplicate) to investigate the dynamics of these complexes. After obtaining the MD trajectories, we analyzed the backbone RMSD and Rg of the complexes to gauge the complex stability and compactness. As shown in Fig. 3, the RMSD of C-II and C-III conversed after 10-15 ns of the production run (Fig. 3C, D), while the C-I showed RMSD with significantly higher deviations (upto 7 Å) (Fig. 3B, 3D). Similar to RMSD, we observed a more stable and conserved gyradius in C-II and C-III compared to C-I (Fig. 3E-3G). In addition, the conformational snapshots of pre- and post-MD snapshots of modeled heterotetramers (Fig. S2) indicated a stable association of the OsL1 and OsS1 subunits in C-II and C-III. To further understand the nature of interactions, an extensive H-bond analysis was carried out at various interfaces: (i) catalytic domain interfaces, (ii) β-helix domain interfaces, and (iii) N-terminal loop interfaces. As shown (in Fig S4), the H-bond analysis at homodimeric β-helix domain interfaces of C-I exhibit a lower numbers of H-bonds compared to the heterodimeric β-helix domain interfaces of C-II and C-III. This emphasizes the importance of interactions between β-helix domains as the primary forces guiding the association of AGPases. And the MD results suggest two different complexes C-II and C-III as the most stable complex.

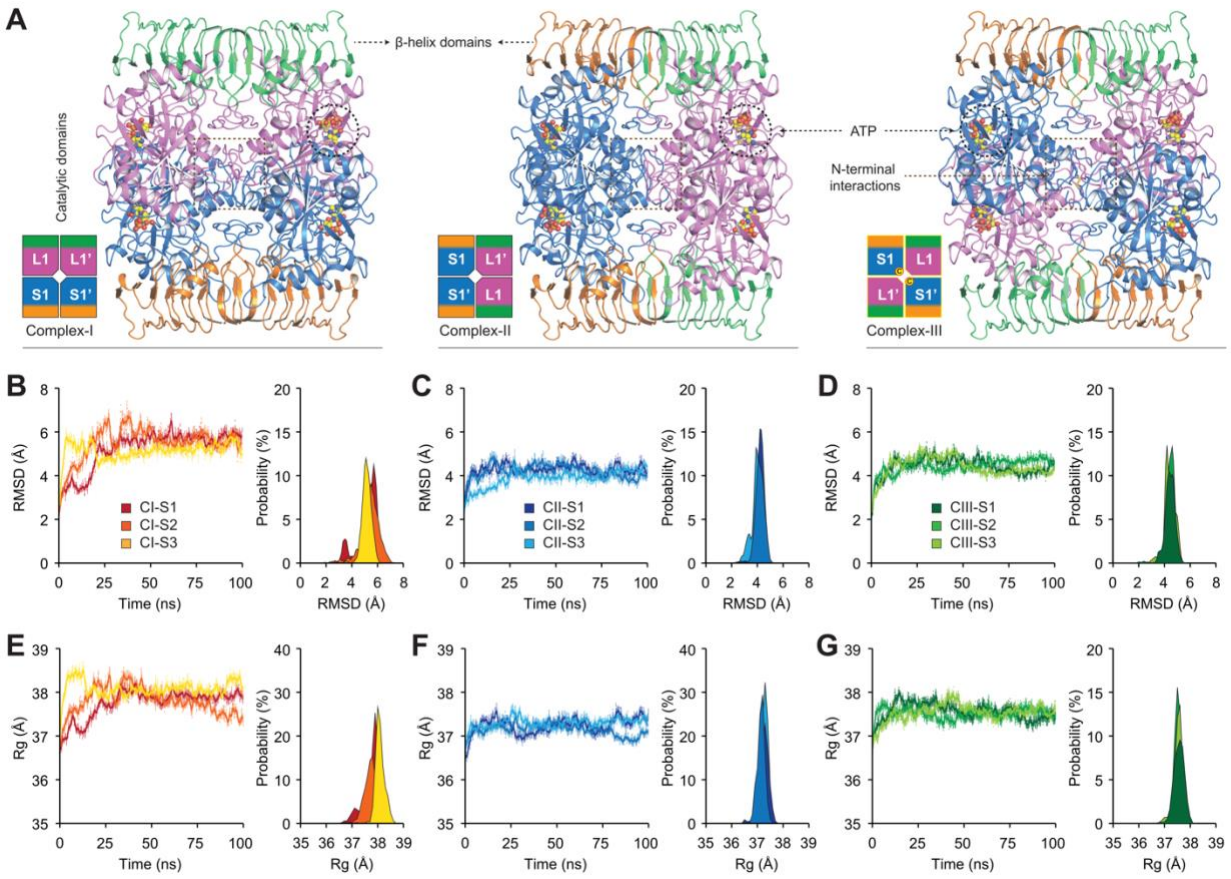


Figure 3. MD simulation of hetero-tetrameric rice AGPase complexes. (A) Overview of modeled hetero-tetrameric complexes (C-I, C-II and C-III) based on potato AGPase SS homotetramer (PDB ID: 1YP3) with highlighted functional domains, ATP-binding (black-dotted circles) and N-terminal loop interaction regions (brown square). (B-D) Comparative overview of backbone RMSD and (E-G) Rg profiles of OsS1:OsL1 heterotetramer complexes, computed from independent trajectories (T1-T3).

3.4. Association of rice AGPase subunits at different interfaces

Due to the lack of available structural data on the native forms (heterotetramers) of AGPases in higher plants, several previous studies have relied on homology models derived from the X-ray crystal structure of the potato AGPase SS homotetramer [29]. Based on the proposed side-by-side and up-side-down model for the heterotetrameric form of potato tuber AGPase [11, 12], similar modeling approaches have been employed for other plant AGPases as well. This approach has been applied to generate models for various plant AGPases based on the structural characteristics observed in the potato tuber AGPase [49].

To investigate the differences in the structural configuration among the models, a yeast-two-hybrid experiment was carried out (Fig. 4A). Reciprocal interactions were observed between OsL1 and OsS1 and between OsS1 and OsL1 of rice AGPase, while no homotypic interactions were observed between OsS1 and OsS1 or between OsL1 and OsL1. Similar interaction profiles were also observed for the potato AGPase StLS:StSS. We also observed that OsS2a, another rice small subunit of rice AGPase, did not interact with either itself or OsS2b (data not shown). Likewise, the cytosolic rice AGPase subunits (OsL2:OsS2b) exhibited reciprocal interactions. While no homotypic interactions were observed for OsS2b, a positive homotypic interaction was observed between OsL2. These findings suggest that the cytosolic rice AGPase possesses a unique structural configuration compared to plastidial rice AGPases, while exhibiting a similarity in configuration with potato AGPases. This validate that the C-III model is physiologically stable, rather than the C-II model. In a previous study [50], unprocessed sequences of OsL1 and OsS1 were used, whereas in our study, we excluded the chloroplast-transit peptides from the sequences to utilize the mature forms of the subunits instead. Nonetheless, the protein interaction profiles for both rice AGPases were consistent with those previously reported [50], suggesting the transit peptides in the sequence does not interfere with protein interaction in the yeast-two-hybrid system. To further elucidate the residual interactions and drive force occurring at the interfaces of homo- and hetero-dimeric complexes, we focused on the highest clustered coordinate from the CIII-T1 trajectory (Fig. S4). The interaction analysis displayed multiple-interface interactions between (i) two catalytic domain interactions, (ii) two β-helix domain interactions at heterodimeric interfaces and (iii) two homodimeric N-terminal loop interactions. At catalytic domain interface, we observed H-bonds between the residues R90, R132, D161, Y145 and A156 of OsL1 and D199, Y200, K192, Q174

and H152 of OsS1 (Fig. 4B). As shown (4C), the β-helix domain interaction majorly dominated by mainchain H-bond interaction networks and the N-terminal regions shows higly dynamic association during dynamics (Fig S6).

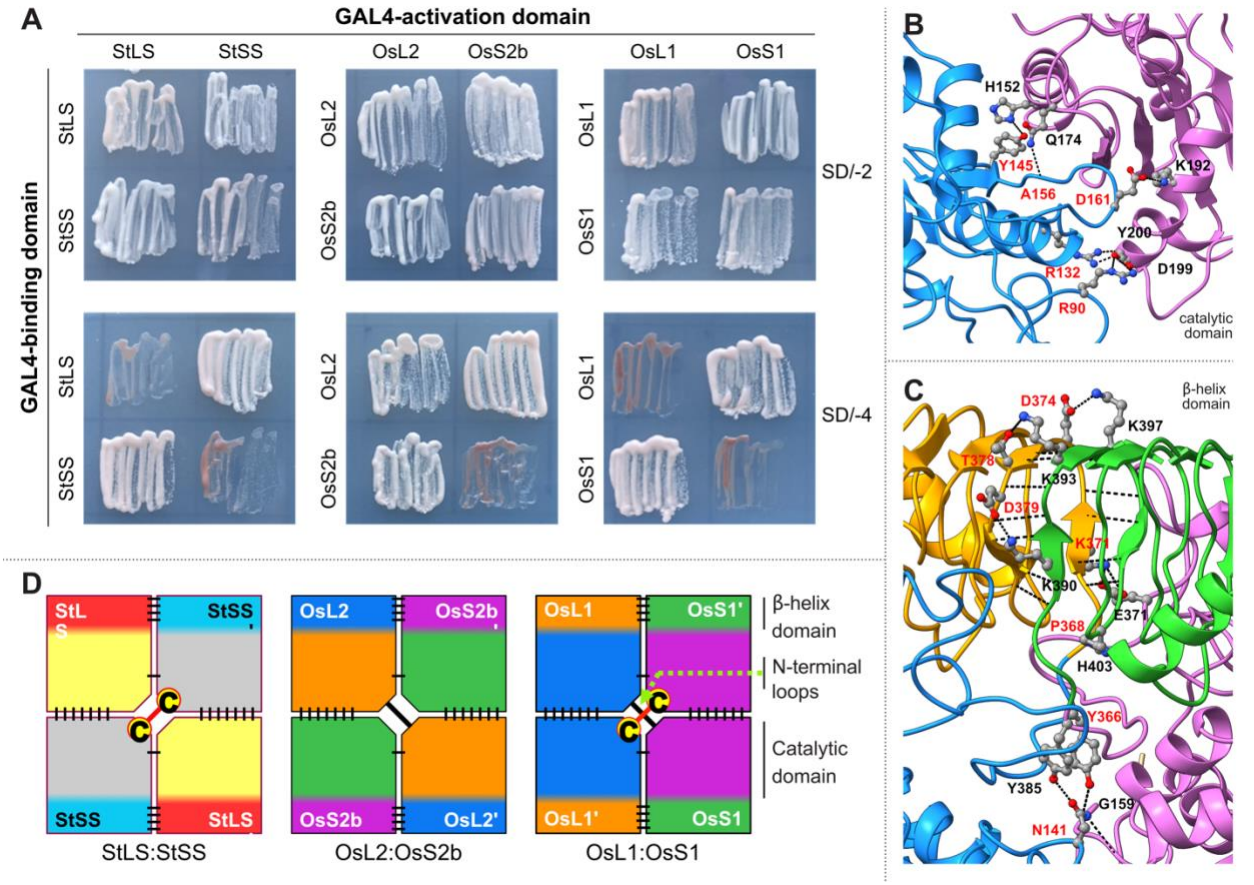


Figure 4. Overview of AGPase interactions. (A) Yeast-two-hybrid analysis of protein-protein interaction. The AGPase subunits were fused to GAL4-binding and GAL4-activation domains, which were then utilized as bait and prey, respectively. The bait and prey constructs were tested in synthetic growth media: SD/-2 (SD medium lacking L-leucine and L-tryptophan) and SD/-4 (SD medium lacking L-leucine, L-tryptophan, adenine, and L-histidine). (B) Molecular interactions between OsL1 and OsS1 at catalytic domain interface and (C) at β-helix domain interface. OsAGPase subunits are displayed in colored cartoons; interacting residues are displayed in gray ball-stick models and annotated with black (OsL1) and red (OsS1) fonts. Intra/intermolecular H-bonds are showed in black dotted lines. (D) Schematic representation of plant AGPase heterotetrametric complexes. The catalytic and β-helix domain regions are colored differently; cysteine residues are displayed in yellow circles and disulfide bonds in red line; and the interactions are denoted by black/dotted lines.

Tang et al. [50] conducted more analysis using truncated forms of the large and small subunits: catalytic and β-helix domains of AGPases OsL1:OsS1 and OsL2:OsS2b. The study revealed that the β-helix domain alone of OsL1 can interact with intact OsS1, and vice versa for OsS1 with OsL1. However, an intriguing observation emerged as the catalytic domain of OsL1 showed interaction with intact OsS1, whereas the catalytic domain of OsS1 did not exhibit any interaction with intact OsL1. This finding implies that the catalytic domains of OsL1 and OsS1 possess distinct configurations. Similar interaction profiles were observed for OsL2 and OsS2b.

In a previous study [51], it was observed that the LS:SS dimer of potato AGPase demonstrated greater stability when the two subunits interacted through their β-helix domains compared to their catalytic domains. Thus, it is evident that the primary interactions between the large and small subunits are mediated by their β-helix domains, while conditional interactions occur between the catalytic domains of the two subunits. Collectively, the proposed models and protein interaction analyses suggest that AGPase OsL1:OsS1 possess a similar structural configuration configuration to OsL2:OsS2b and StLS:StSS. The reason why the small subunits of rice and potato AGPases do not interact with each other in the yeast-two-hybrid system, despite the ability of potato small subunits to form a functional homotetramer [52], remains to be resolved.

In our previous study [27], we proposed the direct association OsL2:OsS2b based on a combination of experimental and computational methods. Furthermore, we demonstrated that the heterotetrametric association of cytosolic AGPases, OsL2:OsS2b [27], exhibits similarities to that observed in potato tuber AGPase [11, 12]. Nevertheless, we discovered a distinct type of heterotetrametric association of OsL1:OsS1. Unlike the previously observed patterns (up-down and side-by-side heterodimers), the OsL1:OsS1 subunits form up-down homodimers and side-by-side heterodimers, presenting a unique arrangement (Fig. 4D).

4. Conclusion

In summary, this study suggests a unique kind of interaction between plastidial AGPases subunits OsL1 and OsS1, where both the subunits form two up-side-down homodimers and two side-by-side heterodimers, which is structurally distinct from OsL2:OsS2b and potato AGPase heterotetramers. Thus, it can be speculated that the plastidial rice AGPases might have a unique

mode of underlying molecular mechanism in comparison to its counterpart OsL2:OsS2b, needs further investigation for its legitimacy. Largely, this study would help in the researcher to manipulate the plastidial AGPase for the development better rice traits.

Authors contribution: JM, SS and MKM formulated the idea and designed the experiment. JM performed the simulations, analyzed the data and prepared the figures. SKH conducted the yeast-two-hybrid experiment. JM, SKH, and DLS wrote the first draft. DP assisted in performing the simulations. TWO, SKH, DP, SS and MKM verified the analyzed data. All the authors finalized and approved the final version of the Manuscript.

Conflict of interest: Authors declare no conflict of interest.

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