A Structure Model Explaining the Binding Between a Ubiquitous Unconventional G-protein (OsYchF1) and a Plant-Specific C2-Domain Protein (OsGAP1) from Rice

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Abstract

The unconventional G-protein OsYchF1 plays regulatory roles in plant defense and abiotic stress responses. We have previously resolved the crystal structures of OsYchF1 and its plant-specific regulator, OsGAP1, and determined the residues on OsGAP1 that are essential for its binding to OsYchF1. In this study, we employed site-directed mutagenesis to identify four critical residues on the TGS domain of OsYchF1 that are critical for its binding to OsGAP1. We also generated a docking model of the OsYchF1:OsGAP1 complex to dissect the molecular basis of their interactions. Our finding not only reveals the roles of the key interacting residues controlling the binding between OsYchF1 and OsGAP1, but also provides a working model on the potential
regulatory mechanism mediated by a TGS domain, particularly in the class of GTPase of the OBG family.

Introduction

We previously solved the crystal structures of an unconventional G protein in rice, OsYchF1, and its regulator, OsGAP1, which is a C2-domain protein [1,2]. OsYchF1 belongs to a group of highly-conserved, ubiquitous P-loop NTPases [3], whereas OsGAP1 is plant-specific and could activate the ATPase/GTPase activities of OsYchF1 [4,5]. OsGAP1 was first characterized as a wounding-inducible gene in the Xa14 rice line (a line harboring the Xa14 resistance gene against the pathogen Xanthomonas oryzae pv. oryzae [Xoo]). The over-expression of OsGAP1 in transgenic rice lines led to enhanced resistance towards Xoo [4]. Through functional characterization of transgenic Arabidopsis expressing OsGAP1 ectopically, OsGAP1 was shown to participate in the well-known plant stress convergent pathway. The transgenic Arabidopsis demonstrated increased resistance towards Pseudomonas syringae pv. tomato DC3000 (Pst DC3000) with increased expressions of both salicylic acid-related (PRI and PR2) and jasmonic acid-ethylene-related (Thi2.1 and PDF1.2) defense marker genes. In addition, OsGAP1 ectopically expressed in the Arabidopsis npr1-3 mutant line did not show enhanced resistance upon Pst DC3000 inoculation, implying OsGAP1 is positioned upstream of the key plant stress response regulator, NPR1 [4]. Furthermore, OsGAP1 also plays a role in abiotic stresses. OsGAP1-transgenic Arabidopsis displayed increased salt tolerance with less leaf chlorosis, less reactive oxygen species generation and ion leakage, and higher induction of salt-inducible genes (RD22 and RD29a) upon salt treatment [5]. Meanwhile, OsYchF1 is a negative regulator of both plant defense and abiotic stress responses in plants, with OsGAP1 playing a positive role by down-regulating the biological functions of OsYchF1 [4–6]. OsGAP1 could inactivate OsYchF1 through stimulating its GTPase/ATPase activities and turning it into the inactive GDP/ADP-bound form. Besides, OsGAP1 could also regulate the subcellular localization of OsYchF1 by recruiting OsYchF1 to the plasma membrane from the cytosol upon wounding [6]. In addition, OsGAP1 was shown to compete for the binding site of 26S rRNA on the TGS domain of OsYchF1 [6]. Therefore, deciphering the molecular interactions between OsYchF1 and OsGAP1 is important in understanding the mechanisms of both plant defense and abiotic stress responses.
We previously showed that OsGAP1 binds to the TGS (Threonyl-tRNA synthetase, GTPase, and SpoT proteins) domain of OsYchF1 [6]. Based on the analysis of the primary structures of OsGAP1 plant homologs and using site-directed mutagenesis, we also successfully determined the amino acid residue clusters on one surface of OsGAP1 that are critical for the interaction with OsYchF1, and those residues critical for the interaction with phospholipids on another surface of OsGAP1 [2].

To identify hot spots on OsYchF1 that are also important for the interaction with OsGAP1, we applied the same strategy by aligning the primary structures of 30 OsYchF1 homologs from different plant species to identify the conserved residues that are likely candidates for the binding hot spots with GAP1 due to the structural conservation [7–10]. We specifically selected the conserved charged and aromatic residues for testing as they would be more electrochemically reactive. Using this approach, we successfully identified the residues of OsYchF1 that are responsible for the interaction between OsYchF1 and OsGAP1. By integrating all structural data, we proposed a high-confidence model to explain the molecular interactions between OsYchF1 and OsGAP1.

Materials and methods
Selection of the potential OsYchF1 amino acid residue candidates involved in binding OsGAP1
Using Protein-BLAST, 30 YchF1 homologs from various plant species were selected for alignment analysis by the Consurf server (http://consurf.tau.ac.il/) [7–10], taking into account the four OsYchF1 crystal structures (apo-OsYchF1 at pH 6.5 [PDB ID: 5EE0], apo-OsYchF1 at pH 7.85 [PDB ID: 5EE1], AMPPNP-OsYchF1 [PDB ID: 5EE3] and GMPPNP-OsYchF1 [PDB ID: 5EE9]) previously published by our group [1]. The most conserved amino acid residues in the alignment that are also presented on the surface of the protein molecule based on our 3-D structures, with side chains most likely to be involved in protein-protein interactions, were selected for site-directed mutagenesis. Targeted residues were mutated to alanine to minimize the possible effects on the overall protein structure.

Site-directed mutagenesis
The plasmid pGEX-4T-1-OsYchF (6kb), which contained an OsYchF1 cDNA fragment amplified from the cDNA pool of rice cultivars (SN1033 and JG30) in the pGEX-4T-1 vector,
was used as the mutagenesis template [6] following the QuikChange site-directed mutagenesis protocol (Agilent, United States). The primers were designed using the online QuikChange Primer Design program (https://www.agilent.com/store/primerDesignProgram.jsp) (Supplementary Table S1) and were synthesized by Invitrogen. The point-mutated fragments were amplified with Phusion™ High-Fidelity DNA polymerase (Thermo Fisher Scientific, United States). The PCR products were verified by electrophoresis in 1% agarose gel to be of the right size (6kb) and then were incubated with DpnI (New England Biolabs, United States) at 37 °C overnight. Five microliters of the final reaction were transformed into E. coli DH5α competent cells and selected on Luria–Bertani (LB) agar plates containing 100 mg/L ampicillin for positive transformants [11,12]. The positive transformants were verified by PCR and sequencing.

Mutants of the Arabidopsis homolog (AtYchF1) were made by site-directed mutagenesis via PCR. Primers were designed to replace the codon for the native targeted residue with that for alanine. Partial fragments with mutated sequences sharing overlapping ends were pooled and further amplified with primers for the full-length gene. Phusion™ High-Fidelity DNA polymerase (Thermo Fisher Scientific, United States) was used for all cDNA amplifications to minimize amplification errors. Primers, PCR cycling protocol and sub-cloning steps used in this study were reported in Supplementary Table S1.

Expression, purification of fusion proteins and in-vitro pull-down experiments
Native and mutant OsYchF1 constructs were subcloned into the pRSETA-HisSUMO vector for HisSUMO fusion protein expression in E. coli BL21 (DE3) cells. Protein expressions were induced by adding a final concentration of 0.5 mM isopropyl β-D-thiogalactopyranoside (IPTG) to the LB medium with 100 mgL⁻¹ ampicillin and grown at 20 °C overnight. Around 20 mL of bacterial culture of each fusion protein construct was used in the in-vitro pull-down experiment. For HisSUMO-tagged native and mutated OsYchF1 proteins, His Mag Sepharose™ Ni Magnetic Beads (GE Healthcare 28-9673-90) were used for purification and in-vitro pull-down. In brief, 100 μL magnetic beads were used to bind the expressed HisSUMO-OsYchF1 proteins in 20 mL of E. coli culture, followed by washing with equilibration buffer (20 mM sodium phosphate, 500 mM NaCl, 50 mM imidazole, pH 7.4) for three times. Before elution, around 10 μg in 30 μL purified DE3-expressed GST-OsGAP1 protein (purified with the MagneGST™ protein
purification system [Promega V8603]) was added as prey and the mixture was incubated at room temperature for 30 minutes. The magnetic beads were then washed with 1 mL equilibration buffer for three times before being eluted in 50 μL elution buffer (20 mM sodium phosphate, 500 mM NaCl, 500 mM imidazole, pH 7.4). The eluted pull-down products were subjected to western blot analysis with anti-GST antibodies for prey detection.

Meanwhile, GST-AtGAP1 fusion protein was applied as bait to examine the binding with the MBP-fused AtYchF1 and AtY104 mutants with the MagneGST™ protein purification system (Promega V8603). First, all fusion proteins were expressed in E. coli using the same protein expression procedure as above. One milliliter of cell extract in MagneGST™ cell lysis reagent (provided in the kit) obtained from 5mL GST-AtGAP1-expressing DE3 culture was allowed to incubate with 200 μL equilibrated MagneGST beads in 1 mL MagneGST™ binding/wash buffer (provided in the kit) as described in user manual. After three rounds of washing, GST-AtGAP1-bound MagneGST beads were used to pull down purified MBP-AtYchF1 or MBP-AtY104 mutant proteins, in which around 10μg MBP fusion proteins purified with SpinClean™ MBP Excelllose® spin kit (Mbiotech 23020) were added as preys. After three further rounds of washing with buffer, final elution was made in 50μL volume. Then, it was subjected to western blot analysis with anti-MBP antibodies for prey detection. All bait protein expressions and extraction efficiencies were examined beforehand to make sure even protein loading in the in-vitro pull-down experiments and western blot analyses. Most procedures were followed as described in the user manuals unless specifically stated above.

Transgenic plant materials
Native rice and Arabidopsis YchF1 homologs (OsYchF1 and AtYchF1) and their corresponding mutants (i.e., OsY104 and AtY104) that were unable to bind the respective rice and Arabidopsis GAP1 proteins were ectopically expressed in tobacco BY-2 cell suspensions, driven constitutively by the cauliflower mosaic virus 35S promoter in the binary vector, V7 or W104 [13]. The BY-2 transformation protocol was as described previously [14]. For the construction of transgenic Arabidopsis, the same binary construct was transformed into AtYchF1-knockdown mutant (from Arabidopsis Biological Research Centre [stock# CS855214]). Protocols of transformation and selection of positive transformants were as previously reported [4,6].
Salt treatment of BY-2 cells and cell viability staining with trypan blue
Salt treatment of BY-2 cell suspension was performed by cultivating the cells with Murashige and Skoog (MS) solution supplemented with 100 mM NaCl for 16 h at room temperature. The cells were then drawn into microcentrifuge tubes and stained with 0.4% trypan blue solution (Sigma-Aldrich T8154) for 10 min before being observed and photographed under a light microscope (Nikon E80i). Around 100 cells over 10 photos were counted for each sample. Cultivating the suspensions with MS only was used as the mock treatment.

Stress treatments of transgenic Arabidopsis
Salt treatment of transgenic Arabidopsis with native OsYchF1 and OsY104 ectopic expressions was performed by transferring 10-day-old seedlings grown on regular MS agar plates to new MS agar plates or MS agar supplemented with 150 mM NaCl. Expressions of salt stress marker genes (RD22 and RD29a) were examined with samples harvested one day after treatment. Phenotype characterization was performed after ten days of salt treatment by photo taking and chlorophyll measurement [15].

For pathogen inoculation test, 4-week-old Arabidopsis seedlings cultivated in a growth chamber (22–24 °C; 70–80% relative humidity; 80–120 microeinstiens light intensity on a 16-h light/8-h dark cycle) was inoculated with Pseudomonas syringae pv. tomato DC3000 (Pst DC3000). Pathogen titer determination at 3 days post-inoculation were performed using a plate count method [16]. Expressions of defense marker genes (PR1 and PR2) were examined with samples harvested three days after inoculation.

Gene expression analysis by reverse transcription-quantitative polymerase chain reaction (RT-qPCR)
RNA extraction, reverse-transcription and real-time PCR were performed according to a previous report [4]. The relative gene expression was calculated using the $2^{-\Delta\Delta CT}$ method [17] and normalized with the Arabidopsis thaliana UBQ10 gene (AY139999; [18]). Detailed primer information for real-time PCR were previously reported [5,6].

Statistical analysis
Quantitative analyses of BY-2 cell survival upon salt treatment, chlorophyll measurement, pathogen titer determination and marker genes expression of transgenic Arabidopsis upon treatments were performed using the Statistical Package for Social Sciences (SPSS; ver. 15.0). The treatment means were analyzed using one-way analysis of variance (ANOVA) followed by the Games-Howell post hoc test.

HADDOCK docking

The docking of OsGAP1 to OsYchF1 was performed using the data-driven docking server HADDOCK 2.4 (https://wenmr.science.uu.nl/haddock2.4/) [15]. The crystal structures of OsGAP1 (PDB ID: 4RJ9) and OsYchF1 (PDB ID: 5EE1) were used as the protein input templates. To generate the Ambiguous Interaction Restraints (AIRs) for HADDOCK, residues L5, L8, K37, K39, K41, R43, T58, and S60 were input as the active residues of OsGAP1, while E345 alone, or K325, H334, E345 and E354 together was/were input as OsYchF1 active residue(s). Passive residues that are close to the active ones were defined by the HADDOCK server automatically. All HADDOCK runs were performed with 1,000 structures for rigid body docking and 10 rounds of rigid body minimization. The best 200 structures were subjected to semi-flexible refinement. The refined structures were clustered using Fraction of Common Contacts (FCC) with a cut-off of 0.6 and a minimum cluster size of 6.

Molecular dynamics (MD) simulation

The crystal structure of OsYchF1 (PDB ID: 5EE1) and the HADDOCK docking structure of the OsYchF1:OsGAP1 complex were employed to initiate MD simulations. Missing residues in OsYchF1 were modeled using Modeller 9.23 [19] with its crystal structure resolved at pH=7.85 (PDB ID: 5EE0) as a template. MD simulations for the complex were initialized by superimposing OsYchF1 and OsGAP1 (PDB ID: 4RJ9) to the HADDOCK docking result. Four replicates of 1-μs MD simulations were performed using GROMACS 2020.2 [20] with the CHARMM36 force field [21] for OsYchF1 alone and the OsYchF1:OsGAP1 complex respectively. The proteins were placed in a dodecahedron box and solvated by TIP3P water [22]. Sodium chloride was added at a concentration of 0.1 M to neutralize the system. All systems were subjected to energy minimization followed by a 1-ns NVT equilibration and a 1-ns NPT equilibration with position restraints on the heavy atoms of the protein backbone. In all simulations, van der Waals interactions were smoothly switched from 8 Å to 9 Å.
interactions were evaluated employing the particle mesh Ewald (PME) method [23] with a grid spacing of 1.2 Å, a PME order of 4, and a cutoff of 9 Å. The system temperature was kept at 300 K using a velocity-rescaling thermostat [24], whereas the system pressure was kept at 1 bar by Berendsen’s algorithm [25]. All bonds with H-atoms were constrained using LINCS algorithm [26] to allow an integration time step of 2 fs. After simulations, atomic fluctuation of OsYchF1 was evaluated by the B-factor of each atom calculated from all four replicates. PyMOL 2.4.0 [27] was then used for the visualization of B-factor-colored structures.

Results

Amino acid residues in the TGS domain of OsYchF1 were responsible for the binding affinity towards OsGAP1 in in-vitro pull-down

By aligning OsYchF1 with homologs from 30 other plant species, and incorporating the knowledge from the four previously reported crystal structures of OsYchF1 ([1], Figure 1), highly conserved amino acid residues on the protein surface with active sidechains were identified using the Consurf server. Then the amino acid targets were narrowed down to those in the G domain, helical domain and TGS domain of the YchF1 protein (Figure 1). Eleven OsYchF1 mutants were thus generated with a total of 16 amino acid substitutions. Targeted residues were all mutated to alanine by site-directed mutagenesis (Figures 1 and 2A).

OsYchF1 mutants were expressed in E. coli as HisSUMO-fusion proteins. Purified proteins were then loaded onto His Mag Sepharose™ Ni Magnetic Beads as bait in in-vitro pull-down experiments. An equal amount of purified GST-OsGAP1 was added as prey in each pull-down experiment, followed by western blot analysis to detect the presence of GST-OsGAP1 in the pull-down product using anti-GST antibodies (Figure 2A). HisSUMO-native OsYchF1 and HisSUMO tag-only were included as positive and negative controls, respectively. Among the 11 OsYchF1 mutants, OsY100 (K325A), OsY86 (H334A), OsY104 (E345A) and OsY117 (E354A) lost the binding affinity for OsGAP1, compared against native OsYchF1 (Figure 2A-B).

Coincidentally, these residues are all part of the TGS domain. To examine whether the changes in protein-protein interactions were the results of perturbed protein folding of the OsYchF1 mutants, circular dichroism (CD) measurements were carried out. All the OsYchF1 mutants that lost OsGAP1 binding exhibited CD spectra similar to that of the native OsYchF1, indicating that
the mutations did not alter the overall three-dimensional structure of these proteins. Thus, residues K325, H334, E345 and E354 are likely located at the protein-protein interaction interface and mediate direct contacts with OsGAP1 (Supplementary Figure S1). However, only OsY104 (E345A) lost binding toward OsGAP1 alone while the other three mutants, OsY100 (K325A), OsY86 (H334A) and OsY117 (E354A), also lost binding toward other known interacting partners (unpublished data). Given its specificity, we focused on the Y104 (E345A) mutant for further functional characterization to illustrate the biological significance of mutating this residue on the interaction between YchF1 and GAP1.

To determine if this specific mutation also has the same effect on other YchF1 homologs, a corresponding Arabidopsis mutant (AtY104 [E345A]) was generated. The MBP-AtY104 mutant protein was expressed in E. coli, purified, and used as prey in *in vitro* pull-down experiments with GST-AtGAP1 as bait (Figure 2C). MBP-native AtYchF1 and MBP tag-only were included as positive and negative controls, respectively. Western blot analyses of the pull-down products using anti-MBP antibodies could not detect the MBP-AtY104 mutant protein (Figure 2C). Therefore, this specific glutamic acid residue at position 345 in both OsYchF1 and AtYchF1 appears to be responsible for binding the respective GAP1. Hence both OsY104 and AtY104 were employed to characterize the significance of GAP1 binding to YchF1 in stress responses.

OsY104 and AtY104 mutants with the E345A mutation resulted in enhanced sensitivities towards salt stress compared to native YchF1

OsY104 and AtY104 mutant constructs were constitutively expressed in tobacco BY-2 cells under a cauliflower mosaic virus 35S promoter. Salt treatment with 100mM NaCl in MS medium for 16 h was performed. Wild-type BY-2 cells, native OsYchF1 and AtYchF1 over-expressers were included for comparisons. Upon salt treatment, BY-2 cells ectopically expressing native OsYchF1 and AtYchF1 exhibited more sensitive phenotype when compared to wild-type cells, with significantly lower survivorship (Figure 3). However, BY-2 lines expressing OsY104 and AtY104 presented even more sensitive phenotypes when compared to their corresponding native-YchF1 expressers, with significantly lower survivorship (Figure 3).

Enhanced salt sensitivity and pathogen susceptibility in transgenic Arabidopsis ectopically expressing native OsYchF1 were previously reported [5,6]. In this study, we compared the
effects of salt stress and pathogen invasion on transgenic Arabidopsis ectopically expressing native OsYchF1 versus OsY104. To minimize the effect of endogenous AtYchF1 protein, transgenic lines were constructed in the AtYchF1-knockdown mutant background (Arabidopsis Biological Research Centre stock# CS855214). Three independent homozygous lines ectopically expressing OsY104 were obtained (named OsY104 A11, C14, and D16) and compared against the native OsYchF1-expressing line, also in the AtYchF1-knockdown genetic background. In the following stress responses characterization, the untransformed AtYchF1-knockdown mutant and its wild-type (Col-2) were also included as controls.

Salt treatment was achieved by transferring 10-day-old seedlings onto MS agar supplemented with 150 mM NaCl and mock treatment was carried out by transferring onto MS agar only. Salt stress symptoms could be observed after 10 days of treatment. There was more extensive leaf chlorosis in the transgenic lines with ectopic expression of OsY104 than those expressing native OsYchF1, which in turn exhibited more extensive leaf chlorosis when compared to the wild-type (Col-2). On the other hand, the untransformed AtYchF1-knockdown mutant displayed a salt-tolerant phenotype as previously reported [5], and showed the least amount of chlorosis compared to the other genotypes. The extent of leaf chlorosis under salt stress was quantified in terms of chlorophyll contents (Figure 4A-B). The expressions of salt-inducible genes (RD22 and RD29a) were quantified with RNA extracted one day after treatment by RT-qPCR, and these two genes were found to have minimal induction after salt treatment in the transgenic lines expressing native OsYchF1 and were even less induced in the lines expressing OsY104, when compared to the wild-type (Col-2). These genes, however, were highly inducible in the AtYchF1-knockdown mutant (Figure 4C). As a quality control, the levels of transgene expressions were examined and those of native OsYchF1 and OsY104 were found to be comparable (Supplementary Figure S2A).

To examine the defense responses of transgenic Arabidopsis ectopically expressing native OsYchF1 and OsY104 against Pst DC3000 infection, 4-week-old seedlings were inoculated with Pst DC3000 via syringe infiltration. Phenotype characterization including disease symptoms (Figure 5A), pathogen titer (Figure 5B) and expressions of defense marker genes (Figure 5C) was carried out at day 0 and day 3 of inoculation. OsY104-expressers exhibited enhanced susceptibility towards Pst DC3000 with more severe disease symptoms, higher pathogen titers
and failure to induce defense marker genes when compared to the native OsYchF1-expressers (Figure 5). Transgene expressions of native OsYchF1 and OsY104 upon Pst DC3000 were examined and comparable expressions were confirmed (Supplementary Figure S2B).

Docking of OsYchF1 to OsGAP1

Our mutational studies clearly indicated that the TGS domain, but not the G domain or the helical domain, of OsYchF1 is critical for OsGAP1 binding. We have previously identified two clusters of amino acid residues, namely L5, L8, T58, and S60 on strands $\beta$1 and $\beta$4, and K37, K39, K41, and R43 on strand $\beta$3 of OsGAP1, that serve as the determinants for the interaction with OsYchF1 (Figure 6A) [2]. Surface rendition of the crystal structure of OsGAP1 revealed that these two clusters lie on the same molecular surface, potentially serving as the interaction interface of the protein complex with OsYchF1 (Supplementary Figure S3). By combining this prior knowledge with the new results from the pull-down experiments here, we aimed to gain structural insights on how these key residues in the two interacting proteins contribute to the complex formation. We performed data-driven molecular docking using the HADDOCK 2.4 server to predict the structure of the OsYchF1:OsGAP1 complex [28]. The 3-D structural model of OsGAP1 was directly retrieved from PDB ID 4RJ9. The aforementioned clusters of amino acid residues were selected as the active residues that participate in the interaction. On the other hand, since the E345 of OsYchF1 is located at the TGS domain that does not undergo conformational changes upon AMP-PNP or GMP-PNP binding, we selected the nucleotide-free apo-protein (PDB ID: 5EE1) as the OsYchF1 model for the in-silico docking experiment. We selected either E345 alone or a combination of K325, H334, E345, and E354 as the active residue(s) to conduct different independent HADDOCK docking runs. All HADDOCK runs were performed with 1,000 structures for rigid-body docking with a fraction of common contacts (FCC) cut-off value of 0.6 for clustering.

Surprisingly, according to the cluster analysis using the highest HADDOCK scores (Supplementary Table S2), our docking simulations using either E345 alone (model 1) or a combination of K325, H334, E345, and E354 (model 2) as the active residues in OsYchF1 produced highly similar poses of the OsGAP1:OsYchF1 complex (Figure 6B-C), indicating the simulated binding mode is highly favorable. The two models were superimposed by the ProFit server (Martin, A.C.R., http://www.bioinf.org.uk/software/profit/) using the C$\alpha$ backbone of
OsYchF1, and the root-mean-square deviation (rmsd) of OsGAP1 between the two models was calculated to be 3.2 Å (Figure 6D). In both docking poses, OsGAP1 docks on the same molecular surface of OsYchF1 formed by the β-strands and the α12b and α14 helices of the TGS domain. Since a larger surface area is buried in model 2 than in model 1 (1766±78 Å² vs. 1605±120 Å²), we selected model 2 for more detailed analysis and this model is hereafter referred to as the complex model of OsGAP1:OsYchF1.

Nearly all selected active residues in our model are located at the interaction interface between the two proteins except K325 and E354 of OsYchF1 (Figure 7A). These two residues interact intra-molecularly with the side-chain of K366 and the carbonyl backbone of T373 at the loops flanking strand β11. Such interactions might be important for maintaining and stabilizing the conformation of strand β11 and Y367 which is in direct contact with OsGAP1 (Figure 7B). In addition, analysis of the crystallographic B-factors, which indicate the dynamic mobility of atoms, of residues within the loops and strand β11 of the OsGAP1 crystal structure revealed higher dynamics of the molecule in this region (Supplementary Figure S4), underscoring the potential need for stabilizing, a task fulfilled by K325 and E354.

Our model reveals that K37, K39 and K41 of OsGAP1 may play the most critical roles in the complex formation. Specifically, the side-chain of K41 forms ionic interactions with the key OsYchF1 residue, E345, and E358 of helix α14 (Figure 7C). The position of OsGAP1 within the complex is further stabilized by a hydrogen bond with the side-chain hydroxyl group of Y367 and van der Waals interaction with F383 of strand β12 (Figure 7C). The phenyl group of F383 also mediates van der Waals interaction with the side-chain of K39, the ε-amine group of which, along with that of K37, form ionic interactions with the side-chain of E314 of strand β9 of OsYchF1 (Figure 7D). On the other hand, L5, L8, and T58 on strands β1 and β4 of OsGAP1 appear to form a small pocket to accommodate the side-chain of H334 of OsYchF1 (Figure 7E).

We previously demonstrated that the binding of OsGAP1 activates the NTPase activity of OsYchF1, leading to the activation of defense and salt stress responses. Yet the current study revealed that the binding site of OsGAP1 is distal to the NTP-binding pocket of OsYchF1. We speculate that the effect of their interaction on the NTPase activity is likely mediated in an
allosteric manner rather than through direct alteration of the NTP-binding site (Figure 6D).

Comparison of the ATP and GTP binding affinities between native OsYchF1 and OsY104 using Mant-nucleotides also illustrated that OsY104 has NTP affinities comparable to those of native OsYchF1 (Supplementary Figure S5).

Discussion

We previously reported that the higher plant-specific OsGAP1 is a regulator of a ubiquitous ancient unconventional G-protein, OsYchF1, in response to biotic and abiotic stresses [2]. In order to study the biological effects exerted by OsGAP1 on OsYchF1, the OsYchF1 mutants that lose the affinity specifically towards OsGAP1 would be valuable genetic materials for functional characterization. In this report, we successfully identified several conserved amino acid residues on the surface of the OsYchF1 protein by taking both sequence homology and three-dimensional structure into account. Most importantly, mutations of these residues did not affect the protein folding of OsYchF1 (Supplementary Figure S1). Among all the residues tested, E345 is of the highest interest due to its specific and critical role in the interaction between OsGAP1 and OsYchF1. To examine its role in GAP1-binding in other plant homologs, AtYchF1, a dicotyledonous model plant YchF1 homolog, was mutated to generate the AtY104 (E345A) mutant. The AtY104 mutant also failed to bind AtGAP1 in the in-vitro pull-down study (Figure 2B). Thus, we believe that the conserved and solvent-exposed amino acid residue, E345, of the YchF1 protein is the most critical for its interaction with the regulator, GAP1.

To confirm this hypothesis, OsY104 and AtY104 mutants were ectopically expressed in tobacco BY-2 cells and subjected to salt treatment. Both OsY104- and AtY104-expressing transgenic BY-2 cell lines exhibited more salt-sensitive phenotypes when compared to the native YchF1-expressing counterparts, which in turn exhibited higher salt sensitivity than wild-type BY-2 cells (Figure 3). In planta experiments making use of transgenic Arabidopsis ectopically expressing native OsYchF1 and OsY104 also revealed the reduced salt tolerance resulting from OsY104 compared to native OsYchF1 (Figure 4). Besides, OsY104 ectopic expressers show more severe symptoms upon Pst DC3000 inoculation (Figure 5). The expression levels of stress response marker genes in OsY104-ectopic expressers were also much lower than those of the native OsYchF1-expressers upon stress treatments (Figures 4 and 5). This result implied that GAP1
binding is important for inactivating YchF1 in order to lower salt sensitivity and pathogen susceptibility in wild-type plants.

Technological advancements in genomic sequencing and mass spectroscopy in the past two decades have revolutionized the fields of plant genomics and proteomics. However, while the number of newly predicted or identified proteins continues to build exponentially, the functional and structural characterization of these novel proteins is significantly lagging behind. Therefore in most cases their biological roles remain elusive. In particular, structural studies of novel proteins using experimental techniques like nuclear magnetic resonance (NMR) spectroscopy, X-ray crystallography and cryo-electron microscopy usually require large investments of effort and time with no guaranteed success. Our approach of taking sequence homology and protein structure into account to hunt for amino acid residue(s) critical for interactions and combining the findings with data-driven docking simulations seems to be an effective and efficient strategy for functional and mechanistic studies of novel protein complexes. By aligning the sequence of OsYchF1 with 30 other plant homologs, 173 out of 394 amino acid residues were found to be identical among all 31 homologs. By superimposing conserved amino acids with the 3D protein structure, only those conserved amino acid residues exposed on the protein surface needed to be focused on. This greatly helped to reduce the number of candidates to be screened. In fact, four out of the eleven candidates tested either lost or showed significantly weaker interactions with OsGAP1. Such a high success rate not only helped to minimize workload and economize on materials and to quickly identify four hot-spot residues, it also minimized the efforts and time needed to obtain structural information on the protein complex by providing key information for high-confidence protein-protein complex docking.

In recent years, molecular docking techniques have served as important methods to elucidate the structural details of protein complexes that are difficult to be obtained by experimental techniques. The data-driven HADDOCK is one of the best-performing docking approaches due to its ability to integrate biochemical/biophysical experimental data during the docking process [16]. Using HADDOCK, we were able to generate a docking model of the OsYchF1:OsGAP1 complex to study the molecular basis of their interaction. Different docking attempts using either E345 as the only interacting residue on OsYchF1 or together with other conserved TGS domain
residues produced highly similar docking results, suggesting that the modeled binding poses are highly possible and favorable. The high complementarity between the TGS domain of OsYchF1 and OsGAP1 and the favorable van der Waals and electrostatic interactions also provide high confidence in the models (Supplementary Table S2).

Our docking model reveals the possible mode of interaction and explains the importance of the key interacting residues we have discovered in both OsYchF1 and OsGAP1, and provides a working model to explain how the binding of OsGAP1 might affect the NTPase activities of OsYchF1 despite the protein-protein interface being distal to the NTP-binding pocket. According to the model, the OsGAP1-binding surface of OsYchF1 is predominantly formed by the β-sheet of the TGS domain. Strand β3 within this TGS β-sheet directly connects to strand β4, which immediately precedes the G3/switch motif of the G domain of OsYchF1. Therefore, we speculate that the binding of OsGAP1 might induce conformational change(s) of the TGS β-sheet, which could allosterically affect the conformation of the G3/switch II motif and subsequently up-regulate the NTPase activity of OsYchF1. To test our hypothesis, we attempted to perform molecular dynamics (MD) simulation experiments to investigate how the binding of OsGAP1 affect the conformation of OsYchF1. Unfortunately, the complex dissociated during the first 300 ns of our 1-μs experiments, likely due to the weak and transient nature of the interaction between the two proteins. Clustering analysis based on the rmsd of OsGAP1 revealed that it made weak contacts with arbitrary sites on OsYchF1 after the dissociation event. Therefore, no obvious atomic fluctuation on OsYchF1 could be observed (Supplementary Figure S4). The TGS superfamily has long been suggested to play a regulatory role in NTP-binding or hydrolysis but their exact functions and mechanisms remain unknown. Although our MD simulation experiments could not provide further insight into the activation mechanism, our model provides new structural basis for future mutagenic studies to investigate the potential regulatory mechanism mediated by a TGS domain, particularly in the class of GTPase from the OBG family.

We previously reported that OsGAP1 is a wounding-inducible gene in bacterial blight-resistant rice lines harboring the resistant gene, Xa14, and its over-expression leads to enhanced resistance against *Xanthomonas oryzae* pv. *oryzae* in rice [6]. Over-expression of AtGAP1 also increased resistance towards *Pseudomonas syringae* pv. *tomato* DC3000 and enhanced salt tolerance in Arabidopsis [4,5]. These positive effects of the GAP1 protein upon biotic and abiotic stresses...
were exerted via its inactivation of the YchF1 protein [2]. Our previous studies implicated the roles of the OsYchF1-OsGAP1 pair in priming responses and systemic acquired resistance of plants under abiotic and biotic stresses. In this report, we provided important information on the structure-function relationship of this interacting protein pair and proposed a strategy that could dramatically speed up the studies of structure-function relationship of novel plant protein complexes to achieve better understanding of their cellular functions.

Authors’ contributions
HML coordinated and designed the strategy of this research. MYC led the site-directed mutagenesis experiments. JCKN completed the structure model. QJ and ZC participated in data generation and data analysis. YW, TL, and YG performed the molecular dynamics (MD) simulation experiment. MYC, JCKN, and HML wrote the first draft of this manuscript. All authors contributed to the final version of this manuscript.

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Conflict of interest
The authors declare no competing interests.

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Figure legends

Figure 1 Amino acid sequence alignments of OsYchF1 with orthologs from 30 different species using BioEdit [29]. Identical and similar residues were boxed. G-domain, TGS domain and helical domain are underlined in blue, purple and green, respectively. Red arrows indicate residues chosen for mutation to alanine in the corresponding OsYchF1 mutants.

Figure 2 In-vitro pull-down experiments to examine how the binding affinity of YchF1 towards GAP1 is affected by various mutations. (A) Table showing the detailed information of the eleven HisSUMO-tagged OsYchF1 mutant proteins used in the experiments. The last column on the right summarizes the results of the in-vitro pull-down experiments from panel B (“+”: with interaction, “-”: without interaction). (B) Both the native OsYchF1 and the eleven OsYchF1 mutants were each fused with a HisSUMO tag and used as bait by coupling to His Mag Sepharose™ Ni Magnetic Beads (GE Healthcare 28-9673-90) to pull down GST-OsGAP1 (the prey, 45.21kDa in size). Pull-down products were subjected to western blot, and the presence of GST-OsGAP1 was detected by anti-GST antibodies. Pull-down with HisSUMO tag-only as prey was included as negative control. (C) GST-AtGAP1 was used as bait to pull down MBP-fused AtYchF1 and AtY104 (the preys, ~86.9kDa in size), making use of the MagneGST™ protein purification system (Promega V8603). Pull-down products were subjected to western blot and the presence of MBP-fused prey was detected by anti-MBP antibodies. Results indicated that MBP-AtY104 failed to be detected in the pull-down product and therefore the interaction between GAP1 and YchF1 was specific. Pull-down with MBP-only as prey was included as negative control.
Figure 3 Tobacco BY-2 cells ectopically expressing the OsY104 or AtY104 mutant protein demonstrated that OsY104 and AtY104 enhanced the sensitivity towards salt treatment when compared to ectopic expressers of the native OsYchF1 and AtYchF1. (A) Wild-type BY-2 cell line, lines expressing native OsYchF1 or AtYchF1, and three independent lines each of OsY104 and AtY104 over-expressers were included in the study. Three-day-old cells were either cultivated in MS medium only, or in MS medium supplemented with a final concentration of 100mM NaCl. Treatment was carried out for 16 h and cell survivorships were evaluated by trypan blue staining and observed under light microscope. (B) Semi-quantitative analysis was performed by calculating the percentage of cell survivorship. Statistical significance was analyzed using one-way analysis of variance combined with the Games-Howell post hoc test. Error bars represent standard errors. Different lowercase letters above the bars represent statistically distinct groups at p<0.05. Two biological repeats with similar results were obtained.

Figure 4 Transgenic Arabidopsis ectopically expressing OsY104 showed increased salt sensitivity compared to those expressing the native OsYchF1. Ten-day old seedlings grown on MS agar plates were transferred onto MS plates supplemented with 150 mM NaCl (salt treatment) or MS-only plates (mock treatment). The various phenotypes of salt stress were recorded after 10 days of treatment. (A) Leaf chlorosis was present in all lines except the AtYchF1 knockdown mutant. (B) Chlorophyll pigment was quantified to measure the degree of leaf chlorosis. (C) the expressions of salt-inducible genes (RD22 and RD29a) were obtained from samples harvested after 1 day of salt treatment (error bars: standard errors of at least 3 samples). * By analyzing the CT values of RD22 and RD29a after normalization with AtUbi10, their expressions in the three OsY104 ectopic expressers were significantly lower than that of native OsYchF1 line after salt treated for 1 day. All transgenic lines were in the AtYchF1-knockdown genetic background. AtYchF1-knockdown mutant and its wild-type (Col-2) were included as controls. Statistical significance was analyzed using one-way analysis of variance combined with the Games-Howell post hoc test. Error bars represent standard errors. Different lowercase letters above the bars represent statistically distinct groups at p<0.05. Two biological repeats with similar results were obtained.

Figure 5 Pathogen inoculation with Pst DC3000 demonstrated that transgenic Arabidopsis ectopically expressing OsY104 exhibited higher pathogen susceptibility when compared to those
expressing the native OsYchF1. Four-week-old seedlings were inoculated with *Pst* DC3000 via syringe infiltration, and the phenotypes of disease symptoms were recorded 3 days after inoculation photographically. (A) Leaf yellowing and lesions could be observed in all lines except the AtYchF1 knockdown mutant after pathogen inoculation. (B) Pathogen titer (expressed in colony-forming units per unit area) was measured to show the degrees of pathogen susceptibility in different Arabidopsis lines. (C) RNA was extracted from samples harvested 3 days after inoculation and the expressions of defense marker genes (*PR1* and *PR2*) were determined via quantitative RT-PCR (error bars: standard errors of at least 3 samples). * By analyzing the CT values of *PR1* and *PR2* after normalization with *AtUbi10*, their expressions in the three OsY104 ectopic expressers 3-day after inoculation were significantly lower than that of native OsYchF1 line. Statistical significance was analyzed using one-way analysis of variance combined with the Games-Howell post hoc test. Error bars represent standard errors. Different lowercase letters above the bars represent statistically distinct groups at *p*<0.05. Two biological repeats with similar results were obtained.

**Figure 6** Overview of the OsYchF1:OsGAP1 docking model. (A) Domain organization and secondary structural elements of OsGAP1 and OsYchF1. Specific residues selected as the active residues for molecular docking are labeled. (B-C) Overall structures of the models obtained after performing HADDOCK docking simulations using either E345 only (B) or a combination of K325, H334, E345, and E354 (C) as the key residue(s) on OsYchF1. OsGAP1 is represented in light grey (B) and yellow (C) respectively. OsYchF1 is colored dark grey (B) and orange (C) respectively. (D) The structures from the two different models are superimposed on each other. The modes of interaction are similar in both models except for small changes in the orientation of OsGAP1. Residues selected as active residues during the docking simulations are indicated with blue sticks. A red dotted circle in the model on the left denotes the NTP-binding site. The OsYchF1:OsGAP1 interaction interface is distal to the NTP-binding site.

**Figure 7** Potential interactions between OsYchF1 and OsGAP1. (A) Overview of the interaction interface in the docking model of the OsYchF1:OsGAP1 complex. OsGAP1 and OsYchF1 are represented in yellow and orange, respectively. All the residues that are selected for conducting the docking are depicted in blue sticks. (B) Intramolecular interactions of OsYchF1 mediated by E354 and K325. (C) Interactions between K41 of OsGAP1 and the key OsYchF1 residue, E345,
and other potential interacting residues. (D) Interactions between the key OsGAP1 residues, K37 and K39, and OsYchF1. (E) H334 of OsYchF1 docks to a small pocket formed by the side chains of L5, L8, T58 and S60 of OsGAP1.
### Table A

| Construct name | Functional domain | Amino acids mutated to alanine | Retain OsGAP1 binding when compared with native OsYchF1 (+) |
|---------------|-------------------|-------------------------------|----------------------------------------------------------|
| 12            | Before G domain   | 16P                           | +                                                        |
| 22            | G domain          | 33P, 34N                      | +                                                        |
| 34            | G domain          | 55P, 56F, 58T, 61P            | +                                                        |
| 131           | Helical domain    | 129F                          | +                                                        |
| 7             | Helical domain    | 136H                          | +                                                        |
| 151           | Helical domain    | 141V, 142D, 143P              | +                                                        |
| 100           | TGS domain        | 325K                          | -                                                        |
| 86            | TGS domain        | 334H                          | -                                                        |
| 104           | TGS domain        | 345E                          | -                                                        |
| 117           | TGS domain        | 354E                          | -                                                        |
| 123           | TGS domain        | 387N                          | +                                                        |

### Figure B

**Prey:**
- GST-OsGAP1
- His-SUMO-OsYchF1

**Bait:**
- His-SUMO

**GST-OsGAP1**
- 7
- 12
- 22
- 34
- native

Detected by anti-GST antibody; 45.21 kDa

### Figure C

**Prey:**
- MBP

**Bait:**
- GST-AtGAP1
- MBP

Detected by anti-MBP antibody; 86.9 kDa
A

Wild-type

native OsYchF1 over-expressor

OsY104 over-expressor -1

OsY104 over-expressor -2

OsY104 over-expressor -3

native AtYchF1 over-expressor

AtY104 over-expressor -1

AtY104 over-expressor -2

AtY104 over-expressor -3

B

![Graph showing survival rates for different conditions](image)

- Wild type
- native OsYchF1
- AtY104_1
- AtY104_2
- AtY104_3
- native OsYchF1
- OsY104_1
- OsY104_2
- OsY104_3

Survivorship (%)
