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**A MOLECULAR DISSECTION OF THE RUBISCO
ACTIVATION SYSTEM FROM RICE**

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SCHOOL OF BIOLOGICAL SCIENCES

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ACTIVATION SYSTEM FROM RICE**

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School of Biological Sciences

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Abbreviations

2PG	2-Phosphoglycolate
3PG	3-Phosphoglycerate
AAA+	ATPase associated with various cellular activities
ADP	Adenosine 5'-diphosphate
APS	Ammonium persulphate
<i>A. tequilana</i>	<i>Agave tequilana</i>
ATP	Adenosine 5'-triphosphate
ATP _γ S	Adenosine 5'-thiotriphosphate
AtRca	<i>Agave tequilana</i> Rubisco activase
CA1P	2-Carboxy-arabinitol 1-phosphate
CABP	2-Carboxy-arabinitol-1,5-bisphosphate
CAM	Crassulacean acid metabolism
CCM	Carbon concentrating mechanism
CD	Circular dichroism
CO ₂	Carbon dioxide
Δ	Deletion
DTT	Dithiothreitol
<i>E.coli</i>	<i>Escherichia coli</i>
ECM	Carbamylated/activated Rubisco
ER	RuBP-inhibited Rubisco
EDTA	Ethylenediaminetetraacetic acid
EM	Electron microscopy
HEPES	N-(2-hydroxyethyl)piperacin-N'-2-ethanesulfonic acid
His ₆ Ub	Hexa-histidine tagged ubiquitin
IMAC	Immobilized metal ion affinity chromatography
IPTG	Isopropyl β-D-thiogalactopyranoside
LB	Lysogenic broth media
Mg ²⁺	Magnesium ion
MgCl ₂	Magnesium chloride
NaCl	Sodium chloride

NAD ⁺ / NADH	Nicotinamide adenine dinucleotide oxidized / reduced
NADP ⁺ / NADPH	Nicotinamide adenine dinucleotide phosphate oxidized /reduced
Native-PAGE	Native polyacrylamide gel electrophoresis
Ni-NTA	Nickel-Nitrilotriacetic acid
OD	Optical density
<i>O. sativa</i>	<i>Oryza sativa</i>
OsRca	<i>Oryza sativa</i> Rubisco activase
PCR	Polymerase chain reaction
PEP/C	Phosphoenolpyruvate/Carboxylase
PMSF	Phenylmethylsulfonylfluoride
Rbc	Rubisco
RbcL	Rubisco large subunit
RbcS	Rubisco small subunit
Rca	Rubisco activase
RT	Room temperature
Rubisco	Ribulose 1,5-bisphosphate carboxylase/oxygenase
RuBP	Ribulose 1,5-bisphosphate
SDS-PAGE	Sodiumdodecylsulphate polyacrylamide gel electrophoresis
SEC	Size exclusion chromatography
TAE	TRIS-acetate-EDTA
TEMED	N,N,N',N'-tetramethylethylenediamine
Tris	Tris-(hydroxymethyl)-aminomethane
TV	PGemT Vector
Ub	Ubiquitin
Usp2	Ubiquitin specific-protease 2
WT	Wild-type

Abstract

Ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco) forms inhibited complexes with its own substrate RuBP and other sugar phosphates. The AAA+ protein Rubisco activase (Rca) counteracts this issue by removing these inhibitors and thus maintaining Rubisco in its functional state. Inactivation of Rca at moderately high temperatures is associated with the loss of the activation state of Rubisco. Here we present a detailed characterization of the Rubisco activase from rice (*Oryza sativa*). We then compare its properties to a highly functional and thermostable Rca system from the desert plant, *Agave tequilana*. An extensive mutational study identified a number of residues that are likely involved in the Rubisco-Rca interaction. Finally we present a modified model for Rubisco activation in plants. Our results indicate that bioprospecting and careful biochemical characterization of Rca homologues will lead to both mechanistic insights and identification of candidate proteins for engineering enhanced thermotolerance in rice.

1. Introduction

The continuously growing human population, reduction of cultivatable land due to urbanisation, and global climate change are threatening our long-term global food security [1]. Collectively these factors represent major challenges for breeders and farmers to maintain food supply in excess of demand. The primary challenge in agricultural research is to increase crop productivity within the available area to sustain the demand of unsustainably growing population in the future. Past and recent efforts involved the green revolution, traditional breeding, genetic engineering, and so on. However, the productivity gains achieved through them are beginning to lag. An important approach to address this problem is to improve the natural efficiency of crop plant photosynthesis [2, 3].

Photosynthesis is the basis of life on earth which provides humanity with food, energy and materials. Ribulose 1, 5-bisphosphate carboxylase/oxygenase (Rubisco) is a key enzyme of photosynthesis, responsible for CO₂ fixation in the majority of photosynthetic organisms including crop plants. In spite of its important role in nature, Rubisco is a rather slow and confused catalyst [4, 5]. Its inability to differentiate between CO₂ and O₂ gives rise to a set of side reactions called photorespiration, which result in reduced photosynthetic efficiency [6]. Apart from this, Rubisco is also prone to inhibition by its own substrate RuBP (ribulose 1,5-bisphosphate) and other sugar phosphates [7]. In plants, this problem is alleviated by a molecular motor, the AAA+ ATPase Rubisco activase (Rca) [8, 9].

Rca performs remodelling of Rubisco and facilitates the release of sugar phosphate inhibitors in an ATP dependent manner. However, Rca is a thermolabile enzyme and this property is believed to be directly associated with the inhibition of photosynthesis at moderately high temperature [10, 11]. There is strong evidence that expressing more thermostable forms of Rca can lead to improved photosynthetic performance at supraoptimal temperatures [12, 13]. Due to such findings and its critical position in regulating the carbon fixation process, Rca is currently considered as target for crop improvement strategies [14, 15]. However, other opinions suggest that the deactivation of Rubisco at elevated temperature may instead be an important regulatory response to protect the plant during heat stress [16, 17].

The molecular mechanism underlying the thermolability of photosynthesis is of particular interest due to the predicted effects of global climate change [18]. Detailed

biochemical and structural characterization of the system's components will assist in clarifying these issues. A comprehensive mechanistic understanding of an agriculturally relevant Rubisco regulation system in plants like *Oryza sativa* will develop our ability to predict and modify the behaviour of their carbon fixation machinery. Ultimately, this will provide the new avenue for future crop improvement derived from such work.

1.1 Photosynthesis

Photosynthesis is the process of conversion of light energy into chemical energy, resulting in the formation of organic compounds from carbon dioxide and water using energy from the sun. It is one of the fundamental processes on earth, performed by plants, algae and some specialised bacteria [19]. It is not only a source of energy for autotrophs but also for all other non-photosynthetic organisms including ourselves that are unable to produce organic materials from inorganic carbon sources. Thus, almost all living beings directly or indirectly depend on photosynthesis for their survival.

Photosynthesis is a compartmentalized process that takes place in the chloroplast of plants and consists of the light reactions (photosynthetic electron transport) which take place in the thylakoid membranes and the dark reactions (photosynthetic carbon metabolism) in the stroma [20]. During the light reactions, light energy from the sun is captured by chlorophyll and converted into chemical energy in the form of NADPH and ATP, with the liberation of molecular oxygen. The dark reactions, also known as the Calvin-Benson cycle utilize these molecules for the carbon fixation and production of carbohydrates (sugars).

Fig. 1.1: Schematic illustration of the basic process of photosynthesis that takes place in the chloroplast. Modified from [21]

1.1.1 The light reactions

These set of reactions take place in the thylakoid membranes of the chloroplast and generate ATP and reducing equivalents from light energy derived from sun. Light in the form of photons is captured by chlorophyll and other pigment molecules (light harvesting complexes) present in the thylakoid membranes. The energy is then utilized to generate a linear electron flow from photosystem (PS) II to I (reaction centres). The electron transport chain begins with oxidation of water molecule yielding energetic electrons used to reduce NADP^+ to NADPH in the stroma. The electron flow also drives the generation of a chemiosmotic gradient that is utilized to phosphorylate ADP to form ATP (Fig. 1.2A).

The process initiates with the concurrent excitation of electrons from specially-bound chlorophyll molecules in the reaction centres of PSI and PSII. The energy cascaded by the pigments in PSI is used by the special P700 chlorophyll (pigment having absorption maxima at $\lambda \sim 700 \text{ nm}$), to excite an electron to a higher energy level (P700^*), which enables an adjoining electron acceptor molecule to capture the excited electron. Electrons derived from P700 are eventually passed down to adjacent lower energy carrier molecules and reaches the ultimate electron carrier NADP^+ , creating an electron transport chain. Two electrons transported in the system combine with H^+ ions and NADP^+ to form NADPH (Fig. 1.2A). The electron deficient P700^+ is

replenished by electrons originating from PSII. Similar to PSI, light energy excites an electron of the specially-bound P680 chlorophyll molecule so as to be absorbed by the neighbouring electron acceptor molecule. The electrons cascade through a series of carrier molecules in a different electron transport chain to be finally transferred to the $P700^+$ molecule of the PSI and making it functional for the next cycle [19]. The electron transport chain of PSII establishes a proton gradient across the thylakoid membrane which fuels the production of ATP from ADP by chloroplast F_1F_0 ATP synthase [22, 23] (Fig. 1.2A). The electron deficient $P680^+$, which ultimately contributed the electrons to $P700^+$, is replaced by the electrons generated by splitting of water into hydrogen ions (H^+) and oxygen gas (O_2) [24]. These electrons are used to regenerate $P680$ of PSII. Electron transport between two photosystems through the electron transport chain complex to reduce $NADH^+$ is also referred as the “Z scheme” (Fig. 1.2B). Protons are pumped into the thylakoid lumen to generate the proton gradient powering ATP synthesis. This is driven by cyclic or non-cyclic electron transport across the photosystems [22, 23].

B

Fig. 1.2: Light dependent reactions of photosynthesis. (A) Schematic representation of the events taking place during the light reactions. The electron transport chain created across the thylakoid membrane from PSII to PSI ultimately reducing NADP^+ to NADPH. (B) Z-scheme of the linear electron flow across two photosystems. Electrons are derived from the oxidation of water molecules at the oxygen evolving complex (OEC) and pass through PSII to plastoquinone (PQ/PQH₂). Oxidation of PQH₂ occurs at the cytochrome b6f complex (b6f) where half of the electrons are linearly transferred to $\text{NADP}^+/\text{NADPH}$ coupled via plastocyanin (PC), PSI, ferredoxin (Fd), and ferredoxin-NADP⁺ oxidoreductase (FNR). The other half of the electrons returns to the PQH₂ pool. Protons released at the OEC and the cyclic reduction and oxidation of PQ/PQH₂ set up a protons gradient across the thylakoid membrane. This chemiosmotic potential is used to produce ATP from ADP at the chloroplast F_1F_0 ATPase. Modified from [19, 23]

1.1.2 The dark reactions (The Calvin-Benson cycle)

The Calvin-Benson or Calvin-Benson-Bassham cycle, named so after their discoverers [25], consists of a series of enzymatic oxidation-reduction reactions. Fig. 1.3 represents the schematic overview of the pathway. This pathway encompasses three phases: carbon fixation, reduction, and regeneration of substrate. Carbon fixation is the first step in which RuBP is carboxylated in the presence of atmospheric CO_2 to produce two molecules of 3-phosphoglycerate (3PG). This reaction is catalysed by the world's most abundant enzyme, ribulose 1, 5-bisphosphate carboxylase/oxygenase (Rubisco) [26, 27]. The reduction step consists of a series of reactions utilizing the end products from the light reactions (ATP and NADPH) to phosphorylate 3PG to 1, 3-bisphosphoglycerate, which is subsequently reduced and dephosphorylated to glyceraldehydes 3-phosphate (G3P). In the regeneration phase, RuBP is regenerated by using carbon from G3P. This is accomplished by a series of carbon reshuffling reactions. In the final reaction, the precursor ribulose 5-phosphate is regenerated in an ATP-dependent manner to RuBP by phosphoribulokinase. Five-sixths of the G3P is used for the synthesis of sugars, which are then used to make all of the organic molecules needed by the plant. Overall, the Calvin-Benson cycle

generates one molecule of G3P from three molecules of CO₂ at the cost of nine molecules of ATP and six molecules of NADPH [28]. The net reaction of Calvin-Benson cycle is therefore:

Fig. 1.3: The Calvin-Benson cycle. RuBP (5-carbon molecule) with the addition of CO₂ forms an unstable 6-carbon molecule (Carbon fixation phase). This 6-carbon molecule immediately breaks down into two 3-carbon molecules (3-PG) which is phosphorylated to form 1,3-bisphosphoglycerate molecules and subsequently reduced to form G-3-P by the consumption of ATP and NADPH (Reduction phase). Some of the G-3-P formed is shunted out of Calvin-Benson cycle to produce glucose (Carbohydrate formation) and other, more complex organic molecules. The remaining glyceraldehyde-3-phosphate is used to regenerate the 5-carbon CO₂ acceptor molecule by a series of reactions (Regeneration phase).

1.1.3 Photorespiration

Rubisco also catalyzes the oxygenation of RuBP under low CO₂ conditions. This futile side reaction leads to formation of one molecule of 2-phosphoglycolate (2PG) and one molecule of 3PG [29]. For plants, 2PG is considered a harmful compound, which needs to be converted into 3PG via an energy consuming photorespiratory pathway [30]. It is salvaged by a series of reactions taking place in the chloroplast, cytosol, mitochondria and peroxisome (Fig. 1.4) where it is converted into glycerate and recycled back to the chloroplast to be utilized in Calvin-Benson cycle [6].

The cycle begins in the chloroplast stroma where resulting 2PG from the oxygenation reaction is dephosphorylated by 2-phosphoglycolate phosphatase (PGP) into glycolate. This glycolate is transported from the chloroplast to the peroxisome where a peroxisomal enzyme glycolate oxidase (GOX) oxidized it into glyoxylate. The produced glyoxylate is transaminated into glycine by serine-glyoxylate transaminase (SGT) and glutamate-glyoxylate transaminase (GGT). The glycine then enters the mitochondria where two molecules of glycine are converted into serine by the action of glycine decarboxylase (GDC) and serine hydroxymethyltransferase (SHMT). Additionally, one molecule of ammonia and CO_2 is released. The serine formed, is sent back to the peroxisome and produces hydroxypyruvate by the action of SGT which is subsequently reduced to glycerate by hydroxypyruvate reductase (HPR). Glycerate is exported from peroxisome to the chloroplast, where it is finally converted into 3PG by the plastidial glycerate 3-kinase (GLYK), thus completing the cycle.

Fig. 1.4: Schematic overview of the photorespiratory pathway. Rubisco catalyzes the oxygenation of RuBP, a major side reaction leading to formation of 2-phosphoglycolate. In plants, this molecule has to be salvaged back to 3-phosphoglycerate by the photorespiratory cycle. The pathway involves a series of reactions taking place in chloroplast, peroxisome and mitochondria as shown in the figure.

The oxygenation reaction costs one ATP molecule and results in loss of one CO_2 molecule, reducing the net efficiency of photosynthesis. It has been reported that significant improvement in the photosynthetic efficiency could be achieved if photorespiration is repressed by augmenting carbon dioxide in the proximity of Rubisco using free air CO_2 enrichment (FACE) studies [31-33]. Plants grown under a high CO_2 fertilized environment in such studies show repressed photorespiration and

produce more biomass. Also, plants carrying a metabolic bypass for photorespiration demonstrate improved photosynthesis and increased biomass [34, 35]. This seems to provide a promising approach for improving the photosynthetic productivity in many C3 plants that confront a significant drain in metabolic energy due to high rates of photorespiration. However, the growth rate enhancement achieved by plants with repressed oxygenation reactions was smaller than predicted by theoretical models [33], when such plants were grown under a high CO₂ environment. Some researchers suggest that reducing photorespiration will have a negative impact on plant physiology. For instance, several studies have reported the importance of photorespiration in processes such as plant defence and nitrate assimilation, hinting at beneficial aspects of photorespiration [36, 37].

1.1.4 Carbon concentrating mechanisms

The significant loss of metabolic energy caused by operation of the photorespiratory pathway has resulted in a strong evolutionary selection pressure to minimize the oxygenation reactions. Therefore, in spite of contrasting reports on the importance and benefits of photorespiration, many photosynthetic organisms including most aquatic microalgae have evolved efficient carbon concentrating mechanisms (CCM). The multiple independent CCM's are employed by different organisms where Rubisco resides in an environment experiencing an elevated local CO₂ concentration.

Cyanobacteria and many proteobacteria possess a specialised micro-compartment enclosed by a proteinaceous, icosahedral shell known as carboxysomes [38]. The shell self-assembles and contains Rubisco and carbonic anhydrase [39]. Inorganic carbon in the form of bicarbonate is pumped into the cells by active transport. The co-localised carbonic anhydrase converts this bicarbonate into gaseous CO₂ near the site of Rubisco, effectively increasing local CO₂ levels and thus minimises the competing oxygenation reaction.

In most algae, such as *Chlamydomonas reinhardtii*, Rubisco is placed in a distinct stromal microcompartment known as the pyrenoid [40]. In green algae, the pyrenoid is surrounded by starch-like oligosaccharides unlike the carboxysome [41]. Due to difficulties in purification and stabilization of the pyrenoid, the mechanistic details of the CCM in algae are still elusive.

Some terrestrial plants have evolved pathways to concentrate CO₂ at the Rubisco active site in syndromes known as C4 and CAM photosynthesis [42]. C4 plants such as maize possess a characteristic leaf anatomy called Kranz anatomy. Initial CO₂ fixation by PEP carboxylase takes place in specialized cells called mesophyll cells followed by shuttling of fixed carbon in the form of malate or aspartate to bundle sheath cells where the Rubisco is located. Thus Rubisco is isolated from atmospheric oxygen and remains saturated with CO₂ released by decarboxylation of these organic acids. Given the advantages of C4 cycles, initiatives have been taken to incorporate C4 features into C3 plants like rice [43, 44].

CAM plants are adapted to survive in hot and arid conditions. The carbon fixation in such plants takes place during night when ambient temperature is low and the stomata are open allowing CO₂ to enter. They remain closed during day to prevent loss of water. The CO₂ is fixed into oxaloacetic acid by the action of PEP-carboxylase at night. Subsequently, this oxaloacetate is converted into malate and stored in vacuoles. During day, when temperature is high and stomata are closed to prevent water loss, this malate is shuttled into the chloroplasts and decarboxylated into pyruvate, releasing CO₂ which then enters the Calvin-Benson cycle for fixation [45]. CAM photosynthesis reflects a temporal separation of CO₂ uptake and fixation, while C4 photosynthesis represents spatial separation of the two processes.

1.2 Ribulose 1, 5- bisphosphate carboxylase/oxygenase (Rubisco)

Rubisco (EC.4.1.1.39) is believed to be the most abundant protein on earth [26] and is the key enzyme of the dark reactions. It often makes up 50% of the total soluble protein found in plant leaf tissue and accounts for the majority of the protein in some phototrophic microbes [5, 29]. It catalyses the initial steps of photosynthetic carbon fixation by converting RuBP and CO₂ to two molecules of 3PG. Rubisco has slow catalytic properties and due to its inability to differentiate between CO₂ and O₂, it also catalyses the oxygenation of RuBP which leads to formation of toxic 2PG [4, 5]. This compound has to be converted back to 3PG at the cost of energy and CO₂, which reduces the efficiency of photosynthesis [6]. Apart from this, Rubisco is prone to inhibition by its own substrate and other inhibitory sugar phosphates, some of them generated by its own active site, leading to a phenomenon called fallover [7, 46]. All these drawbacks might have compelled its abundant expression in nature. The

manipulation of kinetic properties of Rubisco has been the objective for many years and widely attempted for improving photosynthetic yield in higher plants [47, 48]. Rubisco from different species shows different kinetics and substrate specificity. For instance, Rubisco from red algae like *Griffithsia monilis* show at least three fold higher CO_2/O_2 specificity ($S_{\text{C/O}}$) compared to any other plant Rubisco [49-51].

1.2.1 The structure and classification of Rubisco

The Rubisco enzyme found in higher plants consists of large subunits (RbcL) ~ 52 kDa and small subunits (RbcS) ~15 kDa in a hexadecameric stoichiometry, L_8S_8 [52]. The fundamental structural unit of all Rubiscos is a dimer of large subunits (RbcL_2). Rubisco is classified into four major forms namely form I-IV (Fig. 1.5) of which only the first three catalyze RuBP-dependent carbon fixation reactions (carboxylation/oxygenation) [53, 54]. Form IV enzymes termed as Rubisco-like proteins (RLP) and do not catalyse the CO_2 fixation reaction [55].

Fig. 1.5: Representatives of the four forms of Rubisco. All forms are made up of the functional units of antiparallel dimers of large subunits. Form I forms tetramers of dimers and is the only form that comprises of the small subunits. Form II is made up of dimers or oligomers ($n=2-5$), the dimeric form is shown here. Form III Rubisco forms tetramers or pentamers of dimers, shown here is the pentameric form. Form IV enzymes are comprised of a single antiparallel dimer of large subunits. PDB accession numbers are indicated in brackets under the species name [53, 56].

Out of the four types, only form I has a small subunit and exists as a hexadecameric complex (L_8S_8) of four antiparallel RbcL_2 which is crowned by four RbcS subunits at the top and bottom [53]. This is the most common form of Rubisco and is further classified into four sub groups: Form IA-D. Form IA and Form IB are commonly referred to as green-type Rubisco [53] found in plants, green algae, cyanobacteria and proteobacteria. In plants and green algae the RbcL is encoded by chloroplast, whereas

RbcS is encoded by the nuclear genome [57]. Form IC and form ID are referred as Red-type Rubisco [53] in which RbcL and RbcS are encoded in the same operon [58] and is found in some proteobacteria, red algae and other non-green algae.

The overall fold of the RbcL monomer is well-conserved in all forms of Rubisco. It is made up of a N-terminal $\alpha+\beta$ domain and a larger C-terminal α/β TIM barrel domain ($\beta_8\alpha_8$) [59]. The active site is at the intradimer (RbcL_2) interface between the N-domain of one subunit and the C-domain of the adjacent subunit. Consequently, the functional unit of Rubisco is an L_2 dimer with two active sites per dimer [4].

Although the form I RbcL amino acid sequences are highly conserved, the RbcS sequences are more diverse with only 30– 40% homology between different species [59]. Even though they are not strictly required for enzymatic function, the small subunits are important for maximal catalytic rates and substrate specificity [60-62]. The green and red type Rubisco enzymes differ in several structural features, the most notable being an extension in the C-terminal domains of the small subunits in the red-type enzymes. The C-terminal extensions form a small barrel that protrudes into the solvent channel of the holoenzyme [63]. It is possible that the structural variance between the two subclasses could justify the higher substrate specificity for CO_2 over O_2 of some red-type Rubiscos as compared to green-type enzymes.

Form II Rubisco found in proteobacteria and dinoflagellates is active as RbcL_2 . In many chemoautotrophs Form II often co-exists with Form I Rubisco with different numbers of RbcL dimeric pairs, $(\text{L}_2)_n$. This form is reported to have a poor CO_2/O_2 specificity [52]. Although the residues involved in catalysis are conserved, the sequence homology between Form I and Form II large subunits is only 30 % [52, 64].

Form III Rubisco exists in some thermophilic archaea, which lack the Calvin-Benson cycle but catalyzes the carboxylation reaction of RuBP, which is generated via an alternate pathway [65]. They exist as dimeric structure, however pentamers of dimers (RbcL_{2-5}) were also found as active form [66-70].

Form IV Rubisco are known as Rubisco-like proteins (RLP) which forms antiparallel dimers of large subunits similar to Form II however they are not genuine Rubiscos [56, 71]. Form IV Rubiscos demonstrate dissimilarity in critical residues at active sites and do not catalyse CO_2 fixation [55, 72]. The RLPs from *Bacillus subtilis* and

Geobacillus kaustophilus are reported to be involved in a methionine salvage pathway by performing an enolase-type function similar to Rubisco on a different substrate [71, 73]. The RLP from green sulphur bacteria *Chlorobium tepidum* is involved in thiosulphate oxidation [72].

1.2.2 Reaction mechanism: carbamylation and catalysis

The Rubisco apo form (E) requires two cofactor, CO₂ and Mg²⁺, to bind at the active site to perform its catalytic function [74]. The active site for carboxylation is present at the interface between N-terminal domain of one subunit and the C-terminal domain of the adjacent subunit of the antiparallel RbcL2 [75]. Activation takes place by the binding of a non-substrate CO₂ molecule with the ε-amino group of lysine 201 (spinach numbering) located at the catalytic sites to form a carbamate (EC). The carbamate is stabilized by the addition of Mg²⁺ to form the active complex (ECM) [74, 76]. These spontaneously occurring, reversible reactions are only possible in the ‘open state’ of the enzyme, which allows solvent access to the binding pockets [47]. The addition of Mg²⁺ at the Rubisco active site is important for the positioning of the RuBP for the electrophilic attack of the substrate CO₂ molecule [5]. The substrate RuBP can then proceed to bind at the active site, triggering the closure of α/β TIM-barrel loop 6 over the active site. The extended conformation of loop 6 is then fixed in position by the flexible C-terminal tail of RbcL, excluding the active site from solvent [77, 78]. Rubisco then proceeds with the carboxylation of RuBP. The active site opens and closes during every catalytic cycle, mostly involving movement of loop 6 [79].

During catalysis, in the ‘closed state’, loop 6 forms a latch- like structure on top of the substrate and reaction intermediates that are deeply buried in the active site of the complex. Carboxylation is initiated when the carbamylated K201 abstracts a proton from RuBP to form the enediol isomer of RuBP (enolisation) at the active site [29, 76]. This enediol form makes a nucleophilic attack on the substrate CO₂ molecule to form a ketonic acid intermediate. This unstable 6-carbon product intermediate of carboxylation is subsequently hydrated and cleaved to produce the two molecules of 3PG [80]. The oxygenation reaction takes place when O₂ molecules compete at the active site instead of CO₂ molecules. The mechanism is similar to CO₂ fixation where the enediol isomer makes a nucleophilic attack on the O₂ molecule resulting in an intermediate, which is hydrolysed to form one 3PG and one 2PG molecule [80]. The

energy released from the bond cleavage between the two phosphate substituents is likely required to permit the active site to open, allowing release of the products at the end of catalysis. [81].

1.2.3 Rubisco inhibition and regulation

Rubisco is known to form inhibited complexes with its own substrate RuBP and a number of other sugar phosphates. Binding of RuBP to the decarbamylated form of Rubisco results in an inhibited complex known as ER which is catalytically inactive [83]. Additionally, several misfire products generated during catalysis bind to non-carbamylated Rubisco and prevent catalysis [7]. In many plants other naturally occurring sugar phosphates such as the nocturnal inhibitor 2-carboxy-arabinitol-1-phosphate (CA1P) bind to the carbamylated form to make stable inactive complexes (Fig. 1.7) [84, 85].

The affinity for RuBP is low in the carbamylated state in order to facilitate cleavage and release at the active site, while under decarbamylated state the affinity is high and therefore Rubisco becomes inactive in decarbamylated state [86, 87]. Thus, different inhibitors bind to Rubisco in the carbamylated and non-carbamylated state with different affinities. RuBP and its misfire products like D-xylulose 1,5-bisphosphate (XuBP), glycerol-2,3-bisphosphate (PDBP) favours binding to the non-carbamylated enzyme (E) to form the non-carbamylated inhibited complex (EI). Other inhibitors such as CA1P and the non- physiological transition state analogue 2-carboxy-D-arabinitol 1,5-bisphosphate (CABP) binds to carbamylated active Rubisco (ECM) and form a carbamylated inhibited complex (ECMI) (Fig. 1.7) [88, 89]. Rubiscos with ligands occupying the substrate binding sites in carbamylated and uncarbamylated state are structurally similar, indicating that cofactors do not play a critical role in substrate binding to the active sites [90, 91]. Thus, irrespective of whether RuBP or slowly cleavable misprotonation products like XuBP bind in decarbamylated state or CA1P, CABP binds to either form, there is no favourable route for opening and release of product exists. The satisfactory path only exists for ligand RuBP in carbamylated and Mg-bound state for reopening of closed complexes [81]. For instance, a half-life of 530 days was determined for the release of CABP from carbamylated Rubisco in spinach, much higher than other inhibitors, making the binding almost irreversible [88, 92].

Fig. 1.7: Regulation of Rubisco by Rubisco activase. (A) Decarbamylated Rubisco (E) binds to CO₂ and Mg²⁺ to become the active holoenzyme (E.C.M) which actively fixes CO₂ by carboxylation of its substrate RuBP. E form can also bind substrate RuBP or inhibitors to form a dead inhibited complex (E.I). E.C.M can also become inhibited by its nocturnal inhibitor 2-carboxy-arabinitol-1-phosphate (CA1P) or other substrate analogue, D-glycero-2,3-pentodiulose-1,5-bisphosphate (PDBP), 3-ketoarabinitol-1,5bisphosphate (KABP) to form inhibited complex (E.C.M.I). The helper enzyme Rubisco activase remodels the Rubisco in both situations by removing the inhibitory sugar-phosphates. Modified from [7].

The inhibition of Rubisco activity due to binding of sugar phosphates in the plant is part of the physiological regulation of the enzyme *in vivo*; however activity of activated Rubisco also declines in a non-linear manner under *in vitro* assay conditions. This phenomenon is known as ‘fallover’ [93-96]. It is the outcome of the accumulation of misfire products during catalysis of RuBP (oxygenation or carboxylation) which block the active sites and inhibit Rubisco activity. The presence of non-cleavable inhibitors also blocks Rubisco active sites. Although they are present in much smaller quantities compared to RuBP, they can form slow tightly bound inhibited complexes [96-98]. Therefore, when active photosynthesis takes place, these inactive Rubisco complexes would accumulate and obstruct CO₂ fixation. The fallover is less severe at high temperature due to loose binding of inhibitors in spite of a higher rate of formation of misfire products [99]. This could be the outcome of enhanced flexibility of the Rubisco active site at higher temperature leading to an increased rate of release of inhibitors from the active site.

The tendency of inhibition by such mechanisms differs between Rubisco from phylogenetically diverse sources [82, 100, 101]. Generally there exists a compromise between substrate specificity ($S_{c/o}$) and catalytic rate of the Rubisco in different species [102, 103]. According to the prevailing model, Rubisco enzyme exhibiting higher substrate specificity favours tight binding of transition state intermediates of carboxylation and consequently the cleavage of tightly bound reaction intermediates becomes slower. Tight binding favours carboxylation over oxygenation but the slower rate unfortunately also favours aggravated misfire product formation [103]. Also, highly selective enzymes form slow-tight binding complexes with inhibitors, indicating that the active sites of these enzymes are more rigid. For instance, plant Rubiscos possessing high specificity, forms inhibited complexes with RuBP and display fallover. Rubisco of red algae with high specificity do not exhibit fallover inhibition, but when inhibited complexes are formed, they are extremely stable [82, 100]. Recently, a red algal Rubisco from *Cyanidioschyzon merolae* was reported to form a stable inhibited complex only at high temperature (45 °C) [104] that did not reactivate spontaneously at 25 °C. Rubisco from green algae with intermediate specificity forms the inhibited complex with RuBP but does not show fallover when assayed [101]. Whereas, less specific Rubisco from cyanobacteria neither form inhibited ER complexes nor display fallover [82, 101, 105, 106]. Thus, highly specific Rubiscos exhibiting slower catalytic rates are particularly prone to form highly stable dead-end inhibited complexes.

In order to overcome this problem of inhibition, during the course of evolution, nature has recruited a molecular chiropractor of Rubisco called Rubisco activase (Rca) [9, 107]. Rca utilizes energy from ATP hydrolysis to perform its function; subsequently other phosphatases assist in degradation of non-RuBP inhibitors [85, 108, 109]. Fig. 1.7 shows the schematic representative mechanism involved in Rubisco regulation by Rca. Since Rca is responsible for maintaining the activation state of Rubisco, various evidence has been put forward after its discovery that proves that Rca is critical to maintain Rubisco functionality *in vivo* [7, 8]. Rca has been found to exist in all the plant species surveyed and belongs to the AAA+ family of proteins, which are known to execute chaperone- like functions in other homologous and heterologous systems [29].

1.3 Rubisco activase (Rca)

Rubisco activase was discovered as an act of serendipity, derived from large scale screens of arabidopsis mutants that required high CO₂ for growth. It was observed that the strain was unable to grow at ambient CO₂ level and accumulated RuBP due to lack of activation of the Rubisco, but survived at high CO₂ levels [110, 111]. This strain was therefore named as *rca* for Rubisco activation. Later, it was found that two polypeptide bands were missing on a two dimensional polyacrylamide gel electrophoresis in the *rca* mutant during analysis of soluble polypeptides from the arabidopsis mutants and wild-type strains. Biochemical and genetic analysis confirmed that the two missing polypeptides were responsible for the *rca* mutant phenotype and this protein was later named as Rubisco activase [9].

Identifying the presence of Rca in soluble protein extracts of various C3 and C4 plants species confirmed that Rca-dependent regulation of Rubisco was pervasive in higher plants [112]. In the same year, it was also discovered that the Rca is an ATP-dependent remodeler of Rubisco [113], which promotes the release of bound sugar phosphates from the active site of both carbamylated and decarbamylated forms of Rubisco [107, 109]. Addition of Rca to uncarbamylated Rubisco showed no activity loss or to carbamylated Rubisco showed no fallover. Thus, it applies to Rubisco inhibited by CA1P or other inhibitors that are formed during catalysis apart from RuBP alone. However, Rubisco activase is not directly involved in carbamylation or binding of magnesium ion at the active site [107].

Twenty-five years after the discovery of green-type Rca in plants, in 2011 a red-type Rubisco activase (CbbX) from α -proteobacteria was reported [114]. Last year, a CbbQO complex (purple-type) was found to be functional as Rubisco activase in chemoautotrophic bacteria [115]. Recently, a hetero-oligomeric red-type Rubisco activase was described in a thermophilic red alga *C. merolae* that operates as an hetero-oligomeric complex of a plastid and nuclear-encoded CbbX isoform (CmNP) [104]. Although all these Rubisco activases employ distinct mechanisms, the energy required to remodel inhibited Rubisco complexes is generated by an AAA+ protein module. Therefore, in order to generate a foundation for functional understanding of Rubisco activase, it is necessary to appreciate the basic structural and functional aspects of AAA+ proteins in different systems.

1.3.1 Rubisco activases are members of the AAA+ family of proteins

A sequence comparison with related ATPases revealed that Rca belongs to the family of AAA+ (ATPases associated with diverse cellular activities) ring-shaped p-loop ATPase motor proteins [116]. AAA+ proteins as the name suggests, have been described to perform an array of functions at the cellular level and are ubiquitously found across kingdoms of life. The overarching theme in the function of AAA+ proteins involves conformational remodelling of their target substrate (a macromolecule such as protein or polynucleotides) by utilizing energy from ATP hydrolysis [117, 118]. They have an active role in various cellular processes such as protein folding and degradation, DNA replication, membrane fusion, vesicle trafficking, transcriptional activation, motor activity etc. [117-119]. Class I AAA+ proteins (e.g. ClpA, ClpB, ClpC and HSP104) contain two highly conserved AAA domains, referred to as AAA-1 (or D1) and AAA-2 (or D2), separated by a variable length linker sequence. Class II proteins (e.g. ClpX, HslU), including all known Rubisco activases, contain only a single AAA domain [117, 120].

AAA+ proteins consist of a distinct set of conserved sequences and are characterized by nucleotide binding domain (NBD) consisting of a N-terminal α/β Rossman fold and a less conserved C-terminal 4-helix bundle (hb) subdomain [117]. An α/β Rossman fold forms the core of the N-terminal subdomain with a β -sheet of parallel strands, arranged in a $\beta 5-\beta 1-\beta 4-\beta 3-\beta 2$ fashion at its core (Fig. 1.8B). This large AAA+ subdomain comprises the signature Walker A, B and sensor I motifs [121] essential for nucleotide binding and hydrolysis. The nucleotide binding domain also contains conserved arginine residues called arginine fingers for intersubunit communication which affects ATP hydrolysis and subunit oligomerization [122]. Another typical feature of the AAA+ proteins are the pore loops facing the central cavity that is lined by residues from the individual subunits [117]. The underlying mechanism of action often involves protein threading, where the substrate protein is translocated through a central pore formed by a ring shaped oligomer of AAA+ protein subunits [123-125]. In the α -helical subdomain, a conserved sequence motif called sensor II, characterized by a conserved arginine is essential for nucleotide binding and hydrolysis [121, 122]. In many cases AAA+ ATPases act on a large number of substrate proteins, such as the general protein disaggregation machine Hsp104/ClpB or the motor of the bacterial degradation machinery ClpX. Generally,

AAA+ proteins form homohexameric arrangements with a central pore, stabilised by the presence of nucleotides. The AAA+ modules of class I proteins therefore form two stacked hexameric rings [126, 127], while class II proteins form single hexameric ring [118, 128].

To date, three plant Rca crystal structures have been published. A 3.0 Å model of the tobacco AAA+ domain (residues 68–360 out of 383 total) [129], a 1.9 Å model of the creosote bush C-terminal small domain (residues 250–351 out of 379 total) [130] and a 2.9 Å model of the arabidopsis Rca (residues 65–362 out of 416 total) [131]. These structures reveal the organisation of the apo state and architecture of the AAA+ domain.

Fig. 1.8B shows the crystal structure of Rubisco activase from tobacco in apo state [129]. The structure represents a typical AAA+ topology with large AAA+ domain comprising the nucleotide binding α/β subdomain and small α -helical domain characterized by a 4 helix bundle. Rca also comprises the N-terminal domain (67 residues) and a C-terminal extension (23 residues), which were not resolved in the structure due to flexibility. Also the four flexible loop regions were not resolved in the structure and were extrapolated using dotted lines. The α/β subdomain has a Rossmann-fold typical for the AAA+ proteins, which constitute key signature motifs like Walker A, B and sensor 1 region essential for ATPase function [117]. The small C-terminal domain with 4-helix bundle ($\alpha 6-10$) fold at its core differs from the typical AAA+ fold as helix 8 is elongated followed by small helical insertion ($\alpha 9$) responsible for substrate specificity between Rubisco of the Solanaceae and non-Solanaceae family. Helix 9 is further connected to helix 10 through a linker (16 residues), that is totally separated from the main α -helical sub-domain. The helix 8 in the structure of C-terminal of creosote bush is longer and consists of specificity domain. These models suggested a conformational plasticity in the specificity-determining region that was responsible for differentiation between solanaceous (eg. tobacco) and non- solanaceous (eg. spinach) Rubiscos [47, 132].

Fig. 1.8: Structure and domain architecture of Rca from *Nicotiana tabacum*. (A). Domain architecture of tobacco activase (NtRca) showing the N-terminal domain, AAA+ nucleotide binding α/β sub-domain, α helical small subdomain (hb) and C-terminal extension. NBD: nucleotide binding domain, hb: α -helical subdomain. (B) Ribbon representation of the crystal structure of NtRca (3T15). Disordered loops are indicated by dotted lines. Two views related by 90° are shown. The α/β and the α -helical subdomains are indicated in pale cyan and green, respectively. The canonical AAA+ structural motifs are indicated as follows: Walker A (purple), Walker B (magenta), sensor I (tv blue) and sensor II (orange). The disordered pore loops are indicated as light red dots. The specificity helix (H9) is shown in blue. Another disordered loop (residue 208-218) and chain termini are indicated. (C) A hexameric model of NtRca (3ZW6). Top, bottom and side view of the surface of hexameric ring is shown, overlaid by the cylindrical representation. Each subunit is shown in different colour. The images were generated using pymol software.

1.3.2 Oligomeric state of the Rubisco activase

The structural organisation of Rubisco activase oligomers has been a controversial issue. The published crystal structures do not provide a true functional oligomeric form of Rca.

Rubisco activase from diverse species are variedly polydisperse in nature. The oligomeric state of Rca in solution was observed to be varied in a concentration and nucleotide dependent manner. Various oligomeric sizes (50~600 kDa) from 1-16 subunits were reported, with larger species observed at higher enzyme concentrations [133-135]. The specific ATPase, Rubisco activation activity, and the oligomeric size increased with increasing concentration and in presence of macromolecular crowding agents such as polyethylene glycol [136].

It has been reported that a complex of no more than two to four subunits is required for biological activity of tobacco Rca [135] in a study using small angle x-ray scattering (SAXS) and analytical ultracentrifugation. However, this study was conducted in the absence of nucleotides under various dilutions. It has been hypothesized that Rca may form open spiral structures in solution [135, 137, 138] rather than a closed hexamer. It has also been interpreted that a large oligomer of more than eight subunits and up to sixteen subunits is the active state of activase [133, 134, 139]. However, this appears inconsistent with the canonical active functional oligomeric form (hexamer) of an AAA+ protein [120]. Therefore, it is possible that transient hexamers may exist in solution in the presence of nucleotide, however formation of hexamers and their dissociation is too rapid to be detected by the reported methods and therefore a range of oligomers were observed.

Biochemical analysis of interface mutants in tobacco revealed 2.9-3.7 and 3.5-5.4 as minimal subunits necessary for ATPase and Rubisco activation activity respectively [129] providing evidence for the hexamer as minimal functional unit. Indeed mutating the arginine 294 (R294) residue of tobacco Rca resulted in the formation of stable hexamers in the presence of Mg.ATP γ S as observed by mass spectroscopy [140], negative stain EM [129], and analytical ultracentrifugation [141]. The biochemical analysis of this variant (R294V) revealed it to be fully functional, further providing a

strong evidence in support of the hexamer as active species. Also, recently the α isoform and an equimolar mixture of α and β isoform of spinach was observed to form a stable hexamer in presence of Mg.ATP γ S as the first instance for any wild type Rca [141]. The non-homologous bacterial red-type activase CbbX was observed to change its oligomeric form in response to the presence of an allosteric regulator [114]. Thus it is quite possible that activase may acquire variable oligomeric states in solution but forms transient hexamers as an active species in presence of nucleotide, especially when engaging inhibited substrate Rubisco.

1.3.3 Regulation of Rubisco activase

In most plants, including arabidopsis, spinach, and rice [142-144], Rubisco activase occurs as two isoforms often as a result of alternative splicing of a single transcript. Consequently, the isoforms are essentially identical with the α isoform having a ~40 residue C-terminal extension, which contains two conserved cysteine residues that are essential for its regulation [145, 146]. However, in some plants like cotton, two isoforms are generated by different genes rather than splicing [147]. The activase is constitutively ATPase active and in contrast to many other AAA+ proteins the ATPase activity of activase is not stimulated by its protein substrate [109].

The ATPase activity of Rubisco activase is inhibited by ADP and regulated by the ATP/ADP ratio in the chloroplast stroma (Fig. 1.9A). This modulation of ATPase activity of activase also regulates Rubisco activity in response to light fluctuations [113, 148]. It has been shown that the α isoform is more sensitive to ADP inhibition than the β isoform [146, 148].

The α isoform is also regulated by changes in the redox state of two cysteine residues located at its C-terminal extension by thioredoxin-f [146]. Thioredoxins are a class of small redox proteins involved in several regulatory processes in plants [149]. Apart from Rubisco activase, thioredoxin-f regulates the activity of several other Calvin-Benson cycle enzymes such as fructose 1,6-bisphosphatase, sedoheptulose 1,7-bisphosphatase, phosphoribulokinase, and glyceraldehydes 3-phosphate dehydrogenase [149, 150]. Trx-f is reduced in the light via ferredoxin and ferredoxin-thioredoxin reductase (FTR) using electrons originating from the action of

photosystem I [151]. Reduced thioredoxin-f then diffuses throughout the stroma and reduces oxidized target proteins in the light leading to their activation (Fig. 1.9B).

In vitro, the redox regulation of Rca can be mediated by thioredoxin-f, along with a DTT electron source, which makes the ATP/ADP ratio much less inhibitory, causing an increase in ATPase and Rubisco activation activities of the α isoform. A similar effect was not observed in the presence of DTT and thioredoxin-m. This effect is reversible when DTT is replaced by oxidized glutathione leading to a reduction in activity [146]. Substituting either of these two cysteine residues from the C-terminal extension to alanine reduces the ATP/ADP sensitivity of the activase and the light responsiveness of Rubisco activity *in vivo* and *in vitro* [146, 152].

Fig.1.9: Regulation of Rubisco activase (A) By ATP/ADP ratio. During the day when the light reactions generate ATP, the ATP/ADP ratio is high, which activates activase. In the dark when ATP/ADP ratio is low activase becomes inactive due to inhibition by ADP. (B) By ferredoxin-thioredoxin system. Ferredoxin is reduced by the photosynthetic electron transport chain. The reducing equivalent generated along with ferredoxin reduces the disulphide group in the iron-sulphur enzyme ferredoxin reductase. The reduced ferredoxin reductase reduces the disulfide bond in thioredoxin. The reduced thioredoxin then reduces the disulfide bond of target enzymes to activate them. The activated enzyme gets deactivated by the oxidation thiol groups of reduced thioredoxin in the dark which in turn oxidizes the reduced enzyme. Modified from [153].

Although the α isoform is critical for redox regulation and important in regulating Rubisco activation in response to fluctuating light irradiance, interestingly some species like tobacco lack an α isoform. Plants expressing only the β isoform did not show any downregulation in Rubisco activity during a light-dark transition [152] and the β isoform was not observed to be redox sensitive when assayed alone. The regulation of β isoform by the ADP/ATP ratio varies among different species. For instance, the β isoforms of tobacco is reportedly more prone to inhibition by ADP than the β isoform of arabidopsis [154]. Subunit mixing experiments between α and β isoforms under *in vitro* assay conditions validated a regulatory effect of the α isoform on the β isoform in arabidopsis. At the equimolar ratio, the β isoform gets totally inhibited by the presence of α isoform at an ATP/ADP ratio of 1:3 under oxidizing conditions [155], indicating that the α isoform allosterically modulates the activity of β isoforms.

Under *in vivo* conditions, reducing equivalents increase with light illumination, hence it was assumed that redox chemistry of the α isoform modulates activase activity in response to light and regulates Rubisco activity. Later it was confirmed by a study expressing just a single isoform *in vivo*. Arabidopsis *rca* mutants expressing only the α isoform respond to light fluctuations and Rubisco activation was downregulated under light-limiting condition, while the expression of just the β isoform did not downregulate Rubisco activity in response to light [152]. Based on the mutational and cross-linking study, the C-terminal extension of the α isoform in the oxidized state folds back towards the nucleotide binding pocket, perturbing its activity [155, 156]. Furthermore, there is fluctuation in the diurnal expression of activase in plants with maximum expression at the onset of the photocycle and lowest at night [157], which closely mimics the circadian rhythm of Rubisco activation.

1.3.4 Mutational studies on Rubisco activase

Multiple mutational studies have been performed in the past to elucidate the determinants of activase function, long prior to structural information becoming available. Based on this body of work, some critical residues involved in ATPase and activase function were identified by mutational and biochemical analysis and were consistent with AAA+ domain architecture. Fig. 1.10 provides an overview of the prior mutational studies conducted on Rubisco activase and highlights residues

responsible for particular functions. The work has been reviewed by Portis (2003) and Stotz et al. (2011).

Fig. 1.10: Domain architecture of Rubisco activase with a bird eye view of mutational studies at specific region. Specific regions are mentioned (N-term: N-terminal domain. NBD: nucleotide binding α/β -subdomain. 4-hb: 4-helix bundle subdomain. C-ext: C-terminal extension). Residues are highlighted (tobacco numbering) with their specific effect. Reproduced from [158].

In many AAA+ proteins, the N-terminal domain acts as an adaptor for engaging substrate proteins. Consistently, mutations and truncations of the N-terminal region had little effect on ATPase activity but critically impaired Rubisco activation [159, 160]. The conserved tryptophan (16) residue was critically implicated in Rubisco activation in tobacco and spinach. The deletion of residues upstream of this tryptophan residue preserved the activation function, while in spinach, truncating just the initial 12 (W11 in spinach) residues led to loss of activation function [159, 160]. Furthermore, deletion of the initial 50 residues at the N-terminal domain led to no loss of ATPase function but almost complete loss of Rubisco activation without any observed increase in intrinsic fluorescence [159]. This suggests that the N-terminal domain is associated with Rubisco activation alone and not with ATPase function.

Critical residues were identified to be crucial for ATPase activity in the NBD even before Rca was classified as an AAA+ protein. The residues identified, belong to the featured motifs of typical AAA+ proteins and are important for maintaining ATPase function. For instance, lysine (116) at the Walker A motif was implicated in nucleotide binding using photo-affinity labelling experiments with nucleotide

analogues [148, 161]. Other residues including glutamine (114) and lysine (112) were also found to be important for ATPase activity [148, 162]. The sensor 1 region and Walker B motif was suggested to be involved in nucleotide hydrolysis and in coordination of ATP [163], while arginine finger (241, 244) and a lysine (247) in its vicinity were responsible for intersubunit communication or oligomerization and nucleotide hydrolysis [164, 165]. The mutations of glutamine (114) to aspartate or glutamate led to a stimulation of ATPase activity and enhanced activation. The acidic substitution mutant showed less inhibition by ADP suggesting its involvement in ADP inhibition [162, 166].

Importantly, the two amino acid substitutions (D316K and L319V) targeted at a small helical insertion in the C-terminal 4-helix bundle domain were found to confer Rubisco specificity between Solanaceae and non-Solanaceae plant family [132]. The corresponding interaction partner residues R/P89 and K/D94 (spinach numbering) were later found in Rubisco [167, 168]. Since the residue substitutions involved in specificity reversibility were charge switched mutations, a direct physical interaction was suggested between Rubisco and Rca due to formation of a salt bridge between these residues [132].

The two conserved arginine residues in the vicinity of sensor 2 have been found to be important for ATP hydrolysis. Mutation of arginine to alanine (R294A/R296A) led to reduced ATPase and activation activity [165]. Mutation of R294 also affects oligomerization and another variant of this mutant (R294V) in tobacco was observed to form stable hexamers in presence of Mg.ATP γ S and possessed a WT-like biochemical phenotype [140, 141, 158].

The C-terminal extension of plant Rca is less conserved as compared to central AAA+ subdomains and varies in length in the two Rca isoforms (α and β) in many species. Mutation of the conserved tyrosine (Y361A) completely abolished ATPase and activation activity in tobacco [129]. Truncation of the C-terminal (Δ C363) reduced ATPase activity but stimulated activation rate, while another truncation (Δ C360) led to a total loss of ATPase and activase functions indicating the role of conserved tyrosine in regulation [129, 160]. Furthermore, mutation of two conserved cysteines in the extended C-terminus of the α isoform reduced its sensitivity towards ADP inhibition suggesting the role of C-terminus in redox regulation [146]. The role of the

flexible C-terminus in allosteric regulation has been demonstrated for other AAA+ protein spastin [128].

1.3.5 The role of Rubisco activase in regulating photosynthesis at elevated temperatures

Since Rubisco activase is critically responsible for maintaining the activation state of Rubisco *in vivo*, the mechanism and regulation of Rca are the focus of current research. However, Rca is a thermolabile enzyme and inactivation of Rca often limits photosynthetic efficiency at moderately elevated temperatures [11]. Therefore, activase with improved thermal stability is believed to be result in plants with improved photosynthetic performance and growth phenotypes under moderate heat stress [12, 18, 169]. This hypothesis has been strongly supported by expressing more thermostable variants of Rca in arabidopsis, which resulted in plants with higher rate of photosynthesis and growth at elevated temperature [12, 13].

It has also been argued that deactivation of Rubisco at elevated temperature may be a regulatory response to protect the plant during heat stress [16, 17, 170]. Photosystem II was considered as the major cause of thermal damage in the past [171] however, PS II was reported to be unaffected at temperature that inhibits CO₂ fixation [172, 173]. Other electron transport limitations such as RuBP regeneration, stability of the thylakoid membrane and ATP/ADP levels were also supposed to constrain photosynthesis at high temperature [16, 174, 175]. Also, the solubility of CO₂ and Rubisco specificity ($S_{c/o}$) reduces at high temperature favouring the oxygenation reaction leading to production of more misfire products [99, 176]. Therefore, it was hypothesized that activase may function as a fuse to reduce Calvin-Benson cycle flux as a regulatory mechanism to protect Rubisco and the photosynthetic apparatus at high temperature [16].

It has been mentioned that conformational flexibility of Rca is required for it to remodel Rubisco conformation and this likely be the reason why Rca cannot be thermostable [15]. Furthermore, research comparing a warm-season plant and a cool season plant also showed that there was a trade-off between Rca thermostability and its activity at low temperature [15, 154, 169]. The analysis of different genetic variants of rice yielded similar results where a rice species (*Oryza australiensis*)

endemic to hot and arid zone displayed higher thermotolerance than the crop species (*Oryza sativa*) at the cost of catalytic activity [177].

Fig. 1.11: Activase based model of thermal inhibition of photosynthesis at high temperature. At high temperature, activase denatures and thus fails to maintain the activation state of Rubisco, impairing photosynthesis.

The stability of Rca varies between different species, with α isoform reported to be more thermostable than the β isoform in spinach but not in cotton and arabidopsis [147, 166] where both isoforms have similar thermostability. In spinach, the α isoform also confers its thermostability to the β isoform [141, 178]. Rca shows enhanced thermostability in presence of nucleotide, with ADP and ATP guarding the β isoform of arabidopsis and cotton against heat inactivation [134, 138]. Improved thermotolerance of the α and the β isoform was observed in spinach in presence of ADP, while the α isoform of spinach showed a dramatic increase in its thermal stability in presence of ATP γ S [141, 178]. The molecular mechanism underlying the thermolability of photosynthesis is of particular pertinence due to the predicted effects of global climate change [18]. Further insights from structural and biochemical work may generate the possibility to discover and engineer more thermostable variants.

1.4 Aim and scope of the study

Rubisco activase is critical for maintenance and regulation of photosynthesis in plants. However, the thermolability of activase limits its ability to perform its function at supraoptimal temperature. It has been suggested that improving activase properties would result in plants with improved photosynthetic properties and growth [10, 12, 18]. The mechanism of this AAA+ remodelling enzyme has remained elusive in spite of recently available incomplete three dimensional crystal structures [129-131]. The aim of the current study therefore was to take advantage of newly gained structural and biochemical understanding to obtain a comprehensive mechanistic view of the behaviour of this agriculturally relevant Rubisco activation system in the key crop plant *Oryza sativa* (rice). Additionally, characterization of a Rca homologue from a challenging desert environment was undertaken to explore the possibility of discovering naturally thermostable plant Rubisco activases. This study aimed to generate a comprehensive molecular toolbox to address pertinent questions in plant Rubisco regulation from a biochemical angle. In long term, we aim to build a solid biochemical foundation for the next wave of crop improvement technologies, targeting at enhancing efficiency of photosynthesis to improve crop yield.

2. Materials and Methods

2.1 Chemicals

All the chemicals used in the study were of at least analytical grade. Chemicals other than the ones listed below were purchased from Sigma-Aldrich:

Reagent	Supplier
Glycerol	Affymetrix
Sodium phosphate, monobasic, 98%, ACS reagent grade	
Yeast extract	
Coomassie brilliant blue, G-250	Applied Chemistry
Bacto agar	BD, Difco
Tryptone	BD, Difco
30% Acryl/ bis solution 37.5:1	Bio-Rad
EDTA disodium salt di-hydrate	
Ethidium bromide (10 mg/ml Sol.)	
TEMED	
Tris	
dNTP Set 100 mM solutions	Fermentas
X-Gal	
Bleach 5x concentrate	GB bleach
Kanamycin sulphate	Gibco
Ampicillin	Gold Biotechnology
Chloramphenicol	
IPTG	
SDS, Sodium dodecyl sulphate	Hoefer
SYBR Safe® DNA gel stain	Invitrogen
Acetic acid (Glacial) 100% anhydrous	Merck
Ethanol 99.5-99.8%, absolute, GR grade for analysis	
Methanol, GR, min 99.8%	
N,N-Dimethyl formamide AR	
Sodium chloride	
Sodium hydroxide, analysis grade pellets, 99%	
Magnesium chloride hexahydrate	MP Biomedicals

Surface disinfectant	Pharmacon-Virkon
Ammonium persulphate (molecular grade)	Promega
Proteinase K	
Phosphoenolpyruvate (PEP)	Roche
Creatine Kinase	
DTT, Cleland's Reagent	Thermo Scientific
HisPur™ Ni-NTA resin	
Agarose (molecular grade)	Vivantis
Polyethylene glycol (PEG) 3350 20% (v/v)	Sigma

Table 2.1: Chemicals

2.2 Enzymes and kits

Enzymes	Supplier
Pfu and Taq DNA Polymerase	Fermentas
Restriction enzymes - SacII, Hind III, XbaI, NdeI, BamHI, NotI	New England Biolabs
T4 DNA Ligase	
calf intestinal phosphatase (CIP)	
Pyruvate kinase/ Lactate dehydrogenase from Rabbit muscle	Sigma
Glyceraldehyde-3-P dehydrogenase	
Triose-P Isomerase/Glycerol-3-P dehydrogenase	
Creatine P-kinase from rabbit muscle	Roche
Kits	Supplier
pGem-T vector system	Promega
Wizard® Plus SV MiniPreps DNA purification kit	
Wizard SV Gel and PCR Clean-Up System kit	

Table 2.2: Enzymes and kits

2.3 Instruments

Instruments	Supplier
TB2 Thermo block	BioMetra
Professional TRIO thermocycler	
Mini-protean tetra system	Bio-Rad
Power pac Basic	
Power pac HC	
Thermo mixer comfort	Eppendorf
PL-600 pH/mV/Temp meter	Ezodo
Akta Purifier Frac900, Akta micro	GE health care
Superdex 200 16/600, 10/300, 3.2/30	
MonoQ HR 10/100	
SourceQ 15 and 30	
Minicentrifuge	Green liv
Z300K refrigerated Bench top centrifuge	Hermle
Safe imager 2.0	Invitrogen
Trans-Blot	
Semi dry Transfer cell	
Bench top Microcentrifuge	Kintaro
Excella E25 Incubater	New Brunswick
Innov0 44R	
Rocker 400	Rocker
Vortex genie2	Scientific industries
UV-1800 spectrophotometer	Shimadzu
Cps controller	
Sorval Micro centrifuge	Thermo Scientific
Nanodrop 2000	
Forma 900 (-80 °C)	
MaxQ 6000 incubator	
Cimarec strirrer	
Sonics Sonicator	Vibra cell

Table 2.3: Instruments

2.4 Buffers and media

Media were prepared with deionised, double-distilled water and subsequently autoclaved.

LB medium:

- 10 g/l Tryptone
- 5 g/l Yeast extract
- 10 g/l NaCl
- (15 g/l agar for solid medium)
- pH 7.1 adjusted with NaOH

2.4.1 Buffers composition

Buffers	Composition
Coomassie destaining solution	10% ethanol, 7% acetic acid
Coomassie staining solution	0.1% (w/v) Serva Coomassie Blue R-250, 40% ethanol, 8% acetic acid
5xDNA loading dye	0.1M EDTA, 0.1%(w/v) SDS, 0.025% (w/v) bromo phenol blue, 40% glycerol
Electrophoresis migration buffer	120 mM Tris-HCl, 134 mM glycine, 0.1% SDS
TAE	40 mM Tris-acetate, pH 8.5, 2 mM EDTA
TBS	50 mM Tris-HCl pH 8.0, 137 mM NaCl, 2.7 mM KCl
TBS-T	50 mM Tris-HCl pH 8.0, 137 mM NaCl, 2.7 mM KCl, 0.1% Tween 20
PonceauS staining solution	0.1%(w/v) PonceauS, 5% aceticacid
Lysis buffer A	50 mM Tris-HCl pH8.0, 50 mM NaCl, 10 mM imidazole
Lysis buffer B (Elution buffer)	50 mM Tris-HCl pH 8.0, 50 mM NaCl,200 mM imidazole
Purification buffer A	20 mM Tris-HCl pH8.0, 50 mM NaCl
Purification buffer B	20 mM Tris-HCl pH8.0, 1M NaCl
Rubisco extraction buffer	50 mM Tris-HCl pH 8.0, 50 mM NaCl, 1 mM Na ₂ EDTA, 5 mM DTT, 1 mM PMSF, 12.5% glycerol,

	2% PVPP
Rubisco resuspension buffer	50 mM Tris-HCl pH 8.0, 50 mM NaCl, 1 mM Na ₂ EDTA, 5 mM DTT, 12.5% glycerol
Rubisco purification buffer A	Purification Buffer A + 0.1 mM Na ₂ EDTA, 1 mM DTT, 12.5% glycerol
Rubisco purification buffer B	Purification Buffer B + 0.1 mM Na ₂ EDTA, 1 mM DTT, 12.5% glycerol
Rubisco dialysis buffer	Same as Rubisco purification Buffer A
5x SDS-sample buffer	225 mM Tris-HCl pH 6.8, 50% glycerol 5% SDS (w/v), 5% β-mercaptoethanol 0.01% bromphenolblue (w/v)
Western blot transfer buffer	50 mM Tris-HCl, 20% methanol, 192 mM glycine

Table 2.4: Buffers Composition

Buffers used for protein purification were vacuum filtered and degassed.

2.5 Oligonucleotides

Oligonucleotides used in the study were purchased from IDT (Singapore, Singapore).

Name	Sequence
ΔN67OsRcaSacIIfor	CTCCGCGGTGGTGACTTCGACAACACC
T7 Term	GCTAGTTATTGCTCAGCGG
OsRcaW15Afor	CAGGACAGGGCGAAGGGTCTC
OsRcaW15Arev	GAGACCCTTCGCCCTGTCCTG
OsRcaS23Afor	GCCTACGACATCGCCGATGACCAGCA
OsRcaSS23Arev	TGCTGGTCATCGGCGATGTCGTAGGC
OsRcaS23Dfor	GCCTACGACATCGACGATGACCAGCAG
OsRcaS23Drev	CTGCTGGTCATCGTCGATGTCGTAGGC
OsRcaF68Lfor	ACGTACGACCTCGACAACACCATG
OsRcaF68Lrev	CATGGTGTGTCGAGGTCGTACGT
OsRcaG73Dfor	GACAACACCATGGACGGCTTCTAC
OsRcaG73Drev	GTAGAAGCCGTCCATGGTGTGTC
OsRcaS90Tfor	GTCGTCCACATCACCAAGAACTTCATG
OsRcaS90Trev	CATGAAGTTCTTGGTGATGTGGACGAC

OsRcaT95Nfor	AAGAACTTCATGAACCTCCCCAAC
OsRcaT95Nrev	GTTGGGGAGGTTTCATGAAGTTCTT
OsRcaK115Afor	GGGTCAGGGAGCATCCTTCCAGT
OsRcaK115Arev	ACTGGAAGGATGCTCCCTGACCC
OsRcaA143Vfor	AGCGGCAACGTGGGAGAGCCG
OsRcaA143Vrev	CGGCTCTCCACGTTGCCGCT
OsRcaG144A for	GGCAACGCCGCAGAGCCGGCG
OsRcaG144A rev	CGCCGGCTCTGCGGCGTTGCC
OsRcaE145A for	GGAGCGCCGGCGAAGCTGATC
OsRcaE145A rev	GATCAGCTTCGCCGGCGCTCC
OsRcaK148Afor	CCGGCGGCGCTGATCAGGCAG
OsRcaK148Arev	CTGCCTGATCAGCGCCGCCGG
OsRcaQ152Afor	CTGATCAGGGCGCGGTACCGT
OsRcaQ152Arev	ACGGTACCGCGCCCTGATCAG
OsRcaE156Afor	TACCGTGCGGCGGCAGACATC
OsRcaE156Arev	GATGTCTGCCGCCGCACGGTA
OsRcaD159Afor	GCGGCAGCCATCATCAAGAAGGGG
OsRcaD159Arev	CCCCTTCTTGATGATGGCTGCCGC
OsRcaK162Afor	GCAGACATCATCGCGAAGGGGAAG
OsRcaK162Arev	CTTCCCCTTCGCGATGATGTCTGC
OsRcaD173Afor	CTTCATCAACGCTCTGGACGCGG
OsRcaD173Arev	CCGCGTCCAGAGCGTTGATGAAG
OsRcaY214Afor	CCCGGGATGGCCAACAAGGAGGAC
OsRcaY214Arev	GTCCTCCTTGTTGGCCATCCCGGG
OsRcaN215Afor	GGGATGTACGCCAAGGAGGACAAC
OsRcaN215Arev	GTTGTCCTCCTTGCGTACATCCC
OSRcaK216Afor	ATGTACAACGCGGAGGACAACCC
OSRcaK216Arev	GGGGTTGTCCTCCGCGTTGTACAT
OsRcaE217Qfor	TACAACAAGCAGGACAACCCCGT
OsRcaE217Qrev	ACGGGGGTTGTCCTGCTTGTTGTA
OSRcaD218Afor	AACAAGGAGGCCAACCCCGTGTC
OSRcaD218Arev	GGGGTTGTCCTCCGCGTTGTACAT

OSRcaN219A for	AAGGAGGACGCCCCCGTGTC
OSRcaN219Arev	GACACGGGGGGCGTCCTCCTT
OsRcaP220Afor	AAGGAGGACAACGCCCCGTGTC
OsRcaP220Arev	GACACGGGGCGTTGTCCTCCTT
OsRcaR293Vfor	TTCGGCGCTCTTGTAGCCCGTGTTTAC
OsRcaR293Vrev	GTAAACACGGGCTACAAGAGCGCCGAA
OsRcaK315Dfor	AACATTGGCGACAGGCTGGTGAAC
OsRcaK315Drev	GTTACCAGCCTGTCGCCAATGTT
OsRcaR321Afor	CTGGTGAACTCGGCGGAGGGCCCA
OsRcaR321Arev	TGGGCCCTCCGCCGAGTTCACCAG
OsTrx-fC42S for	TGGTGCGGCCCTTCCAAGGTGATGGCA
OsTrx-fC42Srev	TGCCATCACCTTGGAAGGGCCGCACCA
OsRca Δ 21_SacIIfor	AACCGCGGTGGTGAGGAGGGGCG
OsRca Δ 21_NotIrev	TTGCGGCCGCTTAATCATCTAAATTCTT
pUC57(-Apo1)for	ACGGCCAGTGAAATCGAGCTCGGTA
pUC57(-Apo1)rev	TACCGAGCTCGATTTCACTGGCCGT
OsRca β (G738A)for	GGGCGTATGGAGAAATTCTACTGGGCT
OsRca β (G738A)rev	AGCCAGTAGAATTTCTCCATACGCCC
AtRca α G381_K382ins(S)for	AAATTTCTACGGATCCAAAGCGGCCCAG
AtRca α G381_K382ins(S)rev	CTGGGCCGCTTTGGATCCGTAGAAATTT
OsRca α G381_Q382ins(S)for	TTCTTTCTACGGATCCCAAGGAGCACAG
OsRca α G381_Q382ins(S)rev	CTGTGCTCCTTGGGATCCGTAGAAGGAA

Table 2.5: Primers used in this study

2.6 Molecular biology methods

2.6.1. DNA analytical methods

UV spectroscopy (Nanodrop 2000 (Thermo scientific)) was used to determine the DNA concentrations. The measurements were carried out at 260 nm, where one absorption unit equals to 50 ng/ μ l of DNA. DNA samples were subjected to horizontal agarose gels electrophoresis for separation according to size. The DNA samples to be applied on the gel were mixed with 5X DNA loading dye. Agarose gel electrophoresis was performed in 1 % (w/v) TAE-agarose gels supplemented with

SYBR-SAFE (1:10000) for DNA visualization in 1X TAE buffer. The gels were run at constant 100 V and DNA fragments were visualized by blue light (Invitrogen). Cloned constructs were verified using a restriction enzyme digest analysis with suitable restriction enzymes and DNA sequencing by 1st base (Singapore). For one sequencing reaction, 1 µg of plasmid was needed. Sequencing results were analysed using the programs BioEdit (Ibis Biosciences, USA) and MultAlin (<http://multalin.toulouse.inra.fr/multalin/>) for comparative sequence alignment analysis.

2.6.2 Competent *E. coli* cells preparation and transformation

Chemically competent DH5α and BL21 cells were prepared by the CaCl₂ method [179]. Cells from glycerol stock were revived by streaking onto an agar plate and growing overnight (8-16 h) at 37 °C. A single colony was inoculated in LB media and grown overnight in INNOVA 44R incubator shaker (New Brunswick Scientific) at 37 °C, 220 rpm. The overnight grown culture was used to inoculate 1 l of media and grown to mid-log phase (OD₆₀₀ = 0.5) at 37 °C, 220 rpm. After chilling the cells on ice for 20 min, the culture was centrifuged for 30 min at 4000 rpm at 4 °C and pelleted cells were resuspended gently in 20 ml ice-cold 0.1 M CaCl₂, 15 % glycerol (autoclaved). The stock culture was prepared as stated above and stored at -80 °C until used.

For transformation, 50 µl of chemically competent cells were thawed on ice and mixed with ~100 ng plasmid DNA. After incubation on ice for 30 min, cells were heat shocked at 42 °C for 90 s and allowed to cool down before 200 µl recovery media (LB media without antibiotics) were added. Cells were incubated with shaking at 37 °C and 1200 rpm for 45 min to 1 hr. Cells were spun down at 8000 rpm for 30 sec, 150 µl supernatant media was discarded and the pellet was resuspended in the remaining supernatant to spread on to selection plates (LB-Agar plates) supplemented with respective antibiotics (200 µg/ml ampicillin, 34 µg/ml chloramphenicol, 30 µg/ml kanamycin) and incubated overnight at 37 °C. For T-Vector, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-GAL) was also supplemented to the agar plates for subsequent screening of colonies with insert. A single colony of the transformants was picked and inoculated in liquid medium for further analysis.

2.6.3 Plasmid and DNA-fragment purification

For the amplification of plasmid DNA, *E. coli* DH5 α were grown for 12-16 h in LB medium containing the appropriate antibiotics at 37 °C at 220 rpm. Plasmids were subsequently purified via anion exchange chromatography using the Wizard Plus SV Miniprep DNA Purification System (Promega Corporation, USA), according to the manufacturer's instructions. Concentration of the isolated plasmid was determined by UV spectroscopy by measuring absorbance at 260 nm using the Nanodrop 2000 Spectrophotometer (Thermo Scientific, Singapore). Restriction digestion followed by agarose gel electrophoresis was performed to verify the presence of the insert and plasmid DNA. In order to purify or isolate DNA-fragments after agarose gel electrophoresis or enzymatic reactions, the Wizard SV Gel and PCR Clean-Up system was used according to the manufacturer's instructions.

Reagent	Volume
10x NEB Buffer	2 μ l
Restriction enzyme 1	0.3 μ l
Restriction enzyme 2	0.3 μ l
Plasmid/DNA	1 μ g
Autoclaved water	Variable
Total volume	20 μl

Table 2.6: Restriction digestion reaction mix

2.6.4 Polymerase chain reaction (PCR)

Amplification of DNA fragments with point mutations and insertions was achieved by PCR. To amplify genes from plasmids and after site directed mutagenesis, a high fidelity polymerase with proof reading ability, Pfu polymerase (Thermo scientific, Singapore) was used.

2.6.4.1 DNA amplification by PCR

Plasmid/genomic DNA was used as template for PCR, set up in a total volume of 20 μ l on ice with the appropriate concentration of the constituents as mentioned below.

Reagent	Volume
Pfu 10X Reaction Buffer w/20 mM MgSO ₄	2 µl
10 µM forward primer	0.5 µl
10 µM reverse primer	0.5 µl
Pfu DNA polymerase (1.2U)	0.5 µl
Template DNA	1-30 ng
2 mM dNTP	2 µl
Autoclaved water	Variable
Total Volume	20 µl

Table 2.7: PCR Reaction mix

All reagents were pipetted into a PCR tube kept on ice all the time, mixing of the reagents was done by short centrifugation. PCR thermo cycler (BioMetra) was pre heated to 95 °C before reaction tubes were placed inside the machine. The following PCR thermocycler program was used:

Cycle Steps	Temperature	Time	Cycles
Lid	95 °C		
Initial denaturation	95 °C	2 min	1
Cycle denaturation	95 °C	20 sec	35
Annealing	Variable	30 sec	
Extension	72 °C	1min/kb	
Final extension	72 °C	10 min	1
End	16 °C	∞	

Table 2.8: Temperature cycles for DNA amplification by PCR

For cloning into pGemT vector, polyA tail amplification was done by the addition of 0.2 µl of Taq polymerase and 2 µl of 2 mM dATP to the amplified DNA followed by incubation at 72 °C for 30 min in the PCR thermocycler to introduce a desoxyadenosine (dA) tail via ligating with the desoxythymidine overhang. dA-tailed PCR product was mixed with 6x DNA loading dye (Thermo Scientific, Singapore) prior to loading into 1% TAE-agarose gel (40 ml) supplemented with 4 µl of SYBR Safe DNA Gel Stain (Invitrogen, Singapore). Five µl of GeneRuler 1kb DNA Ladder (Thermo Scientific, Singapore) was loaded to serve as marker. Agarose gel electrophoresis was performed in 1xTAE buffer (40 mM Tris-acetate, pH 8.5, 2 mM EDTA) at 100V for 30-45 min and DNA fragments were then purified from the gel using the Wizard SV Gel and PCR clean-Up system (Promega, USA) according to the

manufacturer's protocol. The product was finally eluted in 50-70 μ l of nuclease free water.

2.6.4.2 Site-directed mutagenesis

Site-directed mutagenesis was used to introduce point mutations and insertions in existing plasmids. Site directed mutagenesis of existing plasmids was done using QuikChange protocol (Stratagene) according to manufacturer's instructions (Table 2.9 and 2.10). Forward and reverse primers were self-complimentary and contained the desired mutations (Table 2.5). After the PCR, 0.5 μ l of DpnI were added to a 10 μ l of the reaction mix and incubated at 37 °C for 1 h to digest the methylated template plasmid before transformation into *E.coli* DH5 α .

Reagent	Volume
Pfu 10X Reaction Buffer w/20 mM MgSO ₄	2 μ l
10 μ M forward primer	0.72 μ l
10 μ M reverse primer	0.72 μ l
Pfu DNA polymerase (1.2U)	0.38 μ l
Template DNA	30 ng
2 mM dNTP	2 μ l
Autoclaved water	Variable
Total Volume	20 μl

Table 2.9: PCR Reaction mix

Cycle Steps	Temp.(°C)	Time	Cycles
Lid	95 °C		
Initial denaturation	95 °C	2 min	1
Cycle denaturation	95 °C	30 sec	18
Annealing	Variable	1 min	
Extension	68 °C	15 min	
Final extension	68 °C	15 min	1
End	16 °C	∞	

Table 2.10: Temperature cycles for DNA amplification for site-directed mutagenesis

2.6.5 Restriction digestion and ligation

Expression vector for cloning or vector containing gene of interest or amplified PCR product (1 μ g DNA) were digested with appropriate restriction enzymes (20-60 U) in 20 μ l reactions with respective buffer according to manufacturer's recommendations.

The digestion reaction was carried out at 37 °C for 3 h or overnight (Table 2.6). Digested plasmid vector DNA was incubated for 1 h at 37 °C with calf intestinal phosphatase (CIP) for dephosphorylation. Restriction digestion of the plasmid produces linearized plasmids (vector) and DNA fragments flanked with respective overhangs. The digests, plasmid, and inserts, were separated by 1% agarose gel electrophoresis and the respective DNA bands were extracted for purification by the Wizard Plus SV gel clean DNA Purification System. Concentrations of the purified insert and plasmid DNA were measured using a Nanodrop2000 (Thermo-scientific, Singapore).

Following purification, ligation of purified DNA fragments (insert with the vector) was carried out at room temperature (RT) for 1 h or overnight at 4 °C. The ligation reaction was performed in a total reaction mix of 10 µl containing ~100 ng of digested plasmid DNA and ~500 ng DNA insert incubated with 200 U of T4 DNA ligase (Thermo-Scientific). The reaction was conducted in T4 DNA ligase buffer. The pGem-T vector system (Promega) was used according to manufacturer's instructions, for ligation of dA tailed PCR products. Following the ligation, the reaction mixture was either heat shock transformed into *E. coli* DH5α cells or DNA precipitation was carried out to prepare the ligated vectors for sequential restriction enzyme digestion. Appropriate constructs were verified by purifying the plasmid DNA from the transformed colonies grown overnight at 37 °C on the selective agar plates and subjecting to restriction digestion followed by sequencing the digest using 1st base (Singapore). The sequencing results were validated by Multalin and BioEdit programs.

2.6.6 General cloning strategy

The PCR product was polyadenylated at both the 5' and 3' ends to allow cloning of PCR product into a vector. The polyadenylated PCR product was run on 1% agarose gel and the DNA fragments were purified from the gel using the Wizard SV Gel and PCR Clean-Up system according to the manufacturer's instructions. Ligation of the polyadenylated PCR product into pGem-T was carried out according to manufacturer's instruction. The ligation mixture was transformed into DH5α and plated on an ampicillin LB plate (200 µg/ml ampicillin) supplemented with 25 µl of 40 mg/ml X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) in

N,N-dimethylformamide (DMF). X-gal enables blue white screening and only white colonies containing insert were inoculated into 6 ml LB medium supplemented with ampicillin. This culture was grown overnight in a shaking incubator at 37 °C, 220 rpm. The plasmids were purified using Wizard Plus SV Miniprep DNA Purification System (Promega Corporation, USA) and their concentrations were measured using a Nanodrop 2000 Spectrophotometer (Thermo-scientific, Singapore) at 260 nm. The plasmids were sequenced by 1st base (Singapore). pGem-T vector with inserted sequences was digested with respective restriction enzymes either simultaneously or sequentially and the purified inserts were ligated into the pHue vector digested with the same enzymes and dephosphorylated by CIP.

In this study, the expression vector pHue was used for the heterologous expression of protein constructs in *E. coli*. Proteins were expressed with a N-terminal fusion to hexa-histidine tagged ubiquitin (His₆Ub), which improves solubility and enables easy protein purification by immobilized metal-ion affinity chromatography (IMAC) [180]. The resultant pHue vector was transformed in DH5 α and screened for ampicillin resistant colonies, which was eventually picked and grown in LB medium supplemented with ampicillin and the correct insert was confirmed by sequencing. Mutations in the inserts were generated by site directed mutagenesis. Cleavage of the tag by the deubiquitinating enzyme Usp2 leaves the native N-terminus of the protein of interest [181].

The coding sequence of the mature α (long) and the β (short) isoform of *Oryza sativa* Rubisco activase (Uniprot accession code: P93431-1 and P93431-2, expressed amino acids: 48-466 and 48-433 respectively), Thioredoxin-f of *Oryza sativa* (Uniprot accession code: Q8S091, expressed amino acids 73-186) and the α (long) and the β (short) isoform of Rubisco activase of *Agave tequilana* (locus 3705 and 27298, expressed amino acids: 57-474 and 58-435 respectively [182]) were synthesised (GenScript, USA) with flanking SacII and HindIII restriction sites at 5' and 3' sites respectively and were cloned in pUC57 plasmid. pUC57 plasmid bearing subsequent insert was transformed into DH5 α cells and plated on ampicillin LB-agar plate (200 μ g/ml). The successful colonies were used for the amplification of plasmid DNA in *E. coli* DH5 α which were grown for 12-16 h in LB medium containing ampicillin (200 μ g/ml) at 37 °C with 220 rpm shaking. The plasmids were isolated as described above

using Wizard Plus SV Miniprep DNA Purification System (Promega Corporation, USA) by following manufacturer's protocol. Both pUC57 and pHue plasmids were double or sequentially digested with SacII and HindIII restriction enzymes. The digested plasmid and plasmid insert was loaded on to 1% agarose gel and purified as described above. Ligation of these insert fragment with linearized plasmids was performed at room temperature for 1 h with a 10 μ l reaction mixture containing 1 μ l T4 DNA ligase buffer, 2 μ l vector, 6 μ l insert fragments and 1 μ l T4 DNA ligase (Thermo-Scientific). The reaction mixture was transformed into *E.coli* DH5 α cells and subsequently the plasmid was purified as described above. Approximately 500 ng of plasmid was test cut using SacII and HindIII, to check the presence of genes of interest. These plasmids were transformed into *E.coli* BL21 competent cells for protein purification.

The coding sequence of the N-terminally truncated Rubisco activase (residue 67-386) from *Oryza sativa* was amplified with the primer pairs Δ N67 OsRcaSacIIfor and T7term from the plasmid pHueOsRca β . The PCR product was subsequently dA tailed, cloned into vector pGemT and then double digested with SacII and HindIII and finally ligated into the vector pHue. The expression plasmid was named as pHue Δ N67OsRca β . Mutations in the inserts were generated by site-directed mutagenesis unless indicated otherwise with the appropriate primer pairs (Table 2.5). All the mutations were confirmed by sequencing (1st base, Singapore).

The WT template plasmids from rice and agave were used to introduced point mutations and insertions by PCR mutagenesis following the Quikchange protocol (Stratagene) for generating chimeric mutants of Rca with swapped domains and regions (pHueRca β AtNOsC, pHueRca β OsNAtC, pHueAtRca α OsTail, pHueOsRca α AtTail) as shown in Fig. 2.1. Primers were designed for the removal of ApoI site from pUC57 backbone of AtRca β and for the insertion of ApoI restriction enzyme site into OsRca β via a substitution mutation without changing the amino acid composition. Similarly, primers were designed for the insertion of serine into AtRca α and OsRca α after amino acid residue G381 to create a BamHI restriction enzyme site (Table 2.5) to facilitate the formation of chimeric mutant. Chimeric constructs between rice and agave were also cloned and expressed as His6-Ubiquitin fusions for protein purification.

Fig. 2.1: Schematic representation of the generation of chimeric mutants. N-terminal domain corresponding to the regions after the lysine 246 in short activase isoform was interchanged to generate two chimeric mutants. Similarly, C-tail extension region after glycine 381 was switched for long activase isoform of rice and agave to generate another two chimeric mutants.

2.7 Protein biochemistry

2.7.1 Protein analytical methods

Protein sequence alignment- Protein sequences were aligned using the programs MultAlign [183] or Esript [184].

Protein structure analysis – Pymol [185].

Protein quantification- Protein mixtures were quantified by Bradford (BioRad) assay using bovine serum albumin as a standard. Two to ten µg of bovine serum albumin (BSA) was added to a mixture of 500 µl autoclaved H₂O and 500 µl Bradford reagent and absorbance of the samples were measured at 595 nm on a spectrophotometer. Pure proteins were quantified by their absorbance at 280 nm by Nanodrop 2000 (Thermoscientific, Singapore) and the concentration was calculated using the extinction coefficients listed in Table 2.11. Extinction coefficients were calculated with the program ProtParam [186]. Extinction coefficients of the point mutations not listed were the same as for the respective wild-type OsRca. Final concentration of the protein is calculated using extinction coefficient and molecular weight.

$$C = (A_{280}/\epsilon) \times \text{Molecular Weight}$$

C- Concentration of the protein in mg/ml

A₂₈₀- Absorbance measured by Nanodrop at 280nm

ϵ – Extinction coefficient $M^{-1} cm^{-1}$

Protein	Extinction coefficient ($M^{-1} cm^{-1}$)	Molecular weight (Da)
OsRca α	45840	46485.5
OsRca β	44350	42961.6
OsTrx-f	12490	12569.7
$\Delta N67$ OsRca β	32890	35563.7
AtRca α	45840	46491.7
AtRca β	41370	42041.9
OsRbc (active site)	103250	67612.1
OsRca β W15A	38850	42846.5
OsRca β Y214A	42860	42869.5
Rca β AtNOsC	42860	42158.8
Rca β OsNAtC	42860	42358.2
OsRca α AtTail	45840	46498.5
AtRca α OsTail	45840	46652.8
$\Delta N21$ OsRca2	48360	42416.3
NtRca	39880	42759

Table 2.11: Extinction co-efficient and molecular weight

2.7.2 SDS-PAGE and Native PAGE

Protein analysis was performed under reducing, denaturing conditions by sodiumdodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) [187]. In this method, the proteins were resolved based on their molecular weight and electrophoretic mobility. Gels were prepared as per standard protocol [179]. Samples were mixed with 5X SDS-sample buffer, boiled for 5 min at 100 °C, and centrifuged for 2 min at RT at 16000 xg. Centrifuged samples were loaded into their respective wells and gel electrophoresis was performed in Mini Protean (Bio-rad) electrophoresis

chambers in SDS-electrophoresis migration buffer (with SDS) at constant voltage of 100 V for 20 min followed by 140-180 V for the rest of the run. The proteins were electrophoretically separated in denatured conditions.

Native-PAGE samples were mixed with 5x native loading buffer (without SDS and β -mercaptoethanol). Samples were not boiled and the gel and tank buffer solutions were without SDS. Samples were loaded into their respective gel and subsequently into Bio-Rad electrophoresis chambers in the presence of their respective running buffer (Table 2.4). Gel electrophoresis was carried out in Mini Protean (Bio-Rad) electrophoresis chambers in the presence of electrophoresis migration buffer employing a starting constant voltage of 100 V followed by 140-160 V for the rest of the run. The proteins were electrophoretically separated in native conditions at 4 °C and at RT in denatured conditions.

2.7.3 Coomassie blue staining of SDS and native PAGE

Proteins that were separated by electrophoresis were briefly washed in water before staining by incubation of the gel in Coomassie Brilliant Blue (40 % methanol, 10 % acetic acid, 0.1 % Coomassie Brilliant Blue R-250). Coomassie blue is a triphenylmethane coloring agent. This dye integrates with cationic, nonpolar, and hydrophobic side chains of a polypeptide. Hence, Coomassie blue staining was used for the visualization of protein bands in polyacrylamide gels. Protein bands were fixed and stained by incubating the gels for 1 h with Coomassie blue staining solution. Consequently, the gel was incubated in destaining solution for 1 h or more to remove the background staining until the bands were clearly visible.

2.7.4 Western blotting and immunodetection

After SDS-PAGE, proteins were transferred onto a nitrocellulose membrane using transfer buffer (50 mM Tris-HCl pH 8.0, 20% methanol, 192 mM glycine) in a semi-dry western blot apparatus (BioRad). This transfer was performed at 15 volts for 15 min or 10 volts for 30 min. The membrane was stained with Ponceau S staining solution (0.1% (w/v) Ponceau S, 5% acetic acid) to ensure efficient transfer and destained with Milli-Q water before proceeding with the immunodetection. The

membrane was blocked with 5% (w/v) skimmed milk powder in TBS (50 mM Tris-HCl pH8.0, 137 mM NaCl, 2.7 mM KCl) for 1 hour at room temperature.

The primary antibody anti-Rca or anti-Rubisco (Rabbit, polyclonal,) (Agrisera, Sweden) was diluted to 1:5000 in TBS-T (TBS, 0.1% (v/v) TWEEN 2.0) and incubated with the membrane for 1 h at RT. The membrane was washed thrice with TBS-T and subsequently incubated in TBS-T for 15 min. The secondary antibody peroxidase-conjugated anti-rabbit was diluted to 1:10000 in TBS-T and incubated with the membrane for 1 h at RT. The membrane was washed again (as above). The HRP coupled antibody was detected by enhanced chemiluminescence (ECL) staining with a 1:1 mix of ECL detection reagent 1 and 2 (Thermo Scientific) on an LAS-3000 Imaging system (Fuji).

2.7.5 Circular dichroism (CD) –spectroscopy

Structural integrity of purified Rca at different temperature was analyzed by CD-spectroscopy. Proteins were desalted into buffer (20 mM K_2HPO_4/KH_2PO_4 pH8.0) using the Bio-Spin6 chromatography column (Bio-Rad) according to manufacturer's instructions. Protein concentration was adjusted to a final concentration of 1 mg/ml and the CD spectra were recorded on a ChirascanTM CD-spectrometer (average of 3 passes, 240 nm to 190 nm, 0.1 nm data pitch, 20-50 °C).

2.7.6 Test expression of proteins

Glycerol stock of the BL21 cells carrying the activase and Rubisco constructs were inoculated in LB media supplemented with respective antibody and grown overnight at 37 °C with shaking (220 rpm) in an Innova44 incubator (New Brunswick Scientific). This overnight culture (50 µl) was subcultured into a couple of fresh 5 ml LB media supplemented with the same antibody. This culture was grown at 37 °C with shaking until the optical density reached ~0.6 at a wavelength of 600 nm (OD_{600}). Two different temperatures (37 °C and 23 °C) were tested for expression of proteins. One culture was moved to 23 °C with shaking, while the other was kept at 37 °C. Both cultures were induced with 0.5 mM isopropyl-D-thiogalactopyranoside (IPTG). For co-expression of construct GroELES, initially 0.2% L-arabinose was added and 30 min later 0.5 mM of IPTG was added to express the protein of interest

in the construct. Cells were harvested by centrifugation at 4000 xg for 10 min after 4 h or 12 h of induction. After removing the supernatant media, the cells were resuspended in 1 ml of lysis buffer (50 mM Tris-HCl pH 8.0, 50 mM NaCl). Cells were lysed on ice by sonication at 30% amplitude for 50 s using 10 s pulses alternated with 50 s of pause. The lysate (50 μ l) was added with 12.5 μ l 5X SDS sample buffer for analysis of total protein expression (total lysate sample). The lysate (300 μ l) was clarified by centrifugation at 6000 xg and 4 °C for 15 min and 50 μ l of the supernatant was added with 12.5 μ l 5X SDS sample buffer for analysis of soluble protein expression (soluble sample). After discarding the remaining supernatant, the pellet was washed with 300 μ l MilliQ water and centrifuged at 16000 xg and 4 °C for 15 min. The pellet was redissolved in 72.5 μ l 2X SDS sample buffer for analysis of insoluble protein expression (insoluble sample). The collected samples were heat denatured using a heat block at 100 °C and centrifuged at 16000 xg and RT for 3 min prior to loading on 12.5% SDS polyacrylamide gel. Five μ l of pre-stained marker (PageRuler Plus Prestained Protein Ladder 10-250 kDa; Thermo Scientific) was loaded alongside the samples. SDS-PAGE gel electrophoresis was performed at a starting voltage of 120 V followed by 160 V for the rest of the run.

2.7.7 Expression and purification

All the activase proteins and its variants used in this study were expressed in BL21 as a His₆Ub-fusion constructs using pHue expression vectors. BL21 cells carrying the respective plasmid construct were grown in LB supplemented with respective antibiotics at 37 °C in an Innova44R incubator (New Brunswick Scientific) at moderate shaking (200 rpm) to an optical density at a wavelength of 600 nm OD₆₀₀=0.6. The culture was then cooled down at 23 °C for ~20 min and induced with 0.5 mM IPTG for ~16 h at 23 °C. Following induction, the culture was allowed to grow for ~16 h at 200 rpm at 23 °C. After incubation, *E.coli* cells were harvested by centrifugation in JA-10 centrifuge with JA 10 rotor (Beckman Coulter) at 4000 xg for 20 min at 4 °C. Cell pellets were either stored at -20 °C or directly lysed as described.

2.7.7.1 Purification of His₆Ub-fusion constructs

His₆Ub-fusion constructs were captured by immobilized metal affinity chromatography (IMAC) and after cleavage of the ubiquitin moiety, further purified by anion exchange and gel filtration chromatography. Harvested cells from 1l of bacterial culture were resuspended in 40 ml lysis buffer A (50 mM Tris-HCl pH 8.0, 50 mM NaCl, 10 mM imidazole) supplemented with 0.3 mg/ml lysozyme to aid lysis and with 1 mM PMSF for protease inhibition just before sonication. The cells were disrupted by ultrasonication on ice for 5 min with a sonicator (15 s bursts with 30 s cooling on ice between bursts). Following removal of cell debris by centrifugation in an Avanti J-25 with JA25.50 rotor (Beckman Coulter) for 40 min at 4 °C and 20000 g, the supernatant was applied to an affinity column packed with Ni-NTA resin (Thermo Scientific) equilibrated with lysis buffer A (Table 2.4). The His₆Ub-fusion protein was eluted using elution buffer (50 mM Tris-HCl pH 8.0, 50 mM NaCl, and 200 mM imidazole). The ubiquitin moiety was cleaved overnight at 4 °C using the deubiquitylating enzyme Usp2. The protein solution was then applied to an anion protein exchanger MonoQ 10/100 column (GE); proteins were eluted with a linear salt gradient of 500 mM NaCl. Fractions containing protein of interest were combined and concentrated. Later, to further purify the proteins, size exclusion chromatography was performed by loading sample onto Superdex 200 16/600 column. Proteins were eluted with purification buffer A (Table 2.4). Fractions containing the protein were pooled and concentrated in an Amicon Ultra-15 centrifugal filter units (50-100 kDa cut-off) (Millipore, USA), 5% glycerol was added and the protein was stored in aliquots at -80 °C after flash-freezing in liquid N₂ for future use.

2.7.8 Purification of Rubisco from the leaves of *Oryza sativa*

Leaves of *Oryza sativa* were a kind gift from Prof. Prakash Kumar (NUS, Singapore). The *Oryza sativa* Rubisco (OsRbcL₈S₈) was purified from the leaves as previously described [188], with slight modifications. Briefly, 8 g of leaves from *Oryza sativa* were homogenized in liquid N₂ using mortar and pestle to make fine powder and extracted with 40 ml of Rubisco extraction buffer (50 mM Tris-HCl pH 8.0, 50 mM NaCl, 1 mM Na₂EDTA, 12.5% Glycerol) containing 1 mM PMSF, one tablet of protease inhibitor cocktail, 5 mM DTT and 2% Polyvinylpyrrolidone (PVPP). The

extract was filtered through four layer of miracloth. The cells were further disrupted by ultrasonication on ice for 5 min with a sonicator (15 s bursts with 30 s cooling on ice between bursts). Cell debris was removed by centrifugation in an Avanti J-25 with JA25.50 rotor (Beckman Coulter) for 30 min at 4 °C at 39000 xg. The supernatant was subjected to 33% ammonium sulphate ((NH₄)₂SO₄) precipitation and centrifuged for 30 min at 4 °C at 39000 xg. The supernatant was further subjected to 55% ammonium sulphate precipitation and centrifuged as earlier. The pellet obtained was dissolved in Rubisco resuspension buffer (Table 2.4). The resuspended solution was dialyzed extensively against Rubisco dialysis buffer overnight and applied to an anion protein exchanger MonoQ 10/100 (GE) column equilibrated with Rubisco purification buffer A. Protein was eluted with a linear salt gradient of up to 500 mM NaCl using Rubisco purification buffer B. Fractions containing Rubisco were combined and concentrated. To further purify the protein, size exclusion chromatography was performed by loading the protein onto a Superdex 200 16/600 column. Protein was eluted with Rubisco purification buffer A (Table 2.4). Fractions containing the protein were pooled and concentrated using Amicon Ultra-15 Centrifugal Filter Units (Millipore, USA). Protein was stored in aliquots at -80 °C after flash-freezing in liquid N₂.

2.7.9 Analytical size exclusion chromatography

Analytical size exclusion chromatography of proteins was performed on a Superdex 200 PC3.2/30 increase column equilibrated with buffer A or buffer A supplemented with 5 mM MgCl₂ and 1 mM ATP using Akta micro system (GE Healthcare). Protein-containing samples (50 µl each) were injected and runs were performed at RT at a flow rate of 0.075 ml/min in respective buffer. UV signal for the proteins were detected at 280 nm. The positive fractions were collected and analyzed with SDS or native PAGE gels. The column was calibrated with globular proteins of known molecular weights like blue dextran 2000 kDa, ovalbumin 44 kDa, conalbumin 75 kDa, aldolase 158 kDa, ferritin 440 kDa, thyroglobulin 669 kDa from HMW gel filtration calibration kit (GE Healthcare).

2.7.10 Enzymatic assay

2.7.10.1 ATPase assay

ATPase activity of the activases was measured spectrophotometrically using a coupled assay monitoring the oxidation of reduced nicotinamide adenine dinucleotide (NADH) [189] in buffer (100 mM Tricine pH 8.0, 5 mM MgCl₂, 2 mM phosphoenolpyruvate, 0.5 mM NADH, 2 mM ATP, 20 U/ml pyruvate kinase, 30 U/ml lactate dehydrogenase) with a final Rca concentration of 5 μM at 25 °C unless otherwise stated. The oxidation of NADH, which is directly coupled to the generation of ADP, was followed by the reduction in the absorbance at 340 nm on a UV-1800 spectrophotometer (Shimadzu). The final volume of the reaction was 100 μl.

Fig. 2.2: Representation of the reactions that occurred during an ATPase activity assay. A decrease in absorbance indicates oxidation of reduced Nicotinamide adenine dinucleotide (NADH), which is proportional to ATP hydrolysis rates.

2.7.10.2 Rubisco assay

Rubisco activity assay was performed as previously described [190] with minor modifications. This assay measures the oxidation of NADH spectrophotometrically in a 100 μl reaction volume using a UV-1800 spectrophotometer (Shimadzu) at 340 nm. The assay was performed in a buffer (100 mM Tricine pH 8.0, 5 mM MgCl₂) with 3 μl coupling enzymes mixture (creatine P-kinase (2.5 U/ml), glyceraldehyde-3-P dehydrogenase (2.5 units/ml), 3-phosphoglycerate kinase and triose-P isomerase/glycerol-3-P dehydrogenase (20/2 units/ml)), 20 mM NaHCO₃, 0.5 mM NADH, 2 mM ATP, 10 mM creatine-P, 1 mM RuBP, with a final concentration of 0.5

μM Rubisco active sites and 5 Rca protomer unless otherwise stated. The oxidation of NADH, which is directly coupled to the generation of 3PG, was followed by the reduction in the absorbance at 340 nm on a spectrophotometer.

Fully carbamylated Rubisco (ECM) complex was made by incubating 20 μM Rubisco active sites with 20 mM NaHCO_3 , 10 mM MgCl_2 in buffer A (Table 2.4). RuBP inhibited Rubisco (ER) complex was made in same buffer by incubating 20 μM Rubisco active sites with 4 mM EDTA for 10 min at 25 °C. Later to this, 1 mM RuBP is added to make the final ER complex. RuBP was synthesised enzymatically from ribose-5-phosphate [191] and purified using anion exchange chromatography [192].

Activity of Rubiscos (ECM, ER) was calculated by the 3PG generated and activation rate of inhibited Rubiscos (ER to ECM) complexes is measured by 3PG produced with the addition of the respective activases. The 3PG produced was plotted against time to determine the activity of Rubisco in the presence and absence of activase. The rate of 3PG produced is directly proportional to the activity of the Rubisco. For quantitative analysis of Rca and its variants, the rate of 3PG produced or the rate ECM formation from ER was measured for the initial 4 min and compared with its wild type activase relative to the background rate of Rubisco activation.

Fig. 2.3: Representation of reactions that occurred during a Rubisco activity assay. For each carboxylation of RuBP, two molecules of 3-phosphoglycerate (3-PG) were produced by Rubisco which were phosphorylated by 3-PG kinase (PGK). Glyceraldehyde-3-P dehydrogenase (GAPDH) reduces the bis-PGA to an aldehyde (Ga-3-P). Triose-phosphate isomerase (TIM) produces di-hydroxyacetone (DHAP) from Ga-3-P, glycerol-p dehydrogenase (GlyPDH) reduces DHAP to glycerol 3-phosphate (Gly 3-P). Four NADH being oxidized per RuBP, leading to a decrease in absorbance at 340nm.

2.7.10.3. Variations of enzymatic assay

To investigate the effects of Trx-f and DTT, 25 μM α isoform was incubated at room temperature with 3.6 μM rice Trx-f and/or 5 mM DTT for 10 min before conducting assays.

Thermostability test was done by heating the Rca at indicated temperature for 10 min then the assays were conducted at 25 °C as described above.

To investigate the effects of temperature on the ATPase activity and Rubisco activation by activases, reaction mixes were incubated at the respective temperatures on a heat block for 10 min prior to measurement. The chamber of UV-1800 spectrophotometer (Shimadzu, Japan) was also heated using its thermostat along with quartz cuvette to the respective temperatures prior to measurement for 10 min. The assay was initiated by mixing the activase to reaction mix after 10 min of heating and measurement was done at respective temperatures.

Subunit doping assays were performed by mixing mutant Rca in varying ratio with WT Rca for 10 min to yield a final concentration of 5 µM total Rca in reaction mix. The Rubisco reactivation assay was then performed as described in previous section. To demonstrate the poisoning effect of subunit doping, WT Rca concentration was kept constant and mutant concentration was varied in different proportion and then the ATPase assay was performed as routine. To observe and analyse the inhibitory or stimulatory effect of mutant Rca on WT Rca, data was fitted to the theoretical plots for the population of hexamers at various ratios of WT to mutant assuming random mixing generated by mathematical modelling as earlier reported for other AAA+ proteins [193, 194]. In this approach, mutant subunits are mixed with WT subunits to generate heterohexamer ensembles. Incorporation of mutant subunits into hexamers occurs according to a binomial distribution dictated by the WT: mutant ratio as:

$$P(x) = \binom{n}{x} p^x (1 - p)^{n-x} \quad (1)$$

Where P is the probability that a hexamer (therefore, $n=6$) contains x mutant subunits and p is the probability that a mutant subunit is incorporated. Experiments demonstrated that mutant and WT subunits have similar probability of being incorporated into a hexamer [193-195]. Consequently, p is calculated as the molar ratio of mutant and WT protein present:

$$p = \frac{\text{Mutant}}{(\text{Mutant} + \text{WT})} \quad (2)$$

Therefore, for any specified percentage of mutant subunits the probability distribution of Rca hexamers containing 0, 1, 2, 3, 4, 5 or 6 mutant subunits can be derived (see section 5). Activity versus p plots could then be generated assuming each WT subunit makes an equal contribution to the total activity ($1/6^{\text{th}}$ per subunit). Consequently, if subunits within the hexamer operate independently then activity should decline in a linear manner upon incorporation of defective mutant subunits. Conversely, if subunits are coupled then a specific number of subunits will be sufficient to eliminate activity. Thus zero activity is assigned to hexamers that are in breach of a specific threshold number of mutant subunits. In this way activity versus p plots could be generated assuming one or more, two or more, three or more, four or more, five or more mutant subunits are required to eliminate activity.

The basic assumptions applied for developing mathematical models can be summarised as follows [194]:

1. The functional unit is hexamer
2. All subunits have similar tendencies to form oligomers.
3. Incorporation of one malfunctioning subunit into a hexamer reduces the activity by one-sixth of the wild type activity.
4. Within the observation window, activity is proportional to the number of active hexamers; thus bigger the fraction of the active units, the more activity is observed.
5. If inter-subunit coupling occurs, a certain number of incorporated mutant subunits into the hexamer causes complete loss of activity.

Assumptions 1-4 account for a linear decrease of activity upon incorporation of subunits as would be expected if subunit activity was independent of its neighbour, whereas assumption 5 includes a basis for inter-subunit coupling that gives rise to steep, non-linear decrease of activity.

3. Biochemical characterization of the Rubisco activation system of the staple crop *Oryza sativa* (rice)

In plants, Rubisco activase (Rca) is an essential molecular chaperone due to its necessary role in maintaining Rubisco activity *in vivo* [110]. Rca is highly sensitive to heat stress which limits its capability to sustain the activation state of Rubisco at moderately high temperature [10, 11]. Studies performed on arabidopsis revealed that improving the thermostability of activase can increase growth and photosynthetic efficiency under moderate heat stress [12, 13]. Therefore, Rca is considered as a promising target for crop improvement, especially considering predicted climatic changes in future [18]. However, almost all detailed biochemical data concerning Rubisco activase function is derived from organisms such as arabidopsis, tobacco and spinach. This work focussed on biochemical characterization of the Rubisco activation system of an economically and socially important global crop species, *Oryza sativa* (rice) which could be genetically modified for facile application and commercialisation derived from such biochemical work.

Rice possesses two isoforms of Rca as a result of alternate splicing of a single gene transcript into a long (α) isoform (46 kDa) and a short (β) isoform (43 kDa). The two isoforms are identical except for the α isoform having an additional 33 amino acids at the C-terminus, also substituting five amino acid immediately preceding this extension [144]. This C-terminal extension consists of a pair of cysteine residues responsible for regulating Rca activity via changes in the redox state of chloroplast, which in arabidopsis is modulated by thioredoxin-f [146]. The distribution and properties of Rca varies significantly among and within species. In most plants, the two isoforms are present in equimolar quantities, however, in rice, the β isoform is much more abundant than the α isoform [112, 144] under non-stressed conditions. Importantly, heat stress in rice leads to dramatic upregulation of Rca α [196]. Also, it was reported to be the most upregulated protein under heat stress in the wild rice species *Oryza meridionalis* [197], thus showing a relative changes in protein abundance and gene expression of the two Rca isoforms with heat. A biochemical characterization of Rca isoforms in rice has recently been reported [177]. In this chapter, we present a complementary detailed characterization of the two Rca isoforms covering various aspects of activase function such ATPase and Rubisco activase activity, redox regulation, thermostability and subunit-subunit interaction to interrogate this Rubisco activation system from the model plant *Oryza sativa*.

3.1 Protein Purification

3.1.1 Purification of *Oryza sativa* Rubisco activase (Rca) isoforms

Long and short isoforms of Rubisco activase from *Oryza sativa* (OsRca α and OsRca β respectively) were cloned and purified as described in materials and methods. All Rca constructs and mutants used in the study were heterologously expressed in *E.coli* BL21 as a N-terminal fusion with a hexa-histidine tagged ubiquitin (His₆Ub) to improve the solubility of the respective protein upon expression [180, 181].

Fig. 3.1: Representative purification of OsRca α and OsRca β . (A and B) 12.5% SDS-PAGE analysis. Successive purification by IMAC (Ni-NTA), followed by USP2 mediated cleavage of N-terminal tag and anion exchange chromatography (AIEC) using a MonoQ 10/100 column yielded pure protein. (M: Molecular Weight Marker, L: Lysate, S: Soluble, Ni-NTA: Fractions after IMAC, U: Unbound, W: Wash, E: Final eluate, Cut: eluate after USP2 mediated cleavage of Ubiquitin. The numbers indicates the SDS samples from respective fractions of AIEC). (C and D) Size exclusion chromatograms of OsRca α and OsRca β respectively using a Superdex 200 16/600 column showing a single peak of each Rca purified. (E) Final purified protein on 16% SDS-PAGE gel.

A representative illustration of the purification of Rca isoforms is shown in Fig. 3.1. Rca isoforms were captured by immobilized metal ion affinity chromatography (IMAC) followed by precise cleavage of His₆-Ubiquitin from the N-terminus of the expressed Rca by ubiquitin specific protease (USP2). Overnight cleaved protein was subjected to anion exchange chromatography (AIEC) to yield highly pure protein as analyzed by SDS-PAGE. Whenever deemed necessary, further purification was performed by size exclusion chromatography (Fig. 3.1C and D). The average yield of protein from 1 l culture of *E.coli* BL21 grown in LB media after induction with IPTG at 23 °C was found to be ~15-18 mg of pure protein.

3.1.2 Purification of *Oryza sativa* thioredoxin-f

Thioredoxin-f of rice (OsTrx-f) was heterologously expressed in *E. coli* BL21 as a N-terminal fusion with His₆Ub similar to that described for the activases above.

Fig. 3.2: Representative purification of OsTrx-f. (A). 16% SDS-PAGE analysis. Successive purification by IMAC (Ni-NTA), followed by Usp2 mediated cleavage of N- terminal tag, dialysis and anion exchange chromatography (AIEC) using a MonoQ column. (M: Molecular Weight Marker, L:Lysate, S:Soluble, Ni-NTA: Fractions after IMAC, U: Unbound, W:Wash, E: Final eluate, Cut : eluate after USP2 mediated cleavage of Ubiquitin., Dial.: fraction after dialysis, Dial. U: unbound fraction from Ni-NTA after dialysis. (B). Size exclusion chromatogram of OsTrx-f using a Superdex 200 10/300 column shows a single peak of purified thioredoxin-f (left). Final purified protein on 16% SDS-PAGE (right).

The protein was purified by IMAC using Ni-NTA resins followed by cleavage by USP2 and dialyzed in buffer containing low imidazole concentration (20 mM Tris-HCl pH 8 , 50 mM NaCl and 10 mM imidazole) to remove excess imidazole. The

protein solution was passed back to Ni-NTA column equilibrated with dialysis buffer to separate the His₆-ubiquitin. The flow through was collected as His₆-ubiquitin would bind to the column and analyzed by 16% SDS-PAGE as shown (Fig. 3.2A). Further purification was done by anion exchange chromatography, however, protein was not found to bind onto the column and collected as flow through which was passed again to the Ni-NTA column to finally yield His₆-ubiquitin free protein. The protein was further purified using size exclusion chromatography yielding a single peak (Fig 3.2B).

3.1.3 Purification of *Oryza sativa* Rubisco

Oryza sativa Rubisco was directly purified from 10-12 week old plant leaves as described previously [188] and in methods (section 2.7.8). Ammonium sulphate precipitation at 33% and 55% was performed. Rubisco was found to be precipitated at 55% ammonium sulphate salt in the pellet. Further steps included the desalting of protein using dialysis in a low salt buffer followed by anion exchange chromatography which yielded highly pure protein as analyzed by SDS-PAGE (Fig. 3.3A). Whenever necessary, further purification was carried out by size exclusion chromatography (Fig. 3.3B). A typical yield from 8 g of fresh rice leaf material was found to be ~10-12 mg of pure protein.

Fig. 3.3: Representative purification of Rubisco. (A) 16% SDS-PAGE analysis. Successive purification by ammonium sulphate precipitation, followed by dialysis and anion exchange chromatography (AIEC) yielded pure protein. (M: Molecular Weight Marker, E: Extract, S: Soluble fraction after ammonium sulphate precipitation: Pellet after ammonium sulphate precipitation., D: Dialyzed fraction, U: Unbound fraction on MonoQ. The numbers indicate the SDS samples from respective fractions of AIEC on a MonoQ 10/100 column. (B) Size exclusion chromatogram of Rubisco on a Superdex 200 16/600 column (left). Purified fractions on a 16% SDS-PAGE clearly indicating large and small subunits of Rubisco (right).

3.2 Recombinant Rca isoforms were biochemically active and can activate inhibited rice Rubisco

In order to test the activity of Rubisco activases to be used further in the study, the biochemical activities of the rice Rca were assessed by their ability to activate inhibited Rubisco complex and hydrolyse ATP. Thus, Rubisco activation as well as

ATPase activity assays were performed using continuous enzyme-coupled spectrophotometric assays at 25 °C [189, 190] as described in section 2.7.10.

The activated carbamylated Rubisco (ECM) complex was generated by incubating 20 μM Rubisco active sites with 20 mM NaHCO_3 , 10 mM MgCl_2 in buffer A (Table 2.4). The RuBP inhibited Rubisco (ER) complex was made in the same buffer by incubating 20 μM Rubisco active sites with 4 mM EDTA to chelate free magnesium ions for 10 min at 25 °C. After this, 1 mM RuBP was added to make the final ER complex. The Rubisco activity was then measured using the spectrophotometric assay at 25 °C [190] in presence of 20 mM NaHCO_3 and 5 mM MgCl_2 in the final reaction mixture (section 2.7.10). The concentrations of Rubisco and activase were chosen within the subsaturating limits at 0.5 μM ECM or ER (active sites) and 5 μM activase protomer. The assay monitors the continuous oxidation of NADH leading to a reduction in absorbance at 340 nm. This reduction in absorbance is directly coupled to production of 3-phosphoglycerate (3PG) and can be plotted against time to determine the activity of Rubisco in presence and absence of activase (Fig. 3.4). The 3PG values after particular time interval could be derived from the absorbance values using the following equation:

$$3\text{PG } (\mu\text{M}) = - (A_T - A_0) / 0.00622 / 2 \quad (1)$$

Where, A_T and A_0 are the absorbance values at time T and zero and 0.00622 is extinction coefficient of NADH in $\mu\text{M}^{-1} \text{cm}^{-1}$. Since formation of one 3PG required oxidation of two NADH in the assay (see scheme in section 2.7.10.2), the amount of 3PG produced will be half of NADH oxidized [190].

Since, production of two 3PG utilizes consumption of one RuBP, the rate of CO_2 fixed or rate of ECM could be determined by:

$$\text{Rate of ECM } (V_{\text{ECM}}) = \text{RuBP consumed } \text{s}^{-1} / \text{concentration of ECM} \quad (2)$$

Where, RuBP consumed will be half of 3PG produced in equation (1) as per assay stoichiometry [190]

The fully activated rice ECM fixed CO_2 at a rate of $0.72 \pm 0.10 \text{ s}^{-1}$ (rate of ECM) (from Fig. 3.4A data). RuBP inhibited complex (ER) also possess residual Rubisco activity

in absence of activase. However, the rate of 3PG production was negligible compared to ECM (Fig. 3.4A).

The Rubisco activase activity was determined using the method of Lan and Mott [198] without fitting the polynomial function to the time course of the Rubisco activity assay. Instead the gradient between the two time points in the data could be directly utilized to obtain the first derivative of the curves. The concentration of ECM was then calculated using the equation below and plotted against time.

$$[ECM] = -[(A_2 - A_1)/(T_2 - T_1)/0.00622]/\text{NADH oxidized}/V_{ECM} \quad (3)$$

Where, $(A_2 - A_1)/(T_2 - T_1)$ is the gradient between two time points, and 0.00622 is extinction coefficient of NADH in $\mu\text{M}^{-1} \text{cm}^{-1}$, NADH oxidized for one second will be 4, thus the value of it will be depend on the difference in time point ($1 \text{ RuBP} = 2 \times 3\text{PG} = 4\text{NADH}$) [190].

A linear function was then fitted through the origin to obtain the rate of ECM formation. The obtained rate of ECM in presence of activase was normalized by subtracting the residual rate of ECM formation by ER complex in absence of activase (Fig. 3.4B) to quantitate actual activase activity for total Rca protomer in reaction ($5 \mu\text{M}$). To obtain the activity per μM Rca protomer in nM ECM min^{-1} as shown in Fig. 3.4C, the normalized values were first divided by 5 and then multiplied by a factor of 1000×60 (conversion factor for μM to nM and second to minute)

Both isoforms of OsRca were found to be capable of activating RuBP inhibited Rubisco active sites (ER) and hydrolyzing ATP but at variable rates (Fig. 3.4A and C). The short isoform (OsRca β) was found to be significantly faster (~ 2.5 -3 fold) in activating ER complex than the long isoform (OsRca α). In terms of ATPase activity also, OsRca β possessed ~ 3 -fold higher ATPase rate than OsRca α under assay conditions. Consistent with these findings, a recent study [177], also reported a comparable trend, where OsRca β displayed more than 2-fold difference in terms of Rubisco activation and ATPase activity under similar concentration of activase.

Fig. 3.4: Biochemical properties of Rubisco activase isoforms from *Oryza sativa*. (A) Rubisco activation assay of RuBP-inhibited Rubisco (ER) of rice by its Rca isoforms performed at 25 °C with 0.5 μM Rubisco active sites and 5 μM activase protomer (left). Representative quantification of activase activity. ECM concentration was calculated using the gradient between two time points and plotted against time. A linear function was fitted through the origin to obtain the activase activity (right). (B) ATPase activity assays (5 μM Rca protomer, 25 °C) and quantified Rubisco activase activities of WT Rca isoforms. Error bars indicate the standard deviations of mean values of three or more independent experiments.

3.3 Redox regulation of the OsRcaα in the presence of thioredoxin-f

The low activity of OsRcaα we observed above was likely attributable to the fact that Rubisco activase in most plant species is regulated via changes in the redox state of two cysteine residues located at C-terminal extension of α isoform [146]. The redox state is modulated by the electron carrier thioredoxin-f, thus providing a mechanism for the light regulation of Rubisco *in vivo* [150, 199, 200].

Fig. 3.5: ATPase activity of OsRcaa was stimulated in presence of DTT and thioredoxin -f. (A) Effect of thioredoxin-f on the ATPase activity of OsRcaa. OsRcaa (25 μ M protomer) was pre-incubated with 5mM dithiothreitol (DTT) and 3.6 μ M rice thioredoxin-f (Trx-f) for 10 min before the measurement. Assays were performed at 25 °C with 5 μ M OsRcaa, 1 mM DTT and 0.72 μ M rice Trx-f in the final reaction mix.

Under *in vitro* conditions, activity of α isoform could be modulated by changing the redox state in the presence of DTT and thioredoxin-f as shown (Fig. 3.5). It was observed that the ATPase activity of the isoform of rice was increased almost two fold in presence of DTT and thioredoxin-f. The redox study performed earlier with α isoform of arabidopsis reported 50% increase in ATPase activity after treatment with DTT and thioredoxin-f [146]. However, DTT and thioredoxin alone were not capable of providing necessary reduction (Fig. 3.5) and works like a reductive relay where DTT acts as electron donor for thioredoxin to function as provided by photosystem-I *in vivo* [151].

3.3.1 Evidence for covalent complex formation between OsRcaa and thioredoxin-f

Reduction of substrate takes place via formation of a transition state heterodisulfide between thioredoxin-f and substrate due to disulphide reduction by reduced thioredoxin [201]. It is possible to trap this interaction due to formation of a stable covalent heterodimers by generating a mutant of thioredoxin-f which replaces one of

the active site cysteine involved in formation of intermolecular mixed disulfide intermediate [202, 203]. Therefore, we replaced one cysteine of rice thioredoxin-f to serine (C42S), which should permit formation of a mixed disulfide between thioredoxin-f and Rca [204].

Fig. 3.6: SDS-PAGE analysis of the reaction mixtures containing OsRcaa and Trx-f. (A) The two proteins were mixed in a ratio of 1:4 OsRcaa:Trx-f/mutant in 10 mM HEPES pH 8.0, 150 mM NaCl, and incubated for 30 min at 25 °C under aerated environment. Aliquots of the incubated mixtures were then separated on 10% SDS- PAGE gel under non-reducing condition without heat treatment. (OsRcaa or α: α isoform of rice Rubisco activase, Trx-f: thioredoxin-f). (B) 12.5% SDS-PAGE gel of the similar samples as A under reducing condition in presence of DTT.

In order to obtain a stable disulphide mixed intermediate between OsRca α and Trx-f the two proteins were mixed in excess of Trx-f and its mutant (C49S) with OsRca α (1:4) under aerated condition for 30 min at room temperature and analyzed using SDS-PAGE. For control, all the proteins were run in isolation. The complex formation was only obtained in presence of the Trx-f mutant, while no complex formation was seen in case of WT Trx-f (Fig. 3.6A). There is formation of additional homo-dimers of Trx-f under non-reducing conditions, which were reversible in nature and could be easily eliminated under reducing conditions in the presence of DTT as reported previously (Fig. 3.6B) [204-206]. This was consistent with the reported structural and functional form of oxidised thioredoxin-f [207, 208]. Also, equilibrium binding study have revealed the evidence for dimer formation and its binding to chloroplastic FBPase in pea [209].

In case of thioredoxin mutant (C42S), we observed an additional band above the dimer band (Fig. 3.6A). The formation of homo-dimer and higher molecular mass protein has been reported earlier for thioredoxin-f of tobacco for recombinant protein as well in planta under various non-reducing conditions, especially in presence of high salt [205]. It has also been reported that these dimers could be separated by high ionic strength into monomers [207]. The evidence presented for the formation of the stable complex between Rca α and Trx-f in this study may facilitate further structural and biochemical studies. For instance, a structural and functional understanding of the specificity between different thioredoxin isoforms and their substrate is still elusive [210, 211]. Recent advancements in cryo-EM technology should permit characterization of such complexes at atomic resolution.

3.4 Characterization of Rubisco activase thermostability

A relatively low thermal stability causes denaturation of Rca at moderately elevated temperature which is believed to be directly responsible for shutting down photosynthesis *in vivo* [11]. However, the biophysical properties of activase vary between species with the Rca α reported to be more thermostable and conferring thermostability to Rca β in spinach [141, 178], but not in arabidopsis [166]. Importantly, heat stress in rice leads to upregulation of Rca α [196] and it was reported to be the most upregulated protein in *Oryza meridionalis* [197]. However, such

difference in relative abundance was not observed in arabidopsis under heat stress [212]. Thus, we performed a thermal characterization of both rice Rca isoforms.

3.4.1 Rice Rubisco activases are also relatively thermolabile

Fig. 3.7 shows the ATPase activity of OsRca α and OsRca β isoforms after a 10 min heat treatment at various temperatures. It was found that in terms of activity, both isoforms possessed similar thermal stability and started losing their activity above 35 °C. While, OsRca β lost 50% of its maximum activity at 40 °C, OsRca α lost 50% of its maximum activity at 37 °C and was completely inactivated at 40 °C. OsRca β lost most of its activity at 42 °C and became completely inactive at 45 °C. A decline in ATP hydrolysis rate was also reported in the recent study performed on rice activases after temperature exceeded 36-40 °C [177].

Fig. 3.7: Decline in ATPase Activity of Rubisco activase upon heat treatment. The activase isoforms (260-280 μ M) were pre-incubated for 10 min at indicated temperature. ATPase activity was then measured at 25 °C with 5 μ M Rca (protomer) in the final reaction mix. Error bars indicate the standard deviation of mean values of three or more independent experiments.

To determine the structural integrity of Rubisco activase at different temperatures, the molar ellipticity of protein samples was determined by circular dichroism (CD). Experiments were performed and spectra were collected as described in materials and methods (section 2.7.6).

The secondary structural elements could be estimated by their unique spectral pattern. For instance, α -helices are characterized by a depression at 222 and 208 nm and an elevation at 193 nm. On the other hand, β -sheets could be identified by a negative bands at 218 nm and a positive band at 195 nm [213]. The secondary structural content of Rca was determined by using the K2D2 algorithm [214]. At 20-25 °C, OsRca α and β predominantly possessed high α - helical content (56 and 58% respectively), while β -sheet content was estimated to be 12% and 7% respectively. The CD spectra reported for the β -isoform of arabidopsis estimated 57-61% α -helix and 7-10% β sheet at 15 °C [134], while tobacco contained 44% α -helix and around 10% β -sheet at 20 °C [158]. At 45 or 50 °C, rice α -isoform possessed only 8% α -helices, while the β -isoform retained 18% α -helices. Earlier study on β -isoform of arabidopsis reported 8-22% α -helical content at 65 °C [134]. Thus, the majority of α -helical content was lost at high temperature consistent with denaturation of the protein.

The mid-point of thermal denaturation was approximately determined by visual inspection of the observed ellipticity versus temperature plot at 208 nm (Fig. 3.8) [134, 138]. We observed, the mid-point for thermal denaturation (apparent T_m) lies at about 35-40 °C for both rice Rubisco activase isoforms. This was similar to the earlier report for arabidopsis β -isoforms, where the apparent T_m was estimated to be about 40 °C using CD and mid-point for loss of activity at around 37 °C [134]. Based on such observations, it was concluded that the loss of activase function is associated with structural instability of Rca at elevated temperature [134]. Under identical incubation conditions (temperature), the ATPase activity of Rubisco activase decreased with increasing temperature, exhibiting a mid-point of about 37 °C and 40 °C for α and β isoform respectively as shown in previous section. This clearly indicates that loss of activity of activase is due to the loss of secondary structure and denaturation of Rca at high temperature which was further confirmed by native PAGE analysis (Fig 3.9A) of the Rca isoforms.

Fig. 3.8: CD spectroscopic profile of Rubisco activases at different temperature. (A and B) CD spectra of OsRca α and β (1 mg/ml) respectively recorded at different temperature (20-50 °C). (C and D) Ellipticity of OsRca α and β respectively monitored at 208 nm as a function of temperature derived from the CD curves.

We observed that at temperature 40 °C or above, Rca appears to form higher ordered aggregates which migrate more slowly on a 6% Native PAGE gel. However, SDS-PAGE and turbidity analysis, along with visual inspection of the Rca protein solution shows that although there is partial loss of activity at temperature above 35 °C but rice activase isoforms remain in solution and form soluble inactive aggregates (Fig. 3.9B and C). Contrary to this, tobacco Rca gets highly turbid when heated at 45 °C which was easily discernible. This was consistent with the earlier study performed on tobacco, where a rapid increase in light scattering was reported when temperature was increased above 40 °C [215]. Another study on leaf tissue of wheat and cotton also demonstrated formation of insoluble higher molecular mass aggregates at similar

temperature using western blot analysis [216]. Although no precipitation was observed for rice activase isoform, ATPase activity was completely lost after heat treatment at such temperature (Fig. 3.7). The previous results from Barta et al (2010) and present data suggested that like in arabidopsis, in rice also, denaturation at high temperature leads to production of soluble inactive higher order aggregates. Should this behaviour extend to the *in vivo* systems, it may indicate different mechanisms of Rca reactivation. For instance, smaller soluble species should be easier to recover than aggregated, inclusion like forms.

A

B

Fig. 3.9: Thermostability analysis of Rubisco activase. (A) 6% Native PAGE of Rca at different temperature. Rca (3 mg/ml) was heated at the respective temperature for 10 mins and 10 μ g of final Rca was loaded on to the gel. (B) 12.5% SDS-PAGE analysis of the soluble (S) and pellet (P) fraction OsRca and NtRca (tobacco) after incubating at respective temperature. (C) Turbidity analysis of the Rca at 45 °C. Rice (OsRca) and tobacco (NtRca) activase were diluted to 3 mg/ml and optical density (600 nm) scan against time was performed at 45 °C (left). Activase samples (3 mg/ml) were incubated at indicated temperature (right) where rice activase isoforms stay in solution while tobacco Rca forms precipitate.

3.5 Subunit mixing with OsRca α reduces the ATPase activity of the OsRca β

In many plants such as arabidopsis and spinach two isoforms are present in equimolar ratio [112] however, in rice, Rca β is much more abundant than Rca α under non-stressed condition [144]. Interestingly, heat stress leads to upregulation of Rca α in rice and its wild species *Oryza meridionalis* [196, 197]. This differential distribution of two Rca isoforms in rice leaves should reflect physiological significance *in vivo*. We therefore hypothesized that in the oxidized state, OsRca α might regulate the activity of OsRca β . The ability of Rca to rapidly exchange subunits has been exploited earlier to study the role of various mutations on activase function and subunit coordination [129, 163, 164]. Mixing of wild-type (WT) with ATPase compromised mutants generally have an inhibitory effect on ATPase activity of WT activase. However, the extent of inhibition may vary with the type or site of mutation determining the relative contribution of that residue or region in activase function [129, 163]. This suggests that it is the nature of inhibition that decides the contribution of particular residue or site towards subunit cooperativity and not just the absence of

activity of a particular mutant subunit. Therefore, subunit mixing experiments were performed with Rca α and β to assess how the interaction of Rca α would affect the activity of the Rca β *in vitro*.

Fig. 3.10: Subunit mixing with OsRca α reduces the ATPase activity of OsRca β . WT OsRca β was pre-incubated with OsRca α at 270-280 μ M Rca protomer concentration range for 10 min. ATPase assays were then performed at 25 °C by keeping the final concentration of Rca β as constant (5 μ M) and gradually varying the concentration of Rca α isoform at different proportion in the final reaction mix as indicated. ATPase is given per Rca active site. The experimental values were calculated using the equation; (ATPase μ M min⁻¹/total conc. of Rca (α + β) in μ M). The experimental results were compared with the theoretical values, assuming subunits behave independently resulting in an additive ATPase rate which can be calculated using the equation; (specific ATPase of Rca α *concentration of Rca α) + (specific ATPase of Rca β *concentration of Rca β)/total concentration of Rca (α + β) in μ M.

The preliminary experiment in mixing indicates a reduction in the specific ATPase activity of the Rca mixture when ATPase assay was performed by keeping the concentration of OsRca β as constant and titrating up the concentration of OsRca α (Fig. 3.10). Since, OsRca α also contributes to the ATPase activity of the hetero-oligomer, ATPase activity was quantified per active site and therefore the experimental activities were shown in comparison to theoretical activities. Theoretical activities were calculated assuming the subunits were behaving independently and ATPase activity was enumerated as an arithmetic addition of the contribution of individual isoform in the mix based on their specific ATPase activity. We observed that the difference in experimental and theoretical values were not so significant at low proportion (20%), however as the proportion of Rca α increased the difference became significant. At the equimolar concentrations (50%), experimental ATPase

activity was reduced to 7 min^{-1} , while the theoretical activity was computed as $\sim 11 \text{ min}^{-1}$. A lower experimental value than the theoretical one at all proportions clearly suggests the interaction between the two isoform subunits. However, the residual activity of OsRca α complicated the interpretation of the experiment. Thus, performing the above experiment with ATPase inactive mutants of Rca α would provide a better insight.

To further characterize the interaction among Rca subunits, mutations were designed covering key residues responsible for proper functioning of Rca in terms of ATPase activity which have been described in next section.

3.5.1 Characterization of the ATPase inactive OsRca mutants

It has been reported, that as typical for AAA+ ATPases, a Walker A lysine residue and a Walker B aspartate residue of Rubisco activase in spinach and tobacco respectively, is crucial for ATP binding and hydrolysis [148, 163]. So we mutated lysine 115 and aspartate 173 to alanine (numbering as per rice Rca) at these critical sites in the ATPase active site (Fig. 3.11A). Site-directed mutagenesis was performed to produce mutants of OsRca α and OsRca β as described in material and methods. The mutations at above positions led to a complete loss of ATPase activity in the mutant proteins as expected (Fig. 3.11B). This permitted a more reliable interpretation of the experimental data in terms of subunit doping.

Subunit mixing experiments were then performed with these mutants as described earlier in section 3.5. This time, a reduction in ATPase activity of the OsRca β at different ratios was observed without any interfering activity of the OsRca α unlike previously. We observed a reduction of 55-70% in ATPase activity (Fig. 3.11C) at an equimolar ratio (5 μM mutant), while a decline of 75-85% was observed when the mutant/WT ratio was increased to 2:1 (10 μM mutant) in the final reaction mix. The previous studies with ATPase inactive mutants such as D231R and D174A in spinach have reported a reduction of 80% and 40% ATPase activity respectively for tobacco Rca at 4:1 mutant/WT ratio [163]. Interestingly, here we found that mixing of ATPase inactive Rca β mutants reduced the activities of the wild-type (WT) Rca β subunits to a similar extent as Rca α mutants (Fig. 3.11C). This suggested that the C-terminal extension of Rca α was not involved in modulating the activity of the oligomer.

A

B

C

Fig. 3.11: Mutations at the Walker A and Walker B motifs of Rubisco activase abolish ATPase activity. (A) Ribbon structure of hexameric model of tobacco Rubisco activase (PDB id: 3ZW6) highlighting K115 and D173 residues targeted for mutagenesis in red and blue respectively. (B) ATPase assays were performed as usual (5 μM protomer, 25 °C) with site directed mutants of Rcaα

and Rca β isoform responsible for ATPase activity. WT (wild-type) Rca α and Rca β isoforms were used as positive control. (C) Effect of subunit mixing of ATPase inactive mutant of the α and the β isoform on ATPase activity of the WT β isoform. WT active OsRca β was pre-incubated with the ATPase inactive mutants at 260-280 μ M Rca protomer concentration range for 10 min. ATPase assays were then performed at 25 °C by keeping the final concentration of OsRca β as constant (5 μ M) and titrating up the concentration of inactive mutant proteins at different proportion in the final reaction mix as indicated.

These results further validate that Rca subunits do not function independently but exhibit subunit cooperativity by rapidly exchanging subunits as reported previously for tobacco Rca [129, 163]. Thus, mechanistically Rca follows a concerted or sub-globally cooperative mechanism of ATP hydrolysis where all or a specific minimal number of subunits are required to hydrolyse ATP simultaneously and oligomers containing less than the required number of active subunits become inactive [193, 194, 217]. These considerations are further discussed in detail in chapter 5. It also provides a mechanism to modulate the ATPase activity of the Rca β subunits by Rca α isoform *in vivo*. By forming rapidly exchanging hetero-oligomeric mixtures of low-activity Rca α and high-activity Rca β isoforms, modulating the concentrations of Rca α allows the activity of the oligomer to be reduced with the concentration of the Rca α .

3.5.2 Further evidence for hetero-oligomerization between Rca isoforms

Rubisco activases are polydisperse in nature and change their oligomeric states with concentration and in presence of nucleotides [133-135, 141]. This property of Rca was exploited to validate the physical interaction between Rca isoforms. Initially, we studied the elution behaviour of the rice activase isoforms using analytical gel filtration chromatography.

Both rice isoforms were eluted as polydisperse oligomers with a size corresponding to the apparent molecular weight of 75-440 kDa depending on their concentrations (Fig. 3.12A and B). Peaks corresponding to the larger oligomeric species were observed at higher concentration of activases and vice-versa. The α isoform eluted earlier than the β isoform at low concentration (5 μ g in 50 μ l, 0.1 mg/ml). Therefore, this concentration of both Rca was chosen for another experimental run in the column to detect the shift in the peaks (Fig. 3.12 C). It was observed that when the same amount of α and β isoform (5 μ g each) were mixed and run together in the same buffer (see

figure legend), the mixture was eluted as a single peak rather than two separate peaks of individual protein (Fig. 3.12C). The peak of the mixture was distinct as compared to the individual peak of each isoform alone (Fig. 3.12C). Moreover, the mixed sample of 10 μg total proteins (5+5) runs almost intermediately to 10 μg of the individual protein (Fig. 3.12D), which clearly indicate the shift in the peak was due to the interaction between two isoform subunits and therefore proteins are behaving as a single entity in the mixture.

This was further validated by running the samples from eluted fractions obtained from the experiment in Fig. 3.12C on the SDS-PAGE (Fig. 3.12E). The SDS PAGE analysis of the earliest fractions of the mixed protein run indicates the presence of both isoform which runs as a single entity in mixture while the β isoform was completely undetectable in the same fraction when eluted individually (Fig. 3.13E). This indicates changes in the elution pattern of the β isoform when run together with the α isoform which is only possible when two subunits interact among each other.

Fig. 3.12: Subunit interaction study using analytical gel filtration and SDS-PAGE: (A and B) Analytical gel filtration chromatography of OsRca α and β at different concentration performed using a Superdex 200 3.2/30 increase column in buffer: 20 mM Tris-HCl pH 8.0, 50 mM NaCl, 5 mM MgCl₂, 0.5 mM ATP. (C and D) Analytical gel filtration chromatography of rice isoforms in mix (α + β) along with control elution peaks in same buffer as A and B. E. 12.5% SDS-PAGE analysis of the samples from the fractions obtained from C (zoomed). Each fraction was 50 μ l in volume correspondingly. Bands have been positioned exactly below the fractions or respective elution volumes (see the magnified image) which were used for SDS- PAGE analysis as depicted (left). Schematic representation of the experiment and predicted outcomes differentiating the situation between interaction and non-interaction among two subunits (right). Scheme is only for illustrative purpose.

3.6 Subunit mixing and redox state does not affect the thermotolerance of the rice activase

Considering the fact that Rca α has been reported to confer thermostability to Rca β in spinach [141, 178] and observed to be the most upregulated protein under heat stress in the wild rice species *Oryza meridionalis* [197], we hypothesised that OsRca α may confer thermostability to rice Rubisco activase, possibly in the oxidized, low activity form. Hence, *in vivo* overexpression of the OsRca α may represent a protective mechanism preventing denaturation of the activase in addition to downregulating CO₂ fixation during heat stress. However, the redox state of the proteins in the earlier experiments was not reported [141, 178, 197]. So, we also hypothesized that the OsRca α may vary in its thermotolerance depending on whether the enzyme is reduced or oxidized.

We conducted subunit mixing experiments between the OsRca α and β at an equimolar concentration at different temperatures (Fig. 3.13A). We also performed ATPase activity assay for OsRca α under reduced condition at different temperatures to test this hypothesis (Fig. 3.13). However, it was concluded that subunit mixing in rice did not further improve the thermal stability of rice Rubisco activase. Also, the change in redox state of Rca α confirms the role of thioredoxin-f in modulating the activity of Rca α isoform but did not improve the thermotolerance.

Fig. 3.13: Effect of subunit mixing and Thioredoxin-f on thermostability. (A) OsRca α and OsRca β were mixed at around 280 μ M Rca protomer concentration and incubated at the indicated temperature for 10 min. ATPase assays were then performed at 25° C at an equimolar concentration (1:1) of 5 μ M Rca protomer each in the final reaction mix. (B) OsRca α isoform (25 μ M) along with DTT (5 mM) and thioredoxin-f (3.6 μ M) were incubated separately and the mixture was heated at the indicated temperature for 10 min. ATPase assays were then performed at 25 °C with 5 μ M Rca protomer, 1 mM DTT and 0.72 μ M thioredoxin-f in the final reaction mix. (OsRca α or α : α isoform of Rubisco activase, d: DTT, trx: thioredoxin-f)

In summary, we have presented a biochemical and biophysical characterization of rice activase isoforms in this chapter. The ability to rapidly assay defined subunit mixtures allows us to probe the function of the enzyme further. These ideas are further explored in chapter 5.

4. Characterization of a thermostable Rubisco activase from *Agave tequilana*

It has been demonstrated earlier and also in the present study (section 3.4) that Rubisco activase is a thermolabile enzyme. This property is believed to be directly responsible for the inhibition of photosynthesis at moderately high temperature [10, 11]. In contrast, higher plant Rubisco has been demonstrated to be thermostable even at elevated temperature, indicating that the loss of photosynthetic CO₂ fixation is the result of thermal denaturation of Rca [10]. It has been predicted that the global temperature may rise by 2-5 °C by the end of this century [218] and an estimation showed that each degree rise in temperature in an average growing season results in up to 17% decrease in yield [219]. Therefore it is necessary to take steps to minimise the effect of heat stress on the efficiency of photosynthesis for increasing crop yield and prepare the crops for the future climatic changes [18].

It has been reported that having a more thermostable Rca variants results in higher photosynthetic rate, improved developmental patterns, higher biomass and increased seed yields [12]. This study was conducted using gene shuffling to generate variants of the β isoform of *Arabidopsis thaliana* followed by screening these mutants for more thermostable variants. Another study on the same plant species demonstrated that transforming the arabidopsis Rca mutant plants with a more thermostable chimeric activase between tobacco and arabidopsis containing the Rubisco recognition domain (α -helical subdomain) from arabidopsis showed a higher photosynthetic rate under moderate heat stress [13]. It has been predicted that introducing Rca from a warm season plants like cotton into a cool season species like *Camelina* would improve the thermotolerance of photosynthesis by as much as 5 °C [15, 220]. Thus, thermal stability of the Rubisco activation process could be enhanced either by generating thermostable variants of Rubisco activase or by introducing or replacing the native Rubisco activase with more thermostable activase from other compatible organisms.

Plants from a high temperature environment may not require a particularly thermostable activase, since they often possess powerful leaf cooling mechanisms, especially transpiration [175, 221, 222]. We noted that this mechanism would not be available to CAM plants growing in a hot desert environment, where Calvin-Benson cycle function will be taking place in the light under high exposure of heat, with the stomata closed to minimize water loss [223]. Consequently we reasoned that, like

other enzymes required during daytime, CAM activases should be relatively thermostable [224, 225]. To test this hypothesis, we targeted an activase system from a CAM plant, *Agave tequilana*.

Fig. 4.1: Schematic diagram of a day night cycle in a typical CAM plant: A partial CAM pathway showing CO₂ uptake and release of water through open stomata during night, while during day stomata remain closed to prevent the exit of internally released CO₂ and water while performing photosynthesis.

In this chapter, we describe a biochemical characterization of a thermostable and highly effective activase system from agave in a comparative study with their rice homologues. This promising thermostable system was then utilized for engineering a more thermotolerant and efficient variant of rice activase and locating the regions and key residues responsible for such properties. This was attained by generating chimeric mutants between rice and agave by inter-swapping their major domains supplemented with site directed mutagenesis. Thus we have attempted to explore the natural diversity of activase proteins from different environments to mechanistically understand their biophysical and biochemical properties. This approach should assist in overcoming the limitations of existing activases like slow activation rate and thermolability.

4.1 Purification of *Agave tequilana* Rca isoforms

The genes encoding α and β isoform of Rubisco activase of *Agave tequilana* (AtRca α and AtRca β respectively) were identified from the transcriptome assemblies of *A. tequilana* [182]. Unlike rice, two Rca isoforms in agave are not the product of alternate splicing as the genes encoding them were found to be present on the different locus in the transcriptome data. Consequently, a few residues among the

main part are different in two isoform (Fig. 4.2). The α -isoform of agave possesses around 40 additional residues as a C-terminal extension. Aligning the amino acid sequences revealed that both isoforms of *A. tequilana* shows ~86% sequence identity with the rice activases (Fig. 4.2).

Fig. 4.2: Sequence alignment of *Oryza sativa* and *Agave tequilana* activase isoforms. Amino acid sequences of two Rubisco activase (Rca) isoforms of *Oryza sativa* and *Agave tequilana* were aligned using ESPrnt [184]. Rice and agave has two Rca isoforms α and β . The two isoform in rice (accession code: P93431-1 and P93431-2) are the product of alternate splicing only differing in the C-terminal sequence as shown. OsRca α has 33 extra amino acids at the C-terminal and also differ in 5 amino acid preceding to this extension. The two isoform in agave are the product of different gene (locus 3705, and 27298) [182]. AtRca α also possesses around 40 extra residues as a C-terminal extension.

Following gene synthesis, *A. tequilana* activases were heterologously expressed in *E. coli* BL21 as a N-terminal fusion with hexa-histidine tagged ubiquitin (His₆Ub) and purified exactly as described for rice Rca. The average yield of protein from 1 l culture of *E. coli* BL21 grown in LB media after induction with IPTG at 23 °C was found to be ~12-15 mg of pure protein.

Fig. 4.3: Representative purification of AtRca α and AtRca β . (A and B) 12.5 % SDS-PAGE analysis. Successive purification by IMAC (Ni-NTA), followed by Usp2 mediated cleavage of N-terminal tag and anion exchange chromatography (AIEC) yielded pure protein. (M: Molecular Weight Marker, L: Lysate, S: Soluble, Ni-NTA: Fractions after IMAC, U: Unbound, W: Wash, E: Final eluate, Cut : eluate after USP2 mediated cleavage of Ubiquitin. The numbers indicate the SDS sample from respective fraction of AIEC). (C and D) Size exclusion chromatograms of AtRca α and AtRca β using a superdex 200 16/600 column, showing a single peak of each Rca. (E) Final purified protein on 16% SDS-PAGE gel.

4.2 Both agave isoforms were biochemically active and can activate inhibited rice Rubisco more efficiently than rice Rca

Once the proteins of interest were in hand, first of all, spectrophotometric Rubisco activation assay was performed between RuBP inhibited rice Rubisco (ER) with agave activases to examine if rice Rubisco could be activated by agave activase. Generally plant activases tend to be broadly compatible, and incompatibility has only been demonstrated between activases and Rubiscos derived from Solanaceae and non-Solanaceae species [226]. Consistently, it was found that agave activases can activate rice Rubisco (Fig. 4.4A). The average activation rates for AtRca α and β were around 42 and 36 nM ECM min⁻¹ respectively compared to ~2.7 and 7-8 nM ECM min⁻¹ for OsRca α and β respectively (Fig. 4.4C). It was surprising to us that their ability to activate rice Rubisco was 4-5 fold higher than the rice activase isoform (OsRca β) under the chosen assay conditions. Similarly, both isoforms of AtRca displayed a relatively high ATPase activity ~32 min⁻¹ (Fig. 4.4C) unlike OsRca, where the β isoform showed a much higher activity (~22-25 min⁻¹) than the α isoform (~6-8 min⁻¹).

Based on the above results one possibility is that agave activases are more efficient due to their inherent high activity rather than enhanced Rubisco-Rca interaction. Alternatively, it could be postulated that agave activases possess higher affinity for the rice Rubisco than the cognate rice activases. To distinguish between these two possibilities, we incorporated polyethylene glycol (PEG 3350) in the Rubisco activation assay (Fig. 4.4B).

PEG is a crowding or solvent excluding reagent which has been described thermodynamically to promote self-association and activity coefficient of proteins [227]. Macromolecular crowding approach using PEG 3350 has been previously demonstrated to stimulate the activase activity for plant Rca and the recently discovered CbbQO-type activase of chemoautotrophic bacteria [115, 136]. Moreover, PEG promotes self-association of Rca which not only increased the specific activity of the activase but also enhances the affinity for ATP and interaction of Rca and Rubisco [136]. The concentration of Rubisco (~200 mg/ml) and Rca (~7-20 mg/ml) in chloroplast stroma is extremely high [8, 107], compared to *in vitro* assay conditions. Therefore, it has been speculated that this crowding environment allows Rubisco and activase to be in close proximity and facilitates them to function efficiently. Thus under *in vitro* assay conditions, crowding agents assist in simulating the *in vivo* environment causing higher interaction between Rca and Rubisco, ultimately leading to enhanced activation.

Consistently, we observed that activase activity of AtRca β and OsRca β was considerably enhanced by inclusion of 5% v/v PEG 3350 in the assay (Fig. 4.4B). The activase activity of the AtRca β increased by ~2.8 fold, while a remarkable ~7 fold stimulation was observed for OsRca β . This indicates that OsRca β has a poor affinity for Rubisco under assay conditions, leading to slow activation which was overcome by the presence of PEG due to the crowding effect. AtRca β on the other hand, already had a higher affinity for rice Rubisco and therefore did not benefit from the macromolecular crowding as much as cognate rice Rca. However, AtRca β still maintained a 1.2 fold higher activation rate than OsRca β . Presence of PEG had no significant effect on the Rubisco activity of activated (ECM) and inhibited Rubisco (ER) complexes (Fig.4.4B) indicating that the stimulation was the outcome of enhanced protein-protein interaction between Rca and Rubisco.

Fig. 4.4: Biochemical properties of Rubisco activase isoforms from *Oryza sativa* and *Agave tequilana*. (A) Rubisco activation assay of RuBP-inhibited rice Rubisco (ER) performed at 25 °C (0.5 μ M Rubisco active sites, 5 μ M activase protomer) (B) Effect of crowding agent polyethylene glycol (PEG) 3350 on activase activity of the OsRca β and AtRca β . Rubisco activation assays were performed as in A with inclusion of 5% v/v PEG 3350 as indicated in the figure legends. (C) ATPase activity assays (5 μ M protomer, 25 °C) and quantified Rubisco activase activities of different Rca isoforms. Error bars indicate the standard deviations of mean values of three or more independent experiments.

4.3 Agave Rca isoforms are highly thermostable

Having established biochemical compatibility, thermal characterization of agave activases were performed in comparison to rice. The ATPase activity of the Rca α and Rca β of rice and agave after heat treatment at various temperatures is shown below (Fig. 4.5A)

It was observed that while both the isoforms of rice were almost completely inactivated at 42 °C, both agave isoforms still maintained their activity at 45 °C. At higher temperatures, both isoforms start losing their activities. AtRca β maintained around 60% of its activity at 50 °C and more than 40% of its activity at 52 °C before getting completely inactivated at 55 °C. AtRca α lost around 70% of its maximum activity at 47 °C, and was completely inactivated at 50 °C. Thus, the agave isoforms represented a highly thermostable Rubisco activase system for plants where each isoform of activase displayed ~5-8 °C higher thermostability as compared to their rice homologues in terms of their *in vitro* activities.

Fig. 4.5: Thermal response of Rca. (A) Comparative thermostability of activase isoforms in terms of ATPase activities. Activase isoforms (200-280 μ M concentration range) were incubated at indicated temperature for 10 min and then ATPase activity was assayed at 25 °C with 5 μ M Rca protomer in final reaction mix. (B) Comparative effect at low temperature. ATPase activity assay was performed at indicated temperature for OsRca β and AtRca β . (C) AtRca β has higher Rubisco activase activity than OsRca β even at low temperature. Rubisco activation assays were performed at 15 °C. (D) Rubisco activation assays could not be measured at 45 °C due to instability of ER complex. Error bars indicate the standard deviations of mean values of three or more independent experiments.

From the above data, it was quite evident that the agave activases are more potent at high temperature than the rice activases. However, it is commonly believed that increased thermostability is likely to trade-off with functionality at lower temperature [228] as previously observed for activases from different species [15, 229]. To test whether this was the case for the agave activases, we analyzed ATPase and activase activity of agave and rice Rca β at a reduced temperature (Fig. 4.5B and C). It was observed that ATPase activity of AtRca β and OsRca β were drastically reduced at 15 °C (around 80%) as expected from physical principles but quantitatively AtRca β still maintained higher ATPase rate (7-8 min⁻¹) than OsRca β (4-5 min⁻¹) (Fig. 4.5B). At 45 °C, OsRca β became completely ATPase inactive, while a much higher ATPase activity (~85 min⁻¹) was observed for AtRca β .

Similarly, when the Rubisco activation assays were performed at 15 °C, the activase activity of the OsRca β was severely reduced and did not significantly improve with the concentration (Fig. 4.5C). While AtRca β could still perform faster activation than OsRca β even at 5 μ M concentration and full activation was achieved by increasing the concentration. It was clear from the above data that despite being thermostable AtRca β proclaims high activity at either low or high temperature. However, Rubisco activation activity of Rca could not be measured at 45 °C due to instability of rice ER complex at that temperature (Fig. 4.5D) as reported previously for tobacco ER [99]. Thus, AtRca β exhibits no compromise in terms of their Rubisco activase activities as well as ATPase activity at low temperature compared to rice and displays superior functional characteristics at the temperatures tested.

4.4 Rice and agave activase subunits form hetero-oligomers displaying intermediate functionality but subunit-specific thermostability

Earlier studies of introducing thermostable activase into arabidopsis were performed using plants where the endogenous Rca had been deleted [12, 13]. In rice such a line is not available. It would be more convenient to express thermostable activase in rice plants containing the endogenous activase. In earlier work, Rubisco activases from maize have been overexpressed in rice containing endogenous activases [230]. However, if the thermostable agave activases were to be engineered into rice plant in

future, it is necessary to examine if activases from the two species interact with each other. And if so, it would be further important to ensure that an interaction between AtRca and OsRca should not result in loss of biochemical function due to the formation of ‘inter-species’ hetero-oligomeric complexes.

Therefore, first we characterized the biochemical properties of OsRca β and AtRca β mixture at an equimolar ratio (1:1). When the functional wild-type rice and agave Rca subunits were mixed and assayed, no reduction in functionality (both ATPase and Rubisco activase) was observed. Rather an intermediate level of activities were measured (Fig. 4.6A and B), suggesting the mixed species oligomers are not functionally impaired. However, it was difficult to distinguish between two distinct populations of homo-oligomers and the formation of functional hetero-oligomers. Therefore, subunit mixing experiments were performed between agave activase isoforms (AtRca α or AtRca β) and ATPase inactive Walker B mutant of rice (OsRca β D173A). As shown previously for rice activase isoforms (section 3.5.1), a strong reduction in the ATPase activity of agave activase isoforms were observed (Fig 4.6C) when the concentration OsRca β D173A was titrated keeping concentration of AtRca α or AtRca β as constant. Taken together these results provide evidence for the formation of inter-species functional hetero-oligomers.

Fig. 4.6: Inter-species subunit interaction between the agave and the rice activase. Rubisco activation assay (A) and ATP assay (B) performed were performed at 25 °C using 5 μM activase protomer (2.5 μM + 2.5 μM for AtRcaβ+OsRcaβ). ATPase is given per active site. (C) Evidence for hetero-oligomerization. AtRca was mixed with increasing amounts of ATPase inactive OsRcaβD173A for 10 minutes (210-270 μM concentration range). ATPase assay was then performed by maintaining the concentration of AtRca as constant (5 μM) and varying the concentration of OsRcaβD173A in the final reaction mix. (D) Incubation at 45 °C restores the ATPase activity of impaired hetero-oligomers to the levels of AtRcaβ wild-type. In (C) and (D) ATPase activity was calculated as turnovers per wild-type active site.

From the above results it was established that Rca functionality was maintained in terms of its activities. We then decided to ask whether hetero-oligomerization would affect the thermostability of the agave subunits. Therefore, we conducted an experiment where a 1:1 mixture of AtRcaβ and OsRcaβD173A was heated together at 45 °C for 10 min before assay as depicted (Fig. 4.6D). It was observed that the

subunit poisoning effect of OsRca β D173A was rescued and AtRca β almost regained its full original activity (Fig. 4.6D). This suggested that upon heating AtRca subunits remained in solution, while OsRca formed inactive higher order aggregates. Following the outcome from this experiment we predict that agave activases could perform their function independently at high temperature if expressed *in vivo* without adversely being affected by the lower thermostability of the rice isoforms.

4.5 Characterization of chimeric mutants localized thermostability and high activase activity to the nucleotide binding domain (NBD) of AtRca β

From the previous results it was clear that agave activases are highly thermostable as compared to their rice counterparts, in spite of high sequence identity (~86%) (Fig. 4.2). This prompted us to attempt to map the determinants of thermostability. Therefore, first chimeric mutants of Rca were generated by interchanging the nucleotide binding domain (NBD) and α -helical C-terminal sub-domain between rice and agave Rca (Fig. 4.7A).

Fig. 4.7: Thermal and biochemical characterization of chimeric mutants. (A) Schematic diagram for the construction of chimeric mutants. (B & C) Thermostability testing of chimeric mutants in terms of their ATPase activity compared to their wild type counterparts. ATPase assay was performed at 25 °C after incubating the proteins (160-280 μM) at indicated temperature for 10 min such that the final concentration of the protein remains 5 μM Rca protomer in the reaction mix. (D) ATPase and Rubisco activase activity of the chimeric mutants along with WT activases. Assayed at 25 °C, 5 μM Rca protomer, 0.5μM ECM and ER. Error bars indicate the standard deviations of mean values of three or more experiments.

As observed for other activase homologues in previous studies [13, 154, 231], chimeric activases could be solubly expressed and purified in fully functional form (Fig. 4.7D). The thermal characterization of chimeric mutants showed that replacing the NBD of OsRcaβ with the NBD of AtRcaβ (RcaβAtNOsC) improves thermostability by 5-8 °C as compared to OsRcaβ (Fig. 4.7B). This indicates that the NBD of agave can confer thermostability to rice activase. However, the improvement in thermostability of agave activase is not same as WT β-isoform of agave, and RcaβAtNOsC was observed to lose its functionality at a lower temperature (by 3 °C) as compared to AtRcaβ. On the other hand, when the C-terminal domain of OsRcaβ was replaced with the C-terminal domain of AtRcaβ (RcaβOsNAAtC), no improvement in thermostability was observed and the resultant chimeric mutants have the same thermostability as OsRcaβ (Fig. 4.7B).

We were also interested to test whether the C-terminal extension of the AtRcaα can confer thermostability to OsRcaα. Swapping the C-tail of AtRcaα and OsRcaα

generated two chimeric activase mutants labelled as AtRca α OsTail and OsRca α AtTail (Fig. 4.7A). When the C-tail of OsRca α was transferred to AtRca α (AtRca α OsTail), the resulting chimeric mutant suffered from reduced thermostability by around 5 °C as compared to AtRca α (Fig. 4.7C). Similarly, when the C-tail of AtRca α was incorporated to OsRca α (OsRca α AtTail), the chimeric mutant displayed around a 2 °C decrease in its thermostability as compared to OsRca α . This indicates that the C-tail of AtRca α could not confer thermostability to OsRca α and swapping of C-tail negatively impacts the thermostability of the respective Rca (Fig. 4.7C). Altogether these results suggested that residues responsible for thermostability can largely be attributed to the nucleotide binding domain. Our observation was consistent with the previous study where a chimeric activase mutant consisting NBD of tobacco and C-terminal of arabidopsis (Tob-Arab) displayed tobacco like thermostability [13].

Additionally, our results indicate that ATPase, and more importantly, Rubisco activase activity of the chimeric mutants were similar to the wild-type Rca whose NBD it bears (Fig. 4.7D). For example, activase activity of Rca β AtNOSC was observed to be around 75-80% of AtRca β and approximately 4 times higher than OsRca β , while Rca β OsNAtC retained 70-75% activase activity compared to OsRca β and only 12-15% of the AtRca β activity. Similarly, activase activities of AtRca α OsTail were also observed to be at a level more similar to AtRca α (60%) than OsRca α (8.5 fold) while OsRca α AtTail possess 60-65% activase activity with respect to OsRca α and only 4-6% activity relative to AtRca α .

Furthermore, Rca β AtNOSC maintained almost full ATPase activity (100%) of AtRca β (Fig. 4.7D), suggesting that the NBD of AtRca β is important for high levels of ATPase activities. Consistently, ATPase activity levels of AtRca α OsTail were also observed at similar level to AtRca α (60-65%). This is expected as AtRca α OsTail also contains the NBD of AtRca α which we believe to be important for high levels of ATPase activities. These results were further supported by the low levels of ATPase activity recorded in our data for both Rca β OsNAtC and OsRca α AtTail as compare to AtRca β and AtRca α respectively. Rca β OsNAtC retained only 60% ATPase activity compared to AtRca β , while OsRca α AtTail meagrely possess only 12-15% activity relative to AtRca α . Collectively, these observations suggest that both high activase and ATPase activity of the chimeric mutants mapped to the NBD. Thus, it was

intriguing to further study the residues in the NBD region in an attempt to precisely identify the determinants of these features.

4.6 ATPase activity of AtRca α isoform was not enhanced in presence of DTT and thioredoxin-f of rice

As described previously (section 3.3), ATPase activity of OsRca α could be modulated by the action of thioredoxin-f (trxf) in presence of a reductant such as DTT. Therefore we investigated the effect of rice thioredoxin-f on AtRca α (Fig.4.8). As a control experiment, a 2-fold increase in ATPase activity of was observed for OsRca α in presence of thioredoxin and DTT (Fig. 4.8A). Contrary to this, ATPase activity of AtRca α was not stimulated in presence of rice thioredoxin and DTT (Fig. 4.8B). This suggests that redox regulation should involve specific intra-protein interactions plausibly between the residues of C-tail and other domains of activase as reported earlier [155, 156].

Interestingly, we have observed that ATPase activity of AtRca α was reduced in presence of DTT and thioredoxin (Fig 4.8B). This behaviour was contradictory to existing literature available on redox regulation of Rca and also to our results with rice activase. Is it really possible that an inverse regulatory mechanism could exist between thioredoxin-f and agave activase? However, the reduction in activity was not significant enough to rule out the probability of experimental error and it would be too early to make a statement at this point. Further investigation is needed to validate the outcome and if so then follow up study should be conducted in future to understand the mechanistic aspect of this observation. The preliminary attempts to purify recombinant thioredoxin-f of agave were not successful. In the near future, further attempts would be made to purify recombinant thioredoxin-f isoform of agave and utilize it to conduct similar experiments. This will be useful to explore if the specificity of thioredoxin-f is a crucial factor or a different regulatory mechanism of regulation may exist for agave as compared to rice.

Fig. 4.8: Comparative effect of rice thioredoxin-f on ATPase activity of AtRcaα. Activases (A) OsRcaα, (B) AtRcaα (25 μM each) were incubated with either 5 mM dithiothreitol (dtl), 3.6 μM rice thioredoxin-f (trxf) or both for 10 minutes prior to conducting assays. Assays were conducted at 25 °C with 5 μM Rca protomer, 1 mM DTT and 0.72 μM thioredoxin-f in the final reaction mix.

4.7 Mutational analysis of various residues at NBD revealed that a single glutamate (217) to glutamine (E-Q) substitution confers high *in vitro* activase activity to OsRcaβ

In order to further identify the residues responsible for higher thermostability and activase activity of AtRcaβ, we initiated a comparative sequence-based mutagenesis study in the NBD of rice. Subsequently, site-directed point mutants were generated in the NBD of rice based on the sequence alignment between rice and agave Rcaβ isoforms. Out of various uncommon residues, only the residues which are different in rice and both the isoform of agave were chosen (Fig. 4.9A).

A

B

C

D

Fig. 4.9: Thermal and biochemical characterization of site-directed point mutants in NBD of rice. (A) Sequence alignment between rice and agave featuring NBD region highlighting mutated residues. (B) Ribbon representation of the hexameric model of tobacco Rca (PDB id: 3ZW6) (left) displaying the locations of the mutated residues in blue (right). *The position of E217 is not resolved in the structure and therefore the residue resolved adjacent to it (E219 in tobacco) has been displayed (C) ATPase assay was performed at 25 °C, and after a 10 minute incubation at 45 °C. (D) Biochemical characterization of the mutants. Rubisco activase activities of WT Rca isoforms measured at 25 °C with 5 μ M Rca protomer, 0.5 μ M ECM and ER (final concentration). Error bars indicate the standard deviations of mean values of three or more experiments.

Thermostability test on these point mutants revealed that all tested mutants possessed similar thermotolerance as OsRca β and none of the point mutations could enhance its thermostability even to a slight extent (Fig. 4.9C). Biochemical characterization of the point mutant showed that all Rca mutants retained more or less similar base level of ATPase except F68L (Fig. 4.9C). The Rubisco activation activity of all the point mutants was also intact except F68L which only possessed <10 % of the activity, while K162A and P220A retained around 50% of the activity. G73D, S90T, and T95N preserved ~90% of the activity compared to WT. Interestingly, E217Q attained a significantly much higher activation activity (~4 fold) as compare to OsRca β and conferred AtRca's high activation rate to OsRca (Fig. 4.9D).

Examination of the structure of Rca from tobacco [129] revealed that area around this residue was not resolved but appears to be a part of disordered element. This flexible loop is predicted to be present on the surface and in close proximity to helix 9 (within 17-22 Å) of the adjacent subunit (Fig. 4.10A) which is responsible for recognition of Rubisco in plants [129, 132]. Therefore, we hypothesized that this residue or loop is crucial for Rubisco remodelling and resulting charge difference due to mutation changes the extent of interaction at the Rubisco interacting sites. However, other variants of this mutant such as E217A, E217K possessed the similar phenotype (Fig. 4.9D). This suggested that this residue may not be directly involved in the protein-protein interaction. Instead, mutation of this residue may cause conformational changes in and around the interacting region, which somehow results in better recruitment or a stronger interaction with Rubisco.

Fig. 4.10: Characterization of OsRcaβE217Q. (A) Ribbon structure of hexameric model of tobacco Rca (PDB id: 3ZW6) highlighting disordered loop which consists the mutated residue (red), along with helix 9 (α_9) region (blue) responsible of Rubisco recognition in plant. The numbering was as per tobacco. Rubisco activation assays for OsRcaβE217Q along with WTs Rca from rice and agave (B), and other E217 variants (C). Assays were conducted at 25 °C with 5 μ M Rca protomer concentration and 0.5 μ M ER and ECM. Error bars indicate the standard deviations of mean values of three or more experiments.

From the above data it can be concluded that mutational analysis of the NBD region could not locate the key residues responsible for thermostability. However a single glutamate to glutamine (E217Q) substitution can impart agave-like properties in terms of Rubisco reactivation activity (Fig. 4.10B). It was a promising outcome which prompted us to further study this unidentified loop in order to precisely determine its role in Rubisco-Rca interaction.

4.8 Mutational analysis of the newly identified surface loop residues revealed a key lysine (216) important for Rubisco-Rca interaction

Following the previous observation, single point mutants were generated (Fig. 4.11A) to test the impact of mutation in this surface loop in terms of ATPase and Rubisco activation activity. Mutating the aromatic tyrosine residue (Y214A) led to around 80% loss of ATPase activity and an almost complete loss of Rubisco activase activity (Fig. 4.11C). Mutation of asparagine residues in the loop (N215A & N219A) led to reduction of both ATPase as well as Rubisco activase activity. A reduction of around 45% and 80% in ATPase rate and decline of approximately 78% and 65% in Rca function was observed for N215A and N219A respectively. Mutating aspartate-218 to alanine was tolerated. However, mutating the lysine residue (K216A) adjacent to the glutamate residue (E217) characterized above led to loss of 85-90% of the Rubisco activation activity, while still retaining ~70-75% of the ATPase activity. This was highly indicative of a possible role of this lysine residue in the Rubisco-Rca interaction. Creating a charge switch mutation of this variant (K216E) displayed an even stronger phenotype with almost complete abolition of activase function, while only maintaining ~50% of the ATPase rate (Fig. 4.11C).

Taken together these observations argued for the importance of this surface loop, especially the lysine-216, in Rubisco-Rca interaction. Since structurally this loop appears to be close to helix 9 (Fig. 4.10A), it is quite possible that this loop might either be directly involved in Rubisco-Rca protein interaction through docking or might play an important role in conformational remodelling of the active site of Rubisco.

Fig. 4.11: Characterization of the newly identified surface loop residues. (A) Sequence alignment between rice and agave of the region targeted for mutational study highlighting the mutated residues. (B) Web logo representation [232] of sequence conservation for Rca residues in and around the surface loop (residues 207-217). Sequence logo was created using an alignment of Rca sequences from more than 200 plant species. Size of the letter denotes the level of conservation for particular residue proportional to other. (C) Biochemical characterization of the residues in the loop. ATPase and Rubisco activation assays were performed at 25 °C with 5 μM Rca protomer, 0.5 μM ECM and ER. Error bars indicates the standard deviations of mean values of three or more independent experiments.

The web logo representation [232] of the sequence elements of this loop demonstrates high conservation level among Rca family in plants except for E217 and D218 (Fig. 4.11B). Glutamic acid (E) at position 217 was observed to be substituted by glutamine (Q) in most of the cases including for tobacco, agave and spinach (Fig. 4.11A and B). Considering the results for E217Q mutants in our study (section 4.7) it could be speculated that this substitution might be an affinity switch among Rca in plants. A comparative biochemical characterization of the other Rca homologues carrying this hypothetical switch needs to be performed in future.

Unavailability of a full length atomic model of Rubisco activase until now, even after several attempts, limits our understanding in predicting the exact mechanism. Solving a full length structure of Rubisco activase would assist in planning other structurally guided mutations to understand the activation process comprehensively. Also additional Rca homologues should be considered for bioprospecting and careful biochemical characterization should be carried out to achieve mechanistic insights and identification of candidate proteins for engineering thermostable activase. Considering the data from this study other CAM plants could be a potential source for such exploration process due to their vast diversity and innate physiological properties. Here we predict that, since CAM has evolved convergently on multiple occasions [42, 233, 234], different molecular solutions to thermostability may have evolved.

**5. Generation of a rice Rubisco activase
molecular toolbox and towards an improved
mechanistic appreciation**

The mechanism by which Rubisco activase remodels Rubisco enzyme to release sugar phosphate inhibitors is poorly understood in spite of several mutational studies and emerging structural information. Recently, strong evidence has been produced that red-type Rca (CbbX) functions by threading the C-terminus of the Rubisco large subunit through the central pore of the disc-shaped oligomer [104, 114]. However, an analogous mechanism for the plant system appears unlikely, as the C-terminus of the plant Rubiscos is significantly shorter. In addition, a key tyrosine residue responsible for the threading in the CbbX pore loop 1 segment is absent in the plant activases [158]. In the past several mutagenesis studies have been conducted towards understanding the mechanism of Rubisco regulation by Rca. Based on these studies some of the critical residues involved in ATP binding, hydrolysis and Rubisco activation were identified (reviewed by [8, 47]).

The results from these studies are taken as basis in this section and discussed in terms of their locations/sub-domains in Rca structure. The mutational works performed on activases in the past were mostly the outcomes of sequence homology models of Rubisco activase to the other AAA+ proteins [8] due to non-availability of a model of Rubisco activase structure. Here, we have utilized both the knowledge from earlier mutagenesis studies and the newly gained structural information on Rubisco activases [129, 131] to demonstrate and compare the effect of already known mutants in rice. We have also introduced new structurally guided mutation to further probe their function in terms of ATP hydrolysis and Rubisco activation. Unfortunately, the three currently available published crystal structures of Rubisco activase are incomplete. Out of them the comprehensive structures of arabidopsis and tobacco Rca are lacking the N-terminal domain, the pore loop regions and the regulatory C-terminus of the long isoforms [129-131]

Furthermore, the function of activase in terms of subunit-subunit interaction and cooperativity is also mostly elusive. Subunit mixing or mutant doping strategy is one of the promising ways to overcome this. A mutant doping strategy is often used to determine the relative contribution of the individual mutant subunits towards ATPase and remodelling activity in AAA+ proteins by biochemically analyzing the properties of mutant/wild-type subunit mixtures [193, 194, 235]. For example, the relative ability to tolerate ATPase inactive subunits reveals whether a concerted or stochastic

mechanism is employed in function [236] as previously shown (section 3.5.1). Rubisco activase also demonstrates ability to rapidly exchange subunits as reported earlier [129, 163, 164] and in the present study (section 3.5.1) and is amenable to the mutant doping strategy. In this section, we have employed this strategy to determine the contribution of individual subunit towards Rubisco activation and measure the relative importance of the tested residues.

In this strategy, usually ATP hydrolysis or substrate binding impaired mutant subunits are mixed with wild-type subunits in variable ratios, keeping the final protein concentration constant. Random mixing between WT and mutant subunits would lead to formation of heterogeneous population of hetero-oligomeric ensembles (Fig. 5.1A). This generates a variable probability distribution of incorporation of particular number of mutant subunit in hetero-oligomer as a function of fraction of mutant subunit present in the mix (WT: mutant) which can be modelled using a binomial distribution (Fig. 5.1A, also see 2.7.10.3). Therefore, for any specified percentage of mutant subunits the probability distribution of hexamers containing 0, 1, 2, 3, 4, 5 or 6 mutant subunits can be derived as shown in Fig. 5.1A.

This distribution is then used to predict how activity could be inhibited at different mutant-wild-type ratio. By plotting a curve between theoretical relative activities against % of mutant, theoretical activities at a particular ratio of WT/mutant could be determined. Different curves are obtained, based on the specific number of mutant subunits (1-6) required to abolish activity of the whole hexamer (Fig. 5.1B) [193, 194, 236].

Fig. 5.1: Theoretical model to predict the effect of subunit doping. (A) Theoretical populations of Rubisco activase hetero oligomeric ensembles containing various numbers of mutant subunits (0-6) as function of the fraction of mutant subunit present in the mixture as represented by different colours. (B) Curves representing the theoretical activity against the % of mutant subunits present, when a particular number of mutant Rca subunits (1-6) are required to abolish the activity of the Rca hexamer.

5.1 Subunit doping experiment with Walker B mutants indicates strong cooperativity between subunits

Using the above model, we can determine how Rubisco activation activity is inhibited at various WT to mutant ratios. Based on that analysis, we can state whether subunit coupling within activase oligomers follows a probabilistic (non-cooperative), sub-

globally cooperative or globally cooperative model by comparing our data with the theoretical plots (Fig. 5.1B). These outcomes were based on the hexamer as the functional unit. Consequently, if subunits within the hexamer are independent and follow the probabilistic model, it means the cooperativity is dispensable (non-cooperative effect) and only one WT subunit per hexamer will be sufficient for activity. In this case, activity should decline in a linear manner with increasing concentration of the mutant subunits in the mix. On the other hand, if subunits are coupled, then a specific number of mutant sub-units are required to abolish the activity of the hexamer, which would be consistent with a subglobally cooperative (if two to five mutant subunits per hexamer are required to eliminate activity) or a globally cooperative model (if only one mutant subunit per hexamer ablate activity). In a globally cooperative model there would be a rapid drop in activity and therefore a steep curve is expected, while in case of a subglobally cooperative behaviour an intermediate curve is anticipated (Fig. 5.1B) [193, 194].

Fig. 5.2: Titration of WT β -Rca with increasing concentration of ATPase inactive Walker B (D173A) mutant. WT active β isoform was pre-incubated with the OsRca β D173A at 258-272 μ M Rca protomer concentration range for 10 min. Rubisco activation assay (left) and ATPase assay (right) were performed by maintaining 5 μ M activase protomer concentration in the final reaction mix at 25 $^{\circ}$ C. Activase and ATPase activity was calculated considering WT activity as 100%. Dotted lines indicate the theoretical activity curves if respective number of subunits abolishes hexamer activity. Error bars indicate the standard deviations of the mean values of three or more independent experiments.

For instance if we take an example of ATPase inactive Walker B mutant (D173A), which was earlier shown to be completely ATPase inactive and followed a sub-globally cooperative mechanism of ATP hydrolysis (section 3.5.1 and Fig. 5.2), was also found to be a strong mutant in terms of abolishing Rubisco activase activity in mixing with WT active subunits (Fig. 5.2). OsRca β D173A itself was completely incapable of activating inhibited Rubisco by its own (> 95%). The titration curve with increasing WT to mutant ratio revealed a significant drop of around 50% of maximal activase activity even at very low proportion (20%) of Walker B mutant. Subunit mixing at an equimolar ratio led to almost 80% loss of activity. Similarly a drop of 40% ATPase activity was observed at 20% mutant concentration (Fig. 5.2). This suggests strong inter-subunit coupling with respect to ATPase as well as Rubisco activation activity for Walker B mutant (D173A) of Rubisco activase in rice. Previously, a similar effect in terms of subunit interaction has been demonstrated for ATPase deficient mutants. For instance, addition of Walker B and D231R mutant with wild-type tobacco activase caused a reduction of 40% and 80% ATPase activity respectively whereas a drop of 75% activase activity has been reported in case of the K247R mutant [163, 164].

Mathematical analysis of our data using the theoretical curves (Fig. 5.1B) corresponds to 1-2 mutant subunits being sufficient to abolish the activase activity of whole hexamer, while 2-3 mutant subunits are enough to ablate ATPase function. Thus, in this case Rca hexamer must contain at least five ATPase functional subunits to catalyze Rubisco remodelling, while a minimal four subunits are necessary for ATPase function. It could also be stated that Rca subunits utilize a highly coupled sub-globally cooperative mechanism to hydrolyse ATP for substrate remodelling and multiple subunits within the hexamer should work together to carry out its function in an efficient manner.

In other homologous activase systems like CbbX and CbbQO [114, 115] also, Walker B mutants was found to be deficient in ATPase as well as activase function. However, the type of subunit cooperativity involved in their functioning was not analyzed in above studies. Walker B mutation in non activase AAA+ system such as ClpX, which follows probabilistic model for ATP hydrolysis displays complete loss of ATPase

function and ability to unfold and translocate its substrate while allowing substrate binding [237, 238].

The double Walker B (DWB) mutation among class 1 AAA+ proteins such as ClpB and Hsp104 which possess two Walker B sites also abolished ATPase and luciferase reactivation function [193, 194, 239, 240]. A strongly coupled mechanism in terms of ATPase and chaperone activity was also reported for the DWB mutant of ClpB, similar to our case [193, 194]. On the other hand, Hsp104DWB follows a probabilistic mechanism for ATPase hydrolysis like ClpX but sub-globally cooperative model for luciferase reactivation activity [193]. However, variations in data have been reported by different groups, possibly implying that these proteins may exhibit operational plasticity in ATP utilization depending on the substrate and requirement of other assisting chaperones to determine the extent of cooperativity [240, 241].

5.2 Validating the phenotype of key OsRca variants, studied previously in other plant Rca systems

The effect of various mutations on ATPase and Rubisco activation activity has been investigated earlier for Rubisco activase of spinach and tobacco [129, 159, 160]. The importance of the N-terminal domain has been reported in Rubisco activation as a putative substrate adaptor domain like other AAA+ proteins [128, 242, 243]. In agreement with the finding by van de Loo and Salvucci (1996), mutation of the conserved tryptophan 15 to alanine (W15A) in the N-terminal domain of rice Rca resulted in unaltered ATPase activity but a >90% loss in Rubisco activation (Fig. 5.3). This indicates its involvement in engaging substrate through hydrophobic interactions with Rubisco. However, in contrast to the result reported for tobacco [129], the N-terminal truncation of 66 residues (Δ N67) resulted in a significantly elevated ATPase rate ($\sim$ 1.7 fold) and around $\sim$ 30% of residual Rubisco activation activity (Fig. 5.3). In tobacco, the equivalent truncation led to a normal ATPase hydrolysis rate and an almost complete loss of Rubisco activation [129].

Fig. 5.3: Characterization of Rubisco activase mutants. ATPase and Rubisco activase activities of WT Rca (β isoform), N-terminal truncation (Δ N67), point mutations W15A (N-terminal), K315D (specificity helix), and D173A (Walker B) as indicated. Assays were performed at 25 °C with 5 μ M activase protomer and 0.5 μ M inhibited Rubisco (ER). Error bars indicate the standard deviations of the mean values of three or more independent experiments.

A small helical insertion (5 residues) in the structure of Rca called helix 9 (α 9) is positioned on the periphery of the hexameric ring [129]. Mutation of the residues aspartate 316 and leucine 319 to lysine and valine respectively (tobacco) at helix 9 reversed the specificity of Rubisco activase from Solanaceae Rubisco to the Rubisco of non-solanaceous plants [132, 168]. Consistently, we found that mutating the lysine at 315 to aspartate (K315D) in rice resulted in a slightly reduced ATPase rate (~80%), but almost no Rubisco activation activity (Fig. 5.3). Thus, this helix must play an important role in substrate recognition and was crucial for Rubisco activation in plants.

5.2.1 Rubisco interaction site mutants display variable levels of importance in context of oligomer function

The results in the previous section reconfirmed the important effect of a number of Rca features, previously reported for homologous systems. In order to determine the relative contribution of individual residues to activase function in context of the

oligomer, we initiated systematic subunit-mixing/mutant doping experiments. These experiments are designed to further clarify how Rubisco activase subunits engage and remodel Rubisco, which is currently poorly understood.

Fig. 5.4: Titration of WT β -Rca with increasing concentration of interaction site mutants. WT active β isoform was pre-incubated with mutants: W15A, K315D (A) and Δ N67 (B) for 10 min to yield the desired proportion. Pre-incubation was done at 240-360 μ M (W15A), 330-390 μ M (K315D) and 250-360 μ M (Δ N67) Rca protomer concentration. Rubisco activation assay (left) and ATPase assay (right) were performed by maintaining 5 μ M activase protomer concentration in the final reaction mix at 25 $^{\circ}$ C. Activase and ATPase activity was calculated considering WT activity as 100%. Dotted lines indicate the theoretical activity curves if respective number of subunits abolishes hexamer activity. Error bars indicate the standard deviations of the mean values of three or more independent experiments.

Mixing of the W15A variant (N-terminal domain) with WT Rca in increasing proportions resulted in a roughly linear reduction in activase activity (Figure 5.4A). Assuming the hexamer as the functional unit, the data indicates that an activase

hexamer can tolerate high number of inactive mutant subunits (W15A) and even a single WT subunit per hexamer is sufficient to remodel Rubisco. The high tolerance of WT Rca to W15A subunits suggests that the protein-protein interaction mediated by this residue need not to be present for all six subunits of activase (Fig. 5.1B and 5.4A). This supports a recruitment and/or orientation function of this residue, rather than an active mechanistic role in Rubisco remodelling. The W15A mutant has an approximately similar ATPase activity as WT and subunit mixing with WT almost has no effect on the ATPase activity of the heterocomplexes (Fig. 5.4A). This indicates that the observed pattern in case of activase activity is not affected by the ATPase rate during experiment.

In contrast, the specificity helix charge switch mutant (K315D) showed high cooperativity in terms of activase activity. The data corresponded to a situation where 2-3 mutant subunits abolished hexamer activity (Fig. 5.1B and 5.4A). If two mutant subunits can abolish the activity of Rca hexamer then four Rca subunits are not enough for activity, hence five are needed. Similarly if three mutant subunits can inactivate Rca hexamer then three subunits are not sufficient for activities, hence four are needed. Thus 4-5 WT subunits per hexamer are required for substrate engagement. This suggests that Rubisco engagement in plants via helix 9 utilises a sub-globally cooperative mechanism to remodel Rubisco by utilising multiple subunits within hexamer to be functional at a time. This might involve interaction with more than one recognition site on the surface of different Rubisco large subunit. Subunit doping between K315D and WT Rca did not alter the ATPase activity of the heterocomplexes (Fig. 5.4A). However, the problem here is that the charge switch used in this case is from a positively charged residue to a negatively charged one, which would cause electrostatic repulsion at the interaction site and thus complicates our analysis. Therefore, for a clearer answer, the K315A substitution mutant should be analyzed in future which might generate less drastic changes. In any case, the observed result suggests that multiple helix 9 moieties of the oligomer interact with Rubisco during remodelling.

Mixing experiments with the N-terminal domain (NTD) deletion mutant (Δ N67) resulted in a counterintuitive outcome. Earlier, we observed that mutation at N-terminal domain (W15A) resulted in a linear decline in activase activity of Rca

hexamer. In contrast, activase activity of WT Rca was stimulated by the Δ N67 mutant (Fig. 5.4B). The Δ N67 by itself possessed around ~30% of activity, but led to an enhanced activase activity compared to WT, when incorporated at any proportion. A more than 2-fold increase in the activity was observed at an equimolar mixing concentration. Even at 80% of mutant concentration, the mix maintained an around 1.5-1.7 fold higher activation rate than wild-type subunits. This result suggests that, like for W15A, only 1 or two NTD subunits are required for interaction with Rubisco and the higher activation rate may be attributed to the higher ATPase rate of the NTD deletion subunits (Fig. 5.4B). This observation also compels us to hypothesize that the ATP hydrolysis rate may be the dominant determinant of activation rather than the Rubisco interaction in this case. Probably, this combination should be working in a complimentary way, where required interaction sites in the oligomeric mixture are provided by small number of WT Rca subunits but the overall high ATPase rate of the oligomer may be responsible for better activation. Similar to our observation, a NTD mutant of the yeast chaperone Hsp104 was also found to possess a higher ATPase rate but reduced disaggregase activity and exhibit a stimulatory effect with other mutants as found for Rca in our study [240, 244]. However the effect of mixing NTD mutant subunits with WT Hsp104 was not reported in these studies.

Next, we performed subunit mixing experiments between Δ N67 and other mutants to determine whether the NTD mutant could also enhance the activity of other activase mutants when included in a hetero-oligomeric complex. These were 1) the Walker B mutant (D173A) which can provide a functional N-terminal domain but no ATPase activity, 2) the N-terminal domain mutant (W15A) which could provide ATPase function but lacks a functional N-terminal domain and, 3) the Helix 9 mutant K315D responsible for species specificity, which can offer both ATPase activity and required N-terminal domain, but is impaired at another Rubisco interaction site (Fig 5.5A and B).

In the case of D173A and W15A, the stimulatory trend seen for the WT subunit was not observed (Fig. 5.5B). Adding the D173A mutant to Δ N67 resulted in a drastic reduction of activase and ATPase function similar to that seen when mixing to wild-type. A drop of around 60% in ATPase and activase activity was observed compared to Δ N67 in the presence of low concentration of D173A (20%). In contrast, at low

proportion of W15A, activase activity of the composite oligomer was reduced and almost resembled the function of the W15A variant alone (100%) at all proportions. Since, the ATPase activity of this oligomeric mixture is not strongly perturbed (Fig. 5.5B), the effect should be the outcome of a missing interaction site due to unavailability of a functional N-terminal domain.

A

B

C

Fig. 5.5: Titration of $\Delta N67$ Rca with increasing concentrations of different mutants. (A) Surface representation of the hexameric model of tobacco Rca (PDB id: 3ZW6) highlighting the locations of mutated residues. The N-terminal domain is not resolved in the structure. The putative position of the N-terminal is indicated as a dotted circle around the first residue in the model (aspartate 67 in rice). (B) $\Delta N67$ Rca mutant was pre-incubated with other mutants at 230-250 μM (D173A), 220-230 μM (W15A), 240-290 μM (K315D) Rca protomer concentrations for 10 min to yield a desired proportion. Rubisco activation assay (left) and ATPase assay (right) were performed by maintaining 5 μM activase protomer concentration in the final reaction mix at 25 $^{\circ}C$. Activase and ATPase activity were calculated considering $\Delta N67$ activity as 100%. (C) Experimental scheme with summarized outcomes.

In stark contrast, addition of K315D led to an enhanced activase activity (around 2-fold) as compared to $\Delta N67$ at low proportions (50% or less). Increasing the concentration of K315D beyond 50% diminishes this stimulatory effect and a drastic reduction in activase activity was observed (Fig. 5.5B). This result could be explained by the fact that this combination contains both the required components for Rubisco remodelling. The necessary N-terminal domain is provided by the helix 9 mutant, while the presence of NTD mutant increases the overall ATPase activity of the

combined oligomer as compared to K315D alone (Fig. 5.5B). Although the activase activity of this combination (Δ N67+ K315D) was higher than that of the individual components, the overall activity was still a fraction of WT (50-60%) which does not lack any interaction site. Thus, even if these residues are not solely responsible for activase function, considering the result from Fig. 5.3, 5.4 and 5.5, we may suggest that all residues considered in this study are important for optimal activase functioning to different extents. Functionality of these mixtures indicates that the NTD and the helix 9 interaction site do not have to be localized on the same subunit to function productively.

5.3 Structure based identification of novel Rubisco interacting residues

Many AAA+ proteins utilize a partial or complete threading mechanism to remodel the conformation of their binding substrates through the central pore of the hexamer, which features a highly conserved aromatic-hydrophobic-glycine pore-loop 1 motif [117, 125, 245]. This typical conserved motif is absent in the pore loop 1 of plant Rca, although the sequences in the corresponding region are highly conserved in the plant Rca family (Fig. 5.6A). Unfortunately, all pore loops were not resolved in the available structures, presumably due to their flexible nature. As mentioned earlier, the bacterial red-type Rubisco activase CbbX appears to utilize a classical pore loop threading mechanism [114], but an analogous mechanism for plant activase could not be established [129]. Nevertheless, extensive site-directed mutagenesis of the pore loop segments of the tobacco activase did implicate the pore in its function, possibly to engage larger structural elements of plant Rubisco [129]. As observed previously for NtRca [129], in OsRca the mutation of alanine 143 at the pore loop 1 to valine resulted in a slightly reduced ATPase rate but completely abolished Rubisco activation activity (Fig. 5.6C) suggesting its possible role in substrate remodelling.

The results regarding the pore loop 1 residue (A143V) indicated that this residue is essential for Rubisco activation. Given that mutating adjacent residues in pore loop 1 did not result in significant phenotypes in the tobacco protein [129], the role of the pore loop 1 in activase function remains to be investigated further. Therefore, we performed a mutational study of the residues further downstream to A143 in rice

(G144, E145), which had not yet been interrogated. Also, we perused the surface of the hexamer hypothesized to face the Rubisco holoenzyme [246] and identified a number of previously untested, highly conserved surface residues. They were the charged surface residues in helix 3 (K148, Q152, E156, D159) adjacent to pore loop 1 and a protruding surface residue near the specificity helix 9 (R321) (Fig. 5.6A and B).

Fig. 5.6: Mutational analysis of pore loop 1, its extension and surface residues. (A) Sequence alignment of activase sequences from different species highlighting the targeted residues for mutation. (B) Ribbon representation of the locations of mutated residues in the hexameric model of tobacco Rca (PDB id: 3ZW6). The position of A143 is not resolved in the structure. (C) ATPase and Rubisco activase activities of WT Rca (β isoform), key pore loop 1 mutant (A143V) extended pore loop 1 mutants (G144A, E145A), surface mutants at helix 3 (K148A, Q152A, E156A, D159A) and protruding surface residue in the structure (R321A), as indicated. Assays were conducted at 25 °C with 5 μ M Rca (Protomer), 0.5 μ M RuBP inhibited Rubisco (ER). Error bar indicates the standard deviations of the mean values of three or more independent experiments.

Mutation in the residues adjacent to alanine 143 (G144A, E145A) led to a 80-90% loss of ATPase activity with a concomitant 90-95% reduction of activase function (Fig. 5.6C). Mutational analysis of the surface residues downstream to pore loop 1 gave mixed results. Mutating lysine in this region (K148A) resulted in a slightly enhanced ATPase activity but almost complete loss of activase function. Similarly mutating the surface arginine (R321) near specificity helix resulted in increased ATPase with ~75% loss of activase function. This implicates these two surface residues in Rubisco interaction. The corresponding residue for arginine in tobacco structure is phenylalanine as shown (Fig. 5.6B). Q152A and E156A resulted in reduction of both ATPase (around 30-50%) as well Rubisco activation activity (around 20-30%). Their role is difficult to interpret as the loss of activase function is likely to be the result of low ATPase rate of the mutants. Surprisingly, mutation of an

aspartate residue in this region (D159A) led to a 40-45% reduction in ATPase activity but stimulated activase activity (~1.6 fold), which suggests a role of this residue in Rubisco remodelling. It is possible that either this residue, as a part of surface helix, interacts with Rubisco directly or may be responsible for the conformational changes of the interacting region due to charge differences. The observed behaviour of this mutant could also be the outcome of enhanced affinity for Rubisco and need to be examined using crowding approach in future.

5.3.1 The key pore loop 1 mutant (A143V) reveals strong subunit coupling with respect to Rubisco activase activity

Earlier reported data from the literature along with the results discussed in previous section argued for the importance of pore loop1 residue in Rubisco activation [129]. Thus, to further assess the role of the conserved alanine residue, we performed subunit doping experiment between WT and A143V.

Fig. 5.7: Titration of WT β -Rca with increasing concentrations of pore loop 1 mutant (A143V). WT active β isoform was pre-incubated with the pore loop 1 mutant (A143V) at 220-340 μ M concentration range for 10 min to yield the desired proportion. Rubisco activation assay (left) and ATPase assay (right) were performed by maintaining 5 μ M activase protomer concentration in the final reaction mix at 25 $^{\circ}$ C. Activase and ATPase activity was calculated considering WT activity as 100%. Dotted lines indicate the theoretical activity curves if respective number of subunits abolishes hexamer activity. Error bar indicates the standard deviations of the mean values of three or more independent experiments.

OsRca β A143V mutant was completely incapable of activating inhibited Rubisco by its own as shown previously (Fig. 5.6C). A sharp drop in activase activity was observed even at a low proportion of pore loop 1 mutant. Loss of ~80% of maximum activity was observed at an equimolar concentration of WT and mutant (Fig. 5.7). Mathematical analysis of this data using theoretical plots suggests that incorporation of just one or two mutant subunits was sufficient to abolish the activity of the whole hexamer. At the same time, ATPase function was not perturbed (Fig. 5.7). Thus, an Rca hexamer in this case must contain all or at least five active subunits with intact pore loop 1 to remodel Rubisco and subunits are coupled in a globally or sub-globally cooperative manner to remodel Rubisco (Fig. 5.1B and 5.7) [193, 194]. Although, the A143V mutant has a very strong phenotype, the nature of the mutation is unusual, in that a small side chain is replaced with a bulkier one. To functionally impair the pore loop, a bulky aromatic residue like tyrosine is generally mutated [114, 117]. Collectively, these results suggest that pore loop 1 in plant Rca is vital for Rubisco remodelling and has to be intact in a majority of subunits per oligomer for unperturbed Rca function. Furthermore, we have identified key surface residues involved in Rubisco interaction which supports the hypothesized model of Rca-Rubisco interaction [246]. In future, the important residues found in this study (K148, D159 and R321) must also be analyzed using the mutant doping strategy to further define their role in Rubisco remodelling.

5.4 Mutational analysis of phosphorylating residue at the NTD indicates its role in Rubisco-Rca interaction and might be an alternate regulatory mechanism in plants under light dark transition

Protein phosphorylation is an important type of post-translational modification which can modulate the activity of an enzyme or protein. Effect of protein phosphorylation has been studied for many AAA+ proteins such as the mitotic factor p60 and p97 [247-249] leading to inhibition of their activity. Recent phosphoproteomics studies have found Rca to be an abundantly phosphorylated protein in photosynthetic tissues. Furthermore, it has been predicted that Rca activity could also be modulated by phosphorylation at a site critical for its activity like ATPase rate or its interaction with Rubisco [250, 251]. Thus, protein phosphorylation could be another regulatory

mechanism in plants in addition to redox regulation under light/dark cycle. However, recently redox changes in chloroplast stroma have been reported as the major trigger behind the phosphorylation of Rca, possibly due to oxidation of thioredoxins [252]. It was also suggested that phosphorylation may not be responsible for downregulating *in vivo* Rca activity in arabidopsis at least in the presence of redox regulation. Nevertheless, it was predicted that phosphorylation might regulate Rca activity when redox regulation is not operating or under other stress related conditions [252].

Rca in arabidopsis was found to be phosphorylated at threonine 78 and serine 172 (numbering includes transit peptide), which are located at the NTD and the Walker A region of Rca respectively [250, 253]. However, only threonine 78 was responsive to dark/light response and found to be more phosphorylated in the dark, while serine 172 remained unmodulated. Thus, it was predicted that phosphorylation of threonine 78 could be associated with inhibition of Rca activity in the dark when the Calvin-Benson cycle is inactive [250]. Since this residue is located at the NTD, it is conceivable that this modification modulates the Rubisco activation state by preventing the Rubisco-Rca interaction. However, this threonine 78 is not conserved and replaced by isoleucine in rice and maize. Also, no phosphorylation was found for Rca in maize but Rca was found to be phosphorylated at serine (S23 after removing transit peptide) adjacent to isoleucine in rice chloroplasts [251].

To study this phenomenon, we produced two mutants at this position, S23A and S23D. Substituting serine with alanine, tests for the function of hydroxyl group at this position. Aspartic acid is a commonly used phosphomimetic substitution similar to phosphoserine. Both variants were found to be fully ATPase active and retained the original activity of WT but lost almost 70-80% activase function (Fig. 5.8). Since NTD is responsible for Rca-Rubisco interaction and not for ATPase activity, it makes sense that the mutants retained full ATPase activity as observed for earlier NTD mutants such as W15A and Δ N67 (section 5.2). It also indicates that the oligomeric structure of these Rca variants is not compromised. The results showed that serine 23 is likely involved in the Rca-Rubisco interaction. We believe protein phosphorylation could be a good target for studying Rubisco regulation in plants in future. Most of the aspects of protein phosphorylation pertaining to Rca are not well characterised. The nature of the kinase (plastid casein kinase 2) performing phosphorylating function has

recently been reported [252] and could be a promising candidate for regulation and mutational study.

Fig. 5.8: Mutation of the phosphorylated residue at the N-terminal domain of rice Rca. ATPase and Rubisco activase activities of S23A and S23D variants along with WT Rca (β isoform). Assays were conducted at 25 °C with 5 μ M Rca (Protomer). Error bars indicate the standard deviations of the mean values of three or more independent experiments.

5.5 Mutation of conserved arginine (R293V) at the Rca subunit interface ablates its ability to exchange subunits

It has been shown using tobacco activase that mutating the conserved arginine-294 (in tobacco) to valine or alanine resulted into formation of stable hexamer in the presence of ATP γ S using techniques such as nanoESI mass spectrometry [140], negative stain electron microscopy [129] and analytical ultracentrifugation/SAXS [141]. Earlier mutagenesis study of this residue showed that mutating arginine 294 to alanine in tobacco led to a reduced ATPase rate and Rubisco activation, while mutating the same arginine to valine resulted in a WT-like phenotype [129, 165]. This suggests that this substitution has locked the enzyme into a functional hexameric active form which should be an ideal candidate for structural studies.

Therefore, we targeted the corresponding residue in rice (R293) for mutagenesis, hoping to obtain a stable hexamer for rice Rca for both α and β isoform. This mutant could have been utilised for comparative structural studies between rice and tobacco and for determining the overall structure of α -Rca along with its C-tail extension, since tobacco only possesses the β isoform. However, mutation of arginine to valine

(R293V) in rice resulted in a completely non-functional phenotype devoid of ATPase as well Rubisco activation activity (Fig. 5.9A). This suggests that Rca in these two plant species could differ in intermolecular contacts due to structural variation in this region, which also led to different oligomerization pattern in the two species.

Analytical gel filtration chromatography of this variant indicated an elution pattern corresponding to the void volume of the column which was concentration independent (Fig. 5.9B). This indicates that either this variant might have formed soluble aggregates or acquired a spiral high molecular weight oligomeric form, which is concentration and nucleotide independent in rice. The secondary structural elements of this variant should be analyzed using CD and if found intact, this variant would be a promising candidate for structural studies using cryo-electron microscopy. It was also observed that this mutant had lost its ability to hetero-oligomerize and exchange subunits with other Rca. The mixing experiment between WT Rca and R293V subunits did not result in subunit poisoning in terms of ATPase activity in our experiments (Fig. 5.9C).

Fig. 5.9: Mutation of conserved arginine at Rca interface: (A) ATPase and Rubisco activase activities of conserved arginine at Rca interface (R293V) along with WT Rca (β isoform). Assays were performed at 25 °C with 5 μ M Rca (Protomer). Error bars indicate the standard deviations of the mean values of three or more independent experiments. (B) Analytical gel filtration chromatography of OsRca β R293V at different concentration performed using a Superdex 200 3.2/30 in 20 mM Tris-HCl pH 8.0, 50 mM NaCl, 5 mM MgCl₂, 0.5 mM ATP. (C) Mixing experiment between OsRca β and Rca interface mutant (R293V) at an equimolar concentration in the final reaction mix (5 μ M each). The two proteins (170 μ M Rca protomer) were pre-incubated for 10 min prior to assay.

In this chapter we have carried out an extensive study of activase variant to validate the mutational phenotypes of various previously reported residues. Moreover, by applying a subunit doping strategy, we generated additional information about the relative contribution of the targeted residues to Rubisco interaction and remodelling. Furthermore, several novel structurally guided mutants were generated and biochemically characterized to add to our appreciation of activase function. Based on our observations, we have left many open questions, while generating a molecular toolbox to address them. In future, our library of mutants will allow the design of additional experiments that will permit inferences to be made about the precise mechanism of Rubisco activation and other functional aspects of the Rca enzyme.

**6. A preliminary characterization of the putative
Rubisco activase isoform (Rca2) of rice**

In chapter 3, we provided a characterization of the two abundant Rca isoforms found in rice. However, genomic studies suggest that these may not represent the entire Rubisco activation machinery in rice or plants in general. In particular, an uncharacterized putative Rubisco activase isoform sometimes designated as Rubisco activase 2 or Rca2 has been found to be encoded by the nuclear genome of almost all plants and algae [8].

The homologue of this protein in arabidopsis (uniprot id: Q9AST9) has been detected as a stromal chloroplast protein in many proteomics experiments [254](Plant Proteome Database, Cornell) [255]. The gene is mainly found to be transcribed in photosynthetic tissue, which seems consistent with its possible role in Rubisco activation. However, its expression level is much lower than that of the orthodox activase (appendix Fig. 1A and B) (arabidopsis eFP browser) [256]. The Rca2 (gene id: At1g73110) shows negligible or almost no expression in non-photosynthetic tissues such as root. Peak expression levels were found in the mesophyll and guard cells after water spray. Under stress conditions such as ABA treatment, the expression level in these photosynthetic tissues was significantly reduced. The patterns of change in the expression levels were similar to the activase under identical conditions however, the expression signal intensity was much less for Rca2 (appendix Fig. 1A and B). This supports a possible functional association between Rca2 and Rca as genes with similar sequence and expression profiles are likely to exhibit a functional relationship [257, 258].

From the phylogenetic tree analysis (17 angiosperm, 1 bryophyte and 4 green algae) it is apparent that Rca2 and Rca diverged prior to the evolution of land plants, probably following a gene duplication event in the green algae (Fig. 6.1). The complete conservation of both isoforms in all examined higher plant and green algal genomes suggests that Rca2 evolved to play an important, albeit unknown role. The tree illustrates that Rca isoforms are in general more highly conserved than Rca2 isoforms, indicating that a more stringent selection pressure is acting on the former, canonical Rubisco activase sequences.

Fig. 6.1: Phylogenetic tree of plant and algal activase and putative Rca2 sequences. The sequences from seventeen higher plants, one bryophyte, and four green algae (appendix Table 1) were used to generate a phylogenetic tree by Neighbour joining using Mega 6 software [259] using the P-distance model and pair wise gap deletion. Bootstrap values are the percentage from 500 replicates where the given node was present.

From a functional perspective, a sequence alignment of Rca2 homologue in rice with the Rubisco activase predicts intact ATPase active site motifs (Walker A and B motifs are intact) along with a conserved arginine finger (R243 in OsRca). However, the known protein-protein interaction site (specificity helix, $\alpha 9$) with Rubisco is not conserved (Fig. 6.2). Further comparative analysis of the sequences from different Rca2 and activase homologues in plants revealed conservation among some additional residues responsible for maintaining ATPase function (Fig. 6.2). Most of the residues shown to be important for activase activity were not conserved except the tryptophan (W15 in rice activase) at the N-terminal domain (NTD) and the alanine (A143) at pore loop 1. Also, the low sequence identity (48%) between Rca and Rca2 (residues 19-395 in the alignment for OsRca2) in rice suggests that Rca2 might not be a functional activase by itself. Nevertheless, the residues predicted to be important for subunit-subunit interactions in activase [129] were found to be conserved between Rca2 and Rca. Therefore, we hypothesized that Rca2 might somehow modulate the activity or function of Rca by forming a hetero-oligomeric complex with it. Interestingly, few regions at the NTD are distinctly conserved in Rca2. This suggests that the NTD of Rca2 may be involved in specific functions such as substrate anchoring as predicted for the NTD of Rca [59, 129].

In this section, we aimed to characterize this Rca2 isoform to elucidate its biochemical function. We were able to purify a truncated form of the protein using the heterologous *E.coli* expression system. Our initial results indicate that Rca2 can oligomerize with OsRca β to form an ATPase functional complex. These results indicate that there is much more to be learnt about the complexity of plant Rca function.

Fig. 6.2: Protein sequence alignment between plant Rca and Rca2 isoforms: Amino acid sequences of different Rubisco activase and putative Rca2 isoforms were aligned using ESPript [184] after removing the transit peptide as predicted by the ChloroP online server. The ATPase active sites in AAA+ domain (Walker A and Walker B) were conserved between the two groups of proteins including other residues responsible for ATPase function. The interaction site with Rubisco (lysine 315 in OsRca β) was not conserved as marked in the alignment along with other interaction sites including the ones discovered in the present study. The Rca-Rca interface residues were also found to be conserved between Rca2 and Rca in plant. The N-terminal domain of contained some distinct region which are conserved in Rca2 but not in Rca. The uniprot and NCBI accession code of the proteins utilized for creating the sequence alignment has been tabulated in the appendix Table 1.

6.1 Purification of OsRca2

The gene encoding Rca2 of *Oryza sativa* (Uniprot accession code: Q7XN85, expressed amino acid 47-441 (after removing transit peptide using the ChloroP online server) was synthesized by GenScript (USA). The Rca2 was heterologously expressed in *E.coli* BL21 as a N-terminal fusion with His₆Ub and purified exactly as Rca (section 3.1.1). However, the expressed protein was completely insoluble even after several attempts. The protein parameters analysis of full length Rca2 using ProtParam tool [186] revealed the protein as unstable based on the computed instability index parameter. Thus, we analyzed a sequential deletion of the N-terminal residues and found that after removing the first 21 amino acids the instability index was within a stable range.

The corresponding construct named pHue Δ N21OsRca2 was cloned and the protein was purified using identical purification steps to other activases (section 3.1.1). Now the protein was found be soluble upon expression and could be easily purified using affinity chromatography and size exclusion chromatography (Fig. 6.3).

Initially, we suspected that probably these deleted residues at the N-terminal region of Rca2 constitute a membrane anchor considering association of Rca2 with thylakoid membrane in the proteomics experiments [254]. Removing them would thus have led to solubilization of protein. However, analysis of the sequence using the TMHMM online server did not predict any transmembrane helices in the given region. Another possibility could be the erroneous prediction of the chloroplast transit peptide by the ChloroP server.

Fig. 6.3: Representative purification of Δ N21OsRca2. (A) 12.5% SDS-PAGE analysis. Successive purification by IMAC (Ni-NTA), followed by Usp2 mediated cleavage of N- terminal tag and anion exchange chromatography (AIEC) yielded pure protein. (M: Molecular Weight Marker, L:Lysate, S:Soluble, Ni-NTA: Fractions after IMAC, U: Unbound, W:Wash, E: Final eluate, Cut : eluate after USP2 mediated cleavage of Ubiquitin. (B) Size exclusion chromatogram of Δ N21OsRca2 showed a single peak of purified protein (left). Purified fractions from gel filtration column on a 12.5% SDS-PAGE gel (right).

6.2 Δ N21OsRca2 migrates as a monodisperse species by gel filtration and native PAGE

In order to assess the oligomeric assembly and elution pattern of the Rca2 at different concentration, we performed an analytical gel filtration analysis of Δ N21OsRca2 at different concentrations followed by native PAGE. We observed that the protein was eluted mostly as a single peak with a minor shoulder at all tested concentrations (Fig. 6.4A), which was further confirmed by native PAGE of the samples (Fig. 6.3B). Δ N21OsRca2 dominantly displayed monodispersity with the major peak eluted at 1.23 ml, which corresponds to a Stokes radius consistent with a AAA+ hexamer as recently reported by our group for the red-type activase from red algae [104]. These results were highly distinct to the canonical activase, which shows high polydispersity with a concentration-dependent size as described earlier (section 3.5.2). Therefore, the monodispersity of Rca2 makes it a promising candidate for structural studies in future. Although the majority of the protein eluted as a hexameric species, we also observed some smaller bands on native PAGE (Fig. 6.3B), which could be the dissociated oligomers as suggested by their cross-reaction to an anti-Rca antibody (Fig 6.9). These results suggest that the oligomeric assembly of Rca2 is intact after the N-terminal truncation and this protein could be utilized for biochemical study.

Fig. 6.4: Rca2 eluted as monodispersed species as analyzed by analytical gel filtration chromatography and native PAGE. (A) Analytical gel filtration chromatography of $\Delta N21OsRca2$ at different concentrations performed using a Superdex 200 3.2/30 in 20 mM Tris-HCl pH 8.0, 50 mM NaCl, 5 mM $MgCl_2$, 0.5 mM ATP. (B) 6% Native PAGE analysis of $\Delta N21OsRca2$ (right) compared to $OsRca\beta$ (left) at different concentrations. Amount of protein loaded has been indicated against each lane.

6.3 $\Delta N21OsRca2$ was ATPase inactive and unable to activate rice Rubisco but forms ATPase functional hetero-oligomers with $OsRca\beta$

Biochemical characterization of the purified $\Delta N21OsRca2$ revealed it to be non-functional in terms of ATPase as well as Rubisco activation (Fig. 6.5). However, as per our hypothesis, Rca2 may not be a functional activase by its own. Therefore, we performed subunit mixing experiments between $\Delta N21OsRca2$ and $OsRca\beta$ to determine whether Rca2 can modulate the activity of $Rca\beta$ in terms of its ATPase or activase function. We observed no significant effect of Rca2 mixing on $Rca\beta$ when analyzed using ATPase and Rubisco activation assay (Fig. 6.5). Generally, ATPase inactive subunits poison the activity of functional subunits as demonstrated previously (section 3.5.1 and 5.1). Contrary to this, Rca2 did not poison the activity of $Rca\beta$, which indicates that $Rca\beta$ can tolerate the presence of Rca2 without being inhibited. Therefore, we again hypothesized that Rca2 may have some modulatory effect on $Rca\beta$ or form an functional active site only in presence of $Rca\beta$ but these small effects might not be detectable given the high activity of wild-type $Rca\beta$.

Fig. 6.5: Biochemical characterization of $\Delta N21OsRca2$. (A) ATPase (left) and Rubisco activation (right) assay of $\Delta N21OsRca2$ along with control WT Rca (β isoform). Assays were performed at 25 °C with 5 μM Rca and $\Delta N21OsRca2$ (Protomer). For mixing experiments two proteins (around 190 μM together) were pre-incubated for 10 min to yield an equimolar concentration of 5 μM each in the final reaction. ATPase activity of the mix sample was calculated in terms of functional Rca β active site.

To resolve this issue and further test our hypothesis, we replicated the subunit mixing experiments using the ATPase inactive Walker B mutant (OsRca β D173A). This experiment examined if Rca2 can form a functional hetero-oligomer with OsRca β D173A. Thus, systematic subunit mixing experiments were performed with $\Delta N21OsRca2$ and OsRca β D173A by keeping the concentration of one as constant at 10 μM in final reaction mix, while titrating up the concentration of other and vice-versa (Fig. 6.6A). As hypothesized, we discovered that mixing the non-functional $\Delta N21OsRca2$ and OsRca β D173A resulted in detection of ATPase hydrolysis (Fig. 6.6A). ATP hydrolysis rate was dependent on the concentration of Rca2. Almost constant ATPase was observed, when Rca2 was kept constant and OsRca β D173A was varied (Fig. 6.6A). However, the observed ATPase activity was relatively low and did not increase proportionally with the concentration of OsRca2. Therefore, data have been presented additionally in terms of ATPase activity per minute for total molar concentration of the OsRca2 present in the reaction as well as specific ATPase activity (Fig. 6.6A and B).

Ten μM of D173A possessed a basal ATPase activity of around 3.3 μM ATP min⁻¹ (0.33 min⁻¹), which increased to 14.4 μM ATP min⁻¹ (1.44 min⁻¹), when mixed with an equimolar concentration of Rca2, which possessed a basal ATPase activity of 4.8 μM ATP min⁻¹ (0.48 min⁻¹). The basal activities of the individual isoforms could be the outcome of contaminant ATPases and their values were below the sensitivity of

the method. When the Rca2 to OsRca β D173A ratio was reduced to below 1 (Rca2: D173A), the ATPase activity was also reduced and minimum activity was detected at 2 μ M of Rca2 (4.77 μ M ATP min⁻¹), while maximum at 20 μ M Rca2 concentration (18.7 μ M ATP min⁻¹) (Fig. 6.6A). Contrary to this, when the concentration of Rca2 was kept constant at 10 μ M and concentration of OsRca β D173A was varied, the ATPase activity almost reached its maximum value at 2 μ M Rca2 (12.26 μ M ATP min⁻¹). Titrating up the concentration of OsRca β D173A further even up to 20 μ M in this case did not increase the ATPase activity of the oligomeric mixture (Fig. 6.6A). We noticed that on one hand the ATPase activity of the hetero-oligomer kept increasing by titrating up the concentration of Rca2, whereas the ATPase activity per OsRca2 active site was reduced during the titration (Fig. 6.6A and B). This indicates that a small number of OsRca2 subunits are capable to restore functionality and more subunits led to a reduction in ATPase activity per active site. There is therefore an optimal ratio of OsRca2:OsaRca β D173A subunits, and increasing the concentration of OsRca2 leads to less functional assemblies.

A

Fig. 6.6: Biochemical characterization of OsRca2. (A) Subunit mixing experiments between Walker B mutant of Rca β (D173A) and Δ N21OsRca2 were performed such that in one experiment concentration of OsRca β D173A was kept constant (10 μ M) in the final reaction mix and Δ N21OsRca2 was varied (left) while in another experiment the conditions were reversed (constant Δ N21OsRca2 and varied OsRca β D173A) (right). (B) Representation of the data from A as activity per Δ N21OsRca2 active site. The two proteins were pre-incubated at 160-220 μ M concentration range (protomer) for 10 min. (C) The subunit mixing experiment at an equimolar concentration of OsRca β D173A and Δ N21OsRca2 (10 μ M each in final reaction mix) with varying incubation time. (D) The subunit mixing experiment of Δ N21OsRca2 with OsRca β K115A and OsRca β R293V at an equimolar concentration (10 μ M each in final reaction mix) along with control rates for 10 μ M protomer. ATPase assay were performed at 25 °C. (E) 6% Native-PAGE gel of OsRca β D173A and Δ N21OsRca2 alone and after mixing. Loaded amounts are indicated against each lane.

This study also revealed that subunit mixing in this case was very slow and ATPase activity of the hetero-oligomeric complex kept increasing with incubation time (Fig. 6.6C). For instance, ATPase activity of $\sim 10.9 \mu\text{M ATP min}^{-1}$ was observed at an equimolar concentration (10 μ M each) without any pre-incubation. After 10 minutes of incubation time, observed ATPase was $14.4 \mu\text{M ATP min}^{-1}$, which further increased to $18.7 \mu\text{M ATP min}^{-1}$ after 60 minutes of incubation (Fig. 6.6C). However, even under subunit mixing condition, no Rubisco activation was observed for the OsRca β D173A/OsRca2 hetero-oligomer, which prevented us from drawing conclusions about its function. It is possible that the low ATPase activity of the hetero-oligomer was not enough to activate Rubisco or the functionality of Rca2 could only be confined to its ATPase function.

Repeating the mixing experiment between Rca2 and another ATPase inactive Walker A mutant (K115A) yielded identical result at an equimolar concentration (Fig. 6.6D). In contrast, OsRca2 did not form a functional hetero-oligomer with the Rca interface mutant (R293V), which appeared unable to exchange subunits in earlier experiments (section 5.5) (Fig. 6.6D). This suggests that the detection of ATPase activity in case of hetero-oligomer was the result of subunit interaction and not an artefact, where OsRca β subunit is restoring the functionality of OsRca2. However, formation of complex between Rca2 and Rca β could not be demonstrated using native-PAGE (Fig. 6.6E). It is possible that either the population of hetero-oligomers were too small to be detected or the hetero-oligomeric band could be masked by the overlapping bands of the individual protein. In future, an optimized study with titrated amount of loaded proteins could provide a clearer picture.

Fig. 6.7: Hypothesized scheme for the formation of functional hetero-oligomer between OsRca2 and OsRcaβD173A. (A) Ribbon and surface representation of the tobacco Rca hexamer highlighting the location of D173 and arginine finger residue R243 (position as per OsRca). (B) OsRcaβD173A and ΔN21OsRca2 were non-functional by their own. The active site is formed by two subunits of the hetero-hexamer when arginine finger comes from OsRcaβD173A. Hexamers are for illustration purpose only.

The restoration of functionality of OsRca2 could be explained on the basis of formation of a hetero-oligomeric active site, where OsRcaβD173A provides its arginine finger (R243 in rice activase) to OsRca2 and makes it functional (Fig. 6.7A and B). The arginine finger in many AAA+ proteins has been implicated in sensing the nucleotide binding state of adjacent subunit and mediating inter-subunit communication [121, 122]. In tobacco Rca structure, R243 is located adjacent to the Walker B site (D173A) of the neighbouring subunit (Fig. 6.7A) and substituting it with alanine in tobacco has been reported to abolish ATPase activity and nucleotide dependent conformational changes [165]. Therefore, we hypothesize that during

hetero-oligomer formation; the arginine finger from OsRca β D173A allows ATP dependent conformational changes in OsRca2, leading to formation of functional active site (Fig. 6.7B). This assumption could be verified in future by performing the subunit mixing with an arginine finger mutant (R243A) of OsRca β , which should not permit rescue of the ATPase function of the hetero-oligomer. We also attempted to generate Walker B mutant of Δ N21OsRca2 (D178A) for biochemical study, hypothesising that mixing experiment in this case should eliminate the function of the hetero-oligomer. However, the protein co-eluted with lot of contaminating proteins, which could not be separated even after several chromatographic attempt.

6.4 Demonstration of a novel complex between Δ N21OsRca2 and OsRca β when co-expressed in *E.coli*

Attempts to demonstrate the functionally observed Rca2/Rca β complex by gel filtration were inconclusive. Therefore, we performed a co-expression of Δ N21OsRca2 and its Walker B mutant (D178A) with OsRca β to observe the interaction behaviour of Rca2 with Rca β . As hypothesized, Δ N21OsRca2 was co-eluted with activase after anion exchange chromatography, which was further confirmed using analytical gel filtration chromatography (Fig. 6.8A and B). Both Rca2 and Rca β appeared in all eluted fractions and could not be separated from each other. However, two overlapping peaks were seen, where the peak of Rca2 eluted at similar volume, while the activase peak kept shifting with concentration (Fig. 6.8B). In spite of this, almost all the fractions contain both the proteins. On the other hand, Δ N21OsRca2D178A also co-eluted with activase; however analytical gel filtration chromatography showed that the mutant forms soluble higher ordered oligomers. Still, co-elution of both, mutant and activase was observed in all fractions here also (Fig 6.8B).

The co-expressed $\Delta N21OsRca2$ and $\Delta N21OsRca2D178A$ with $OsRca\beta$ was also analyzed using native PAGE and western blot (Fig. 6.9A and B). $Rca2$ was detected using the anti-activase antibody; however the western blot signals for $Rca2$ were

much weaker than the activase signal (Fig. 6.9A). In case of co-expressed protein, bands for Rca2 and Rca β were clearly seen using the native PAGE and western blot, where the relative position of Rca2 bands does not change, while a visible shift was observed for Rca β with the concentration (Fig. 6.9B). In case of Δ N21OsRca2D178A, the mutant protein appeared to run higher on the gel and the Rca β bands were seen as usual, suggesting that Δ N21OsRca2D178A was forming higher order oligomers even in presence of Rca β .

A

B

Fig. 6.9: Complex formation between Δ N21OsRca2 and Rca β during co-expression. (A) Native PAGE (left) and western blot (right) analysis of OsRca β and Δ N21OsRca2. (B) Native PAGE (left) and western blot (right) analysis of Δ N21OsRca2 and Δ N21OsRca2D178A co-expressed with OsRca β demonstrating formation of complex between OsRca β and Δ N21OsRca2 through additional band. Loaded amount is indicated above each lane.

Interestingly, in case of these co-expressed proteins, there was an additional band between the original Rca2 and Rca β bands, which could correspond to a complex between Rca2 and Rca β . This band was not observed when Rca2 and activase were run in isolation (Fig. 6.9A). However, some experiments are needed to validate this outcome. Next steps will involve attempts to isolate the complex in a homogenous preparation.

In summary, this section demonstrated a preliminary attempt to characterize the putative Rubisco activase isoform, Rca2. So far, most studies concerning this isoforms were confined to detection in high-throughput approaches. To our knowledge, it was the first attempt where this protein was obtained in purified form and considered for characterization. The results presented here indicate some relationship between Rca2 and Rca β . Although, it would be too early to give a conclusive statement on the function of Rca2, still this work generated a hint towards its functional characterization.

Although we have left many unanswered questions regarding Rca2 due to time constraints, the present study could be taken advantage for the future work in the laboratory to fill the gaps. For instance, further biochemical characterization of the Rca2 with other activase mutants such as interaction site mutants could be utilised to find if Rca2 is a component or modulator of plant Rubisco activase or not. Apart from that the monodispersity of Rca2 projects it as an ideal candidate for structural studies using cryo-electron microscopy. In future, attempts should be made to obtain a high resolution structure of Rca2 alone and also in complex with Rca2-Rca or Rca2-Rubisco for its structural characterization. Also, *in vivo* approaches such as purifying activase from the plant source and analyze whether Rca2 co-purifies with activase using mass spectrometric techniques might provide evidence for *in vivo* interaction among Rca2 and activase. The Rca2 knockout seeds are available commercially and could be used for phenotypic study in plants. Collectively, positive outcomes glean through such approaches would endeavour to characterise Rca2 biochemically and unfold the mystery behind its *in vivo* function.

7. Discussion

Rubisco activase (Rca) is a key photosynthetic enzyme as it maintains the Calvin-Benson cycle flux in plants by remodelling of inhibited Rubisco, using energy from ATP hydrolysis. The thermolability of Rubisco activase appears to limit photosynthesis capacity of plants under moderate heat stress [10, 11]. The increasing global temperature due to climate change will further exacerbate this problem [18]. A comprehensive understanding underlying the mechanistic basis of activase thermostability and function is much needed to counter the predicted adverse effects of global warming.

In this study, we have made an effort to improve our appreciation of plant Rubisco activase function from a biochemical perspective, using rice as a model system. The work presented here has focussed on a detailed characterization of rice Rubisco activase followed by a comparative study with thermostable Rca homologues from a CAM plant, *Agave tequilana*. Moreover, extensive mutagenesis based on previous studies and with aid from available structure were utilised to enhance our mechanistic appreciation of the Rubisco activation system in this economically important crop.

Here, we place our results in context with the newly published results on rice activase [177] and previous studies on other plant systems. Finally, we present an updated model for the mechanism of Rubisco activation .

7.1 Rice activase isoforms exhibit typical regulatory behaviour and have similar thermostability to other characterized homologues

The data generated here represent the detailed biochemical characterization of two Rubisco activase isoforms from the key crop species rice. The biochemical information regarding rice activase was completely unavailable when this study was initiated. However, recently, a biochemical and thermal characterization of two rice species (*Oryza sativa* and *Oryza australiensis*) was published [177]. This study mainly focussed on concentration and temperature dependent activity of Rca. Consistent with this study, we found that both activase isoforms are capable of hydrolysing ATP and remodelling Rubisco with differential ability in the oxidized state. OsRca β was significantly more active than OsRca α in terms of both activities at tested concentration (Fig. 3.4). This trend was also reported in the recent study at

similar concentration ranges [177] and also for arabidopsis in earlier studies [146], where the β isoform possessed higher activities than the α isoform. However, for both rice (Fig. 3.5) and arabidopsis, the α isoform shows enhanced activity under the reducing effect of DTT and thioredoxin-f, while no effect was observed on the β isoform [146]. Thus, the α isoform has the ability to modulate its activity under redox state via thioredoxin-f, providing a mechanism for the light regulation of Rubisco as first discovered in arabidopsis [146]. This property of the α isoform is due to the presence of two conserved cysteine residues at its C-terminal extension, which are capable of forming a disulphide bond.

It has been reported that the thermal stability of Rca varies between different species, and there is a direct correlation between the heat tolerance of photosynthesis and the thermal stability [169]. Our data on the thermal characterization of Rubisco activases revealed that rice exhibits a very similar thermostability within its activase isoforms with the β isoform being slightly more thermostable than the α isoform (Fig. 3.7). The α and β isoforms lost half of their activity at 37 °C and 40 °C respectively. In Scafaro et al's work, the loss of ATPase hydrolysis was also reported after temperature exceeded 36-40 °C [177].

In other species like cotton, the α isoform is found to be slightly more thermostable than its β isoform but thermostability within its activase isoform does not differ greatly unlike spinach, where the α isoform is much more thermostable than its β isoform [141, 147, 178]. These differences in thermostability in rice and other species seem interesting and suggested some physiological significance. The α isoform in rice was reported to be upregulated during heat stress at mRNA level and found to be most upregulated protein in the wild rice species *Oryza meridionalis* [196, 197]. However, similar effects were not observed in arabidopsis [212]. It was hence tempting to speculate that altered α and β isoform abundance in rice with heat could be a mechanism to downregulate Calvin-Benson cycle in response to temperature and redox changes, where low activity OsRca α modulates the activity of OsRca β . Heat stability of the α -isoform did not alter with its redox state as shown in this study (section 3.6). Unlike in spinach, where Rca α confers thermostability to the β isoform [141, 178], no such effect was observed in our study (section 3.6). Thus, the hypothesized role for the α isoform to transfer high thermostability to the β isoform

was not confirmed in rice and the primary role of the α isoform appears to be confined to redox regulation of activase. Similarly, in arabidopsis, the α isoform was not observed to be more thermostable than β the isoform [166].

The structural integrity of Rca isoforms was also assessed by circular dichroism spectroscopy (CD). The CD analysis confirmed that the mid-point for thermal unfolding lies at about 35-40 °C for both Rubisco activase isoforms of rice (Fig. 3.8). The earlier CD studies also denoted the similar thermostability for the β isoform of arabidopsis with mid-point for thermal unfolding falls around 40 °C [134, 138]. The thermal mid-point for protein denaturation was approximated at 45 °C for the β isoform of cotton and tobacco, while 46-47 °C for creosote Rca β in the reported study [138]. All these CD studies were performed in presence of Mg^{2+} and ADP, while we have performed all our experiments in apo form.

It has been reported that nucleotide binding plays a role in Rca stability as ADP and ATP provide protection against heat inactivation to the β isoform of Rca in arabidopsis [134] and cotton [138]. ADP was found to increase the thermal stability of spinach Rca isoforms, while ATP γ S, a non-hydrolysable analogue of ATP, caused a substantial increase in the thermal stability of the α isoform of spinach [141, 178]. Therefore, in future, a similar characterization of the effect of nucleotides and metal ions should be performed for rice. Nevertheless these results along with observations from the native PAGE analysis (Fig. 3.9A) in our study showed a clear relation between the structural instability of Rca at high temperature and its activity in rice.

7.2 Subunit exchange and cooperativity between Rca isoforms

Some AAA+ ATPases including Rca can exchange subunits *in vitro* and therefore subunit mixing experiments were carried out between the isoforms to observe the mutual regulatory effect of the isoforms and to probe the mechanism employed in their function. Consistent with the arabidopsis model [146], it was observed that the ATPase activity of OsRca β was continuously reduced with increasing concentration of OsRca α (Fig. 3.10), clearly indicating the interaction between the two isoforms. However, to deconvolute the results, similar experiments were performed in the presence of ATPase inactive mutants of the both isoforms. Use of these mutants revealed that subunits interact irrespective of their types and follow a concerted or

sub-globally cooperative mechanism of ATP hydrolysis (3.11C). The interaction between OsRca β and OsRca α was further validated in the study using analytical gel filtration chromatography (Fig. 3.12). Previously, the evidence for interaction between Rca α and Rca β has been presented for arabidopsis and spinach using subunit mixing [141, 146, 155, 178]. Recently, interaction between the α and the β isoforms subunits in sweet potato has also been suggested using yeast two hybrid system [260].

In vitro mixing experiment between Rca β and Rca α in rice supports a regulatory influence of α isoform on the β isoform. This provides a mechanism to modulate the ATPase activity of the β isoform by the α isoform *in vivo* by rapidly forming the hetero-oligomeric mixture of low activity Rca α and high activity Rca β . Since in rice, Rca β is much more abundant than Rca α under normal conditions [144], modulating the concentration Rca α allows activity of the hetero-oligomer to be regulated under different redox state and other stressful conditions. In Arabidopsis, two Rca isoforms occurs in 1:1 ratio, while tobacco possesses only the β isoform. This differential stoichiometry of two isoform in plants could have different physiological significance. In arabidopsis, it has been demonstrated that the β isoform gets completely inhibited in the presence of α isoform at the equimolar concentration at an ATP/ADP ratio of 1:3 [155] indicating that the α isoform allosterically regulates activity of the β isoform. While in tobacco, which only expresses the β isoform, ADP shows strong competition with ATP resulting in reduction of ATPase activity [154]. The unusual stoichiometry of rice Rca under normal conditions, and its altered abundance under heat stress reflects that rice might represent a phenotype which could be intermediate to arabidopsis and tobacco. It is possible that either rice Rca may not completely deactivate *in vivo* or both isoform might possess intermediate level of sensitivity towards ATP/ADP ratio.

In summary, contrary to our hypothesis rice isoforms do not exhibit significant difference in thermostability and the upregulation of the α isoform is most likely a mechanism to reduce the activity of the β isoform under abiotic stress conditions. In future, the effect of nucleotides on subunit mixing and behaviour of different Rca under variable ATP/ADP ratio needs to be investigated further to perceive and compare the effects. Also, a high resolution structure of the α isoform alone and the

Rca α -Rca β complex with intact C-tail extension in reduced and oxidized state would be extremely helpful in resolving these issues.

7.3 Comparison between biochemical and thermal properties of rice (C3) and agave (CAM)

This study includes the first characterization of the Rubisco activase system from a CAM plant. The physiological properties of CAM plants suggested the possibility of encountering natural thermostable activases as per our hypothesis. *Agave tequilana* possesses two Rca isoforms like many other plants. However unlike rice and arabidopsis, where two isoforms are the result of alternate splicing [112, 143] the two isoforms in agave are produced from different genes as described in cotton [147].

Studies on activase from spinach and arabidopsis reported that the β isoform possesses a higher Rubisco activation rate than their respective α isoform [146, 148]. Arabidopsis follows similar trend also in term of its ATPase activity, while both isoforms of spinach have almost similar ATPase rate [178]. This comparative study between rice and agave showed that the β isoform of rice was significantly more active than the α isoform in the oxidized state, a phenotype more similar to arabidopsis. Interestingly, in agave both isoforms were almost equally active in terms of their ATPase as well as Rubisco activation activity (rice Rubisco). Agave exhibited higher Rubisco (rice) activation rates *in vitro* compared to the cognate OsRca (section 4.2).

The experiments testing the effects of molecular crowding performed in the study indicated a higher affinity between agave Rca and rice Rubisco as compared to rice Rca itself. Thus, introducing a high affinity activase from agave into rice could be a possible approach in future, as less activase may be required to activate the same amount of Rubisco. However, in the crowded stromal environment high affinity could result in reduced activase activity due to a slow off-rate and these issues therefore need to be carefully evaluated in future.

Subsequent thermal characterization of activases revealed that the β isoform of agave maintained significant ATPase hydrolysis activity at 50 °C, whereas most of the OsRca functionality was lost at 42 °C (section 4.3). Considering the problem

associated with the thermolability of plant activases such outcomes are very promising. The thermostability observed for agave activases in our study was to date the highest for any reported plant activase system isoform in terms of their stability in the apo form (Table 7.1). Recently, a hetero-oligomeric red-type activase from the thermophilic red algae, *Cyanidioschyzon merolae* has been reported to be fully functional up to 55 °C, however it was unable to activate plant Rca [104].

The previous data in the literature on activase thermostability is quite complex and measured in various ways such as in terms of ATPase activity, CD, light scattering thermofluor shift assay etc. Furthermore, the experimental conditions employed in the studies were also variable and complicate comparative analysis. A summary of the information available on activase thermostability in various plant systems has been tabulated in Table 7.1.

Earlier studies reported that the α isoform of spinach, which is a temperate plant, possessed enhanced thermostability with a T_m of 55 °C in presence of ATP γ S, while the increase was not so significant for the β isoform (41 °C) [178]. Similar thermostability profiles were replicated for spinach using the thermofluor shift assay [141]. Thus, spinach Rca α demonstrates a unique property in thermostability among its Rca isoform in the presence of Mg-ATP γ S, as such a substantial difference in the thermostability of the two isoforms has not been reported for any other plant Rca system. However, both the isoforms in spinach were extremely sensitive to a moderate increase in temperature in their apo form with a T_{50} of 30 °C, much lower than agave and other plants species isoforms (Table 7.1). It is interesting to note that the second most thermostable activase so far was found in creosote bush, which is a desert plant, followed by tobacco, the tropical plant, while temperate plants, like arabidopsis possess lower thermostability. Taken together our study on agave, which is a CAM plant, further supported the reasonable assumption that the thermostability of Rca varies from warmer to cooler climatic region [15, 169].

Organism	State	Full ATPase activity (°C)	T ₅₀ (°C)	Onset of protein denaturation (°C)	Apparent T _m (°C) (CD/TF)	Citation
Agave (α)	Apo	40	45	ND	ND	This study
Agave (β)	Apo	45	50	ND	ND	This study
Arabidopsis (β)	Apo	25	30	~32 (Mg-ADP)	~40 (Mg-ADP)	[12, 134, 138]
Cotton (β)	Apo	33	40	~40 (Mg-ADP)	~45 (Mg-ADP)	[138, 169]
Creosote bush (β)	ATP γ S	41	43	~40 (Mg-ADP)	~46-47 (Mg-ADP)	[138, 169]
Antarctic hairgrass	ATP γ S	35	38.5	ND	ND	[169]
Rice (α)	Apo	35	37	~35	~40	This study
Rice (β)	Apo	35	40	~35	~40	This study
Spinach (α)	Apo/Mg-ATP γ S	25/40	30/55	ND	25/30/55 (apo/Mg-ADP/Mg-ATP γ S)	[141, 178]
Spinach (β)	Apo/Mg-ATP γ S	25/40	30/41	ND	25/40/40 (apo/Mg-ADP/Mg-ATP γ S)	[141, 178]
Tobacco	Apo	35	40-42	~40 (Mg-ADP)	~45 (Mg-ADP)	[138, 169]

Table 7.1: Comparison of the *in vitro* data on thermostability of Rca among higher plants.

***ND = not determined, CD=Circular dichroism, TF = thermofluor assay**

It was hypothesized that conformational flexibility of Rca is required for it to remodel Rubisco conformation and this is likely be the reason why Rca cannot be thermostable [15]. Research comparing a warm-season plant (cotton) and a cool-season plant (*Camelina sativa*) also showed that there was a trade-off between Rca thermostability and its activity at low temperature [15, 229]. The recent data published on rice further reported similar compromise between thermostability and activity among its two species [177]. However, this is not true for agave activase and no trade-off was observed in terms of their Rubisco activation activities as well as ATPase activity at low temperature compared to rice (section 4.3).

Our results thus contradict a long standing argument pertaining to the thermostability of Rca and engendered a hope that it is possible to find both thermostable and functionally efficient Rca at the same time in the natural environment. The high functionality of agave activases at both low and high temperature could be related to the North American desert environment, where temperature in the light can fluctuate between broader ranges in summer (20-45 °C) and in winter (2-25 °C) [261]. It is possible that in similar species exposed to such a wide alteration in temperature regime as seasonal and diurnal variation, a strong selection pressure has been acting to maintain activase functionality at both high and low temperatures.

Our work was motivated to explore the future possibility of heterologously expressing more thermostable activases in rice. In earlier work, overexpression of Rca in rice resulted in improved activation state of Rubisco and rate of carbon dioxide assimilation with respect to wild type at fluctuating light conditions [230, 262]. Overexpression of barley Rca in rice plants led to reduced activation state under ideal conditions [262]. On the contrary, overexpression of maize Rca resulted in better activation state and CO₂ assimilation at variable light intensity [230]. This suggests, a more effective and compatible Rca could provide benefit to the host plants.

In our study, we have observed that although rice and agave Rca readily formed hetero-oligomeric complexes, activase functionality was not perturbed in terms of both ATPase rate as well as Rubisco action activity. Upon heating the hetero-oligomer, agave subunits remained in solution and functional while rice Rca formed inactive higher order aggregates (section 4.4). Therefore, we hypothesize that agave

Rca overexpressed in rice plants *in vivo* might replicate similar effects and could result in plants with enhanced photosynthetic performance at elevated temperatures.

There has been a controversy regarding the factors limiting photosynthesis at high temperature. However, thermolability of Rca and limitations of electron transport chain are considered as most debatable [10, 16-18, 169, 171, 263]. Expressing thermostable variants of activases have demonstrated improved growth and photosynthetic potential at supraoptimal temperature [12, 13]. However, it could be argued that in normal C₃ plants like rice, having a more thermostable Rca could actually decrease the efficiency of photosynthesis. This is because as temperature rises, the rate of photorespiration increases faster than the rate of photosynthesis [16]. Unlike C₄ and CAM plants, C₃ plants do not have a carbon-concentrating mechanism (CCM). Thus, when they close their stomata at high temperature to prevent water loss; there will be insufficient CO₂ entering the leaves. Moreover, the solubility of CO₂ and Rubisco specificity (CO₂/O₂) decreases with high temperature favouring production of misfired products [99, 103]. In such a situation, having a functional thermostable activase will result in increased photorespiration which is a wasteful process. Therefore, having a thermolabile Rca may serve as a regulatory mechanism to stop Rubisco from functioning at high temperature [16, 17], especially when the rate of photorespiration exceeds the rate of photosynthesis.

However, under agricultural care, plants can overcome the problem of photorespiration if enough water is provided as they would be able to keep their stomata open at high temperature without being dehydrated. In this situation, the thermostability of Rca could become a limiting factor. Since the problem of photorespiration is ameliorated, having a more thermostable Rca should allow Rubisco to stay active at higher temperature, increasing the efficiency of photosynthesis.

In earlier studies, accumulation of glycinebetaine in chloroplasts and overexpression of the enzyme SBPase have been associated with increased Rca stability in tobacco and rice respectively [264, 265]. Another genetic engineering approach utilised silencing of a specific ω -3 fatty acid desaturase gene, which caused reduction of thylakoid membrane lipid unsaturation. This strategy has also proved to be effective for the host plant by improving its growth and photosynthetic at elevated temperature

[263, 266]. Taken together, our study along with previously existing ones suggested possible solution pertaining to the thermosensitivity of photosynthesis. Expressing agave Rca in rice could provide a good experimental system to further evaluate the *in vivo* impact of such strategies and interrogate whether photosynthesis is more limited by the thermolability of Rca or other factors. In fact a collaboration has been initiated to probe these issues

7.4 Insight into thermostability and Rubisco activase mechanism gleaned through bioprospecting

In an attempt to locate specific regions/residues that confer thermostability, chimeric mutants were generated by interchanging the domains of Rubisco activase between rice and agave (section 4.5). Domain swapping experiments localized thermostability to the nucleotide binding domain (NBD) of AtRca. The chimeric mutant possessing the NBD of agave Rca not only exhibits 5-7 °C higher thermostability but also ~4 fold higher Rubisco activation *in vitro* for rice activase compared to OsRca.

An earlier study generated a chimeric activase by replacing the specificity domain of tobacco with arabidopsis [13]. The chimeric mutant (Tob-Arab) in that study acquired higher thermostability of tobacco, while the rate of Rubisco activation was similar to wild type arabidopsis Rca under standard *in vitro* assay conditions. Thus our strategies have observed to confer dual benefit in terms of thermostability and Rubisco activation rate (*in vitro*). Also the results from the previous study further supported our assumption that the NBD contributes significantly to the thermostable properties of activase. It was interesting to note that one of the residues from the sensor-2 region (C-terminal domain) was observed to affect thermostability in the gene shuffling strategy employed previously [12]. However, these variants were generated as second round mutant, also consisting an amino acid substitution from NBD out of total three residues. The *in vitro* phenotype of variant consisting single residue substitution (T274R in arabidopsis) did not yield a significant result at high temperature compared to WT in the same study. Similarly, no tested single residue substitutions could be identified to be responsible for thermostability in the NBD region in our study (section 4.7). This indicates that thermostability might be the outcome of tighter packing of structural elements and employed larger segments of

protein such as domain or motif rather than a single amino acid difference. This postulate is supported by our study taking together previous studies mentioned in this section.

Interestingly, in such attempts we have found that a single glutamic acid (E217) to glutamine (E-Q) substitution localized at a newly identified surface loop of the activase conferred AtRca's high activation rate to OsRca (section 4.7). Examination of the structure of Rca from tobacco [129] revealed that this loop is not resolved but present on the surface at close proximity of helix 9 (specificity region) of the adjacent subunit. Further mutational analysis implicated this loop in Rubisco-Rca protein-protein interactions and revealed a key lysine residue (K216) adjacent to glutamate (E217), which might be involve in Rubisco-Rca interactions (section 4.8) along with residues from helix 9 as the primary site of interaction. Further bioprospecting and careful biochemical characterization of Rca homologues will lead to both mechanistic insights and identification of candidate proteins for engineering enhanced thermotolerance in rice and other important crops.

7.5 New insights into the Rubisco activation mechanism

Our mechanistic understanding of the green-type Rca function is still poorly defined. Various models pertaining to Rubisco-Rca interaction have been proposed in the past based on structural and mutational studies. When the structure of the activase was unknown, a model was proposed where large oligomers of the activase surround the equator of the Rubisco holoenzyme [8]. This early model was superseded by a more detailed structural and biochemical study which proposed the transient hexamer as the functional unit for activase functioning [129]. Based on the structural considerations and mutational data obtained, a model for Rubisco activation was proposed, where the N-terminal domain and helix 9 (specificity helix) are involved in initial recognition of Rubisco as primary site of interactions [59, 129]. Furthermore, partial threading of larger Rubisco segments through central pore was proposed. This model was further supported by another study performed on interspecies-hybrid Rubisco containing large subunit from *Chlamydomonas* and small subunits from tobacco, spinach and arabidopsis [246]. Based on the biochemical characterization of the chimeras, two hypothetical models were proposed, where a side on model of the Rubisco-Rca interaction was preferred over the top-on geometry as proposed earlier [59, 129].

In this work, we have performed a reasonably extensive mutational study to validate previously reported mutational phenotypes in rice, and identified some novel residues that are likely involved in the Rubisco-Rca interaction. The sequence alignment between activases from different plants has been displayed in Fig. 7.1 highlighting the residues mutated in this study. Table 7.2 compiled the list of mutations performed in the present study along with the observed outcome on the functionalities.

We have observed that mutation of residues in the N-terminal domain (W15A, S23A and S23D) and helix 9 (K315D) eliminate activase function supporting the previously proposed mechanism where these domains mediate initial interaction with Rubisco [59, 129]. Mutations in pore loop 1 (A143V) completely abolished activase function further strengthening the role of pore loop 1 in Rubisco remodelling. We have identified new residues (K148, D159) located further downstream to pore 1 at the surface helix 3, involved in the Rubisco-Rca interaction (section 5.3) along with the surface arginine (321) located at the periphery of the hexamer. Characterization of the newly identified surface loop implicates a key lysine (216) residue in activase/Rubisco interaction (section 4.8).

We have also attempted to rank the relative importance of the key residues by cooperativity analysis using the mutant doping strategy (chapter 5). Our results are consistent with the notion that mutations in the residues or domain thought to be involved in activase-Rubisco interaction can be tolerated more easily (W15, Δ N67) compared to the mutant subunits containing lesions at mechanistically important sites such as pore loop 1 motif (A143). In the near future, similar mixing experiment will be performed with all newly identified residues to generate a more detailed picture.

Fig. 7.1: Protein sequence alignment of Rubisco activase from selected species. Amino acid sequences of selected Rubisco activases (Rca) from plants and green algae were aligned using ESPript [184]. The functional motifs and residues analyzed using mutagenesis are indicated using coloured bar and triangles respectively. The secondary structure elements of the tobacco activase (PDB id: 3T15) has been displayed above the sequence.

Mutant (OsRca β)	Inference		Location
	ATPase activity	Activase activity	
W15A	✓	↓	N-terminal domain
S23A/S23D	✓✓	↓↓	N-terminal domain
F68L	↓	↓	N-terminal domain
Δ N67	↑	↓	N-terminal domain
G73D	✓	✓	N-terminal domain
S90T	✓	✓	Helix 0 (α 0)
T95N	✓	✓	Between α 0- β 1
K115A	↓	↓	Walker A
A143V	✓	↓	Pore loop 1
G144A	↓	↓	Pore loop 1
E145A	↓	↓	Helix 3 (α 3)
K148A	↑	↓	Helix 3 (α 3)
Q152A	↓	↓	Helix 3 (α 3)
E156A	↓	↓	Helix 3 (α 3)
D159A	↓	↑	Helix 3 (α 3)
K162A	✓	↓	Between α 3- β 3
D173A	↓	↓	Walker B
Y214A	↓	↓	Unresolved loop
N215A	↓	↓	Unresolved loop
K216A/K216E	✓↓	↓↓	Unresolved loop
E217Q/E217K/E217A	✓✓✓	↑↑↑	Unresolved loop
D218A	✓	✓	Near β 4
N219A	↓	↓	Near β 4
P220A	✓	↓	β 4
R293V	↓	↓	Rca-Rca interface
K315D	✓	↓	Helix 9 (α 3)
R321A	↑	↓	Surface near (α 9)

✓	✓
Normal	Slightly reduced
↑	↑
Highly stimulated	Slightly stimulated
↓	↓
Strongly reduced	Moderately reduced

Table 7.2: Summary of rice activase mutants evaluated in this study

Taken together the information from the previous study and the data generated in this study, a modified model for Rubisco activation is proposed (Fig. 7.2B). We would like to present a hypothesis, where the newly identified flexible surface loop (red) might directly be involved in Rubisco-Rca interaction as primary site of contact as proposed for helix 9 ($\alpha 9$). This loop is also positioned close to the rim of the pore (~ 17 - 22 Å), however the mechanism of C-tail pulling through the pore as described for the proteobacterial red-type Rca appears unlikely for plant activase (see 5.3). Therefore, another possibility might be that the new surface loop in conjunction with other surface residues (helix 3) should play an important role in conformational remodelling of the active site by positioning the Rubisco towards central pore for partial threading, leading to release of inhibitor. The distribution of the newly discovered interaction residues suggests that many more protein-protein interactions between Rubisco and activase exist than described so far. The engaged AAA+ ring could be anticipated to bury a significant area of the surface of the Rubisco holoenzyme.

Fig. 7.2: Mechanistic model for Rubisco activation in plant. (A) Surface representation of the six fold symmetry of the Rubisco activase highlighting previously described helix 9 (blue), important for Rubisco interaction. The regions containing newly discovered residues in this study are highlighted in red. (B) Helix 9 ($\alpha 9$, in blue) and N-terminal domain (not resolved in the crystal structure) might form the initial site of interaction as hypothesised earlier [59, 129]. Residues critical for recognition by Rca are indicated in magenta on Rubisco surface [145]. The flexible surface loop (red) identified in the study might either be involved in Rubisco-Rca interaction as proposed for helix 9 ($\alpha 9$) or in remodelling of the active site by affecting the conformation of the C-tail. Helix 3 (cyan) along the pore axis and protruding surface residue (green) might form additional site of contacts for positioning the Rubisco towards central pore for partial threading or bringing about conformational change in Rubisco active site leading to release of inhibitors. This model suggests that Rca involved larger section of the surface element of the Rubisco holoenzyme rather than just C-tail unlike found for RsCbbX in proteobacteria [114]. Surface representation of Rubisco and Rubisco activase from spinach were shown (PDB id: 1RCX and 3ZW6). Rubisco's large subunits were shown in dark and olive green while small subunits were shown in brown. C-tail was displayed in light blue. Rca subunits were shown in grey and orange colour. The solid arrow represents the known Rubisco-Rca interaction site whereas the dotted arrows indicate the hypothesized Rubisco-Rca interaction site.

7.6 Biochemical insights into the regulation of Rca by phosphorylation

Redox regulation by the ferredoxin-thioredoxin system has already been well established as the mechanism for regulating the activity of Rca under dark/light cycle and variable ADP/ATP ratio [146, 152, 155, 156]. Recently published proteomics data have indicated that in the dark Rca activity could also be regulated by phosphorylation of some residues situated at regions important for Rca function [250, 251].

Mutating the phosphorylated serine to its phosphomimetic counterpart, aspartate, in our study led to normal ATPase rate, while resulting in almost a 80% loss of Rubisco activation (section 5.4). Thus, this residue appears to be important for Rubisco-Rca interaction. However, its role in inhibiting Rca activity under dark is not conclusive [252]. This is because the phosphorylation pattern is poorly conserved in higher plants. For instance, the phosphorylated threonine in arabidopsis is replaced by isoleucine in rice and maize. On the other hand, in rice, the adjacent serine is phosphorylated, while in maize no phosphorylation was observed [251]. These facts still argue the role for phosphorylation for downregulating photosynthesis by inactivating Rubisco activase in the dark. Conversely, the phosphorylated residue might differ in different species as observed earlier at position, as well in terms of the number of phosphorylation sites. Therefore, it is possible that Rca in different species exhibit different phosphorylating response to modulate its activity under limiting light conditions. Also, unlike redox regulation, the residues for phosphorylation are common in both Rca isoforms and it was found that both the isoforms were evenly phosphorylated at nights [252]. However, any attempt to demonstrate an effect of phosphorylation on Rca activity in the study was not conclusive.

Identifying the phosphorylating targets in other Rca homologues followed by mutagenesis study should provide better insight into regulation process. It is plausible that phosphorylation might possess as important a role as redox regulation in regulating Rca activity. Also, other post translational modifications could be anticipated to be involved in Rubisco regulation and need to be investigated in future.

7.7 Distant classes of Rubisco activase have convergently evolved to remodel their respective Rubisco

Three classes of Rubisco activases have been described so far. The green-type Rca found in higher plants/green algae [9, 129], a red-type Rca (Cbbx) for α -proteobacteria/red lineage phytoplankton [104, 114] and the recently discovered CbbQO (purple-type Rca) in chemoautotrophic bacteria [115]. All three activases belong to the superfamily of AAA+ proteins and utilise energy of ATP hydrolysis to remodel Rubisco. However, they have poor sequence homology among them and employ different mechanisms of Rubisco activation.

CbbX functions by transiently threading the C-tail peptide of Rubisco large subunits through the central pore of the hexameric ring to remodel Rubisco, similar to widely studied AAA+ homologues including the Hsp100 proteins, ClpB and ClpX [267, 268]. A conserved key tyrosine residue in the pore loop was found to be indispensable in CbbX function, while the pore loop 1 in plant Rca was different to classical pore loop found in AAA+ proteins and shows an absence of tyrosine or aromatic residues. Also, the C-terminus of plant Rca was shorter than that of red-type Rubisco and extending the length of C-terminus in plant does not alter the ability of the enzyme to be activated [129, 269]. On the other hand, plant Rca interacts with two surface residues on the large subunit of Rubisco via helix 9 residues, additionally involving N-terminal domain as described earlier [129, 145, 167, 168]. However, mutational analysis from the previous study [129] and the present study also supports the role of pore loop and surface residues lining the pore loop 1 in Rca remodelling. Additionally, the newly identified surface loop in our study might also implicate itself in the mechanistic function as described in the previous section.

Apart from this, CbbX only forms active hexameric complex in presence of ATP and RuBP and shows stimulatory ATPase rate in presence of substrate Rubisco [114]. Plant Rca was found to be formed hexamer in presence of Mg-ATP or Mg-ATP γ s [129, 141] and is constitutively ATPase active without RuBP. Stimulation in presence of its substrate does not occur for plant Rca unlike CbbX [109] and its activity is regulated by ADP/ATP ratio and redox state of α isoform. Also, a recently discovered XuBP phosphatase, called CbbY also functions in cooperation with CbbX to degrade

XuBP, arresting its rebinding to Rubisco. The homologous CbbY gene is encoded by all photosynthetic organisms [108].

A

B

Fig. 7.3: Mechanism of Rubisco activation in different system. (A) Model of Rubisco activation by the Red type Rca, CbbX. The CbbX hexamer docks onto inactive Rubisco and transiently pulls the extended C-terminal sequence of red-type RbcL into the central pore of the hexamer. This action is mediated by the stimulated CbbX ATPase and destabilizes the Rubisco active site, releasing the inhibitory sugar. (B) Model of Rubisco activation mechanism by CbbQO (purple-type) for form I Rubisco. CbbQO binds to inhibited rubisco complexes via the acidic surface residue (Asp 82, coloured yellow) of the rubisco large subunit using the MIDAS binding site located on CbbO. The AAA+ hexamer formed by CbbQ may also interact with rubisco directly, possibly via the C terminus of the large subunit (indicated by yellow dashed double arrow). Productive binding, which involves both Asp 82 and the C terminus, results in a stimulation of ATPase activity. This provides the energy used to remodel the inhibited rubisco active site allowing release of the inhibitor. Adopted from [114, 115]

The newly discovered third type of Rubisco activase system, CbbQO, in chemoautotrophic bacteria deploys a mechanism, whereby the hexameric AAA+ ATPase CbbQ forms a complex with a large protein CbbO ($CbbQ_6O_1$). The later acts as a substrate adaptor for inhibited Rubisco via its von Willebrand Factor A (VWA) domain [115, 270]. The MIDAS motif of the VWA domain of CbbO interacts with

the acidic surface residue of the Rubisco (Asp 82). The corresponding surface residue (Pro 89) was found to be crucial for activation of plant Rubisco by Rca [167]. The activation process required necessary involvement of the C-terminus of Rubisco large subunits like CbbX, however, the role of the pore has not been established [115]. The interaction with Rubisco leads to a stimulation of ATPase activity for CbbQ as found for CbbX. The energy produced from the hydrolysis of ATP may be transmitted directionally from CbbQ to the VWA domain through the CbbO linker to disrupt the active site of Rubisco. CbbQO class of Rca does not respond to RuBP, similar to plant Rca, and contrary to CbbX. Thus, the regulation of ATPase by the Calvin-Benson cycle metabolite RuBP appears specific to the red-type activase system for now. Stimulation of ATPase by inhibited substrate Rubisco is observed for the chemoautotrophic CbbQO activases and CbbX but is absent in plant activase systems [104, 109, 114, 115].

Comparing the three activase systems, it could be concluded that the contact points between activase classes are surface residue and the C-terminus, which remain conserved. The secondary structure elements of large subunits of Rubisco are highly homologous [5, 53], however their respective activases demonstrate poor sequence homology among them except the AAA+ core motifs. This suggests that during the course of evolution the activases were recruited from the general AAA+ molecular chaperone machinery in different lineages and are hence a product of convergent evolution. It appears likely that additional convergently evolved Rca systems are yet to be discovered.

7.8 Conclusions and future perspectives

Considering the problem of population explosion and climate change there is an obligate need to take measures to ensure food security for the coming generations. Optimization of photosynthetic carbon fixation has been the current focus, since the benefits gained from the previous approaches have already reached their saturation limit [2, 15]. Rubisco being the key enzyme of photosynthesis has always been a potential target for improvement in terms of its catalytic efficiency. However, this approach suffers from a variety of limitations owing to complexities involved in recombinant expression of more efficient algal Rubisco in the higher plant chloroplast due to chaperone compatibility issues [49, 271]. However, success is anticipated in

resolving these issues due to rapid advancements in understanding the factors involved in assembly and biogenesis of green-type Rubisco [59, 63, 272, 273].

Improving just Rubisco might not solve the problem as Rubisco is highly prone to inhibition by sugar phosphates and requires its helper protein, Rubisco activase, to dissociate them from the active site. Although Rubisco is quite thermostable at higher temperature, photosynthesis ceases at moderately high temperatures [174]. This thermal intolerance of photosynthesis is believed to be attributed to the thermolability of plant Rubisco activase which gets denatured and fails to maintain the activation state of Rubisco under heat stress [10, 11, 169]. To extract full benefits for enhancing photosynthetic efficiency, Rubisco manipulation strategies should be co-integrated with activase engineering strategies. Addressing the issue pertaining to thermal stability of activase should provide preparatory measures to deal with the problem associated with the future climatic changes [12, 18].

Our work focussed on the biochemical characterization of the rice Rubisco activase to develop a more detailed mechanistic appreciation of the Rubisco activation system. In parallel, we have also discovered and presented a detailed characterization of the highly thermostable and effective Rca from the CAM plant, *Agave tequilana*. This system was later utilised to engineer thermostable rice-agave activase chimeras. The *in vitro* biochemical data suggests that such thermostable activases could be introduced into host plants *in vivo* directly or as chimera without any compatibility issue. If so, this might address the problem of thermolability associated with most of the plant activases and improve functionality at elevated temperature.

Attempts to exactly locate residues or motifs responsible for thermostability in the study were not successful, as the point mutations tested in this study did not yield informative results. A future approach should be to target small domains or motifs for mutagenesis. Nevertheless, the knowledge obtain through our result might be crucial for engineering not only thermostable but also functionally more effective activase variants of rice and other crops.

The mutational studies performed in this thesis have provided an additional layer of information towards the mechanistic understanding of the Rubisco activation process. The interesting behaviour of some mutants at different locations in the structure

requires further follow up and will serve as framework for future studies. A major milestone in plant Rubisco activase research would be the establishment of conditions that favour a stable Rca-Rubisco complex. Structural and biochemical characterization of such a complex would then allow rapid progress to be made in our understanding of the activation process. There is some evidence for trap-mutants in other AAA+ systems [239] and a complex between Rubisco and its assembly chaperone RbcX has been obtained [274]. Also the crosslinking experiments with the red-type Rca of *Rhodobacter sphaeroides* have been successful (unpublished results from Oliver Mueller-Cajar), but as of now there is no solid evidence of a stable plant Rca-Rubisco complex, likely due to the transient nature of the interaction between them. Discovery of high affinity activase-Rubisco combination as shown here for agave-rice will be useful in this context. Additionally, other biophysical techniques such as H/D exchange by mass spectrometry should be attempted to identify residues involved in Rubisco-Rca interaction. Furthermore, structural characterization of the intact N-terminal domain which is presumed to be an adaptor for recruitment of Rubisco would be useful to validate its role.

We have successfully demonstrated the formation of covalent complex between the α -Rca of rice and a thioredoxin-f mutant. Attempt should be made to purify such a complex to homogeneity for biochemical and structural studies. In the absence of structural information the mechanism of redox regulation is not well understood. Obtaining a structure of Rca α in oxidised and reduced state would aid in a detailed understanding of activase regulation.

A major obstacle in interrogating protein-protein interactions between Rubisco and Rubisco activase is our inability to produce recombinant plant Rubisco in *E.coli* or other microbial hosts [275, 276]. This limits our ability to generate Rubisco mutants, which could allow us to test hypotheses on activase function by modifying substrate protein. The prokaryotic Rubiscos that are closely related to plants are recombinantly produced as part of parallel projects in our lab and are amenable to extensive mutagenesis to make them compatible. A favourable outcome would be if such Rubiscos could be activated by plant activases. The directed evolution study using artificial evolution systems should facilitate this outcome in future [276] (unpublished data of Zhijun Guo). Successful advances in such an approach would greatly expand

our ability to further test our hypothesis concerning the mechanism of activation as successfully done in other activase classes [114, 115]. Beside this, a co-variation study of multiple Rca and Rubisco sequences should be performed using co-evolution-based computational tools [277-279] to predict contact points between the two proteins, which could be further verified experimentally.

In summary, this study generated a comprehensive molecular platform to address pertinent questions in Rubisco regulation and maintenance from biochemical angle. Our results indicate that further bioprospecting and careful biochemical characterization of Rca homologues will lead to both mechanistic insights and identification of candidate proteins for engineering enhanced thermotolerance in rice and other crops. Such genetically modified variants of crops are expected to show better adaptability in the predicted future climatic conditions, eventually leading to enhanced agricultural productivity. The importance of such work is difficult to overstate, considering the significant sustainability challenges encroaching on our species in the coming decades. We hope our work will inform that of others who are similarly approaching the same problem from multiple angles [280-282].

8. Appendix

A

At1g73110 262377_at

Arabidopsis eFP Browser at bar.utoronto.ca
Winter et al., 2007. PLoS One 2(8): e718

B

At2g39730 2:45061_at RCA

Arabidopsis eFP Browser at bar.utoronto.ca

Winter et al., 2007. PLoS One 2(8): e718

Fig. 8.1: Tissue specific expression level of Rca2 (A) and Rubisco activase (B) as obtained from The arabidopsis eFP browser [256].

Organism	Rca2	Rca
<i>Agave tequilana</i>	Locus 30142	Locus 27298
<i>Arabidopsis thaliana</i>	Q9AST9	P10896
<i>Cajanus cajan</i>	A0A151S6K1	A0A151R088
<i>Capsella rubella</i>	R0IAN8	R0HC86
<i>Chlamydomonas reinhardtii</i>	A8JIJ0	P23489
<i>Chlorella variabilis</i>	E1ZCY8	E1Z378
<i>Cucumis sativus</i>	A0A0A0K6Q9	Q01587
<i>Daucus carota</i>	A0A164U7N2	A0A166DCY4
<i>Glycine max</i>	I1M608	D4N5G0
<i>Gossypium arboreum</i>	XP_017603330.1	A0A0B0Q068
<i>Micromonas commoda</i>	C1EGP0	C1E4S4
<i>Musa acuminata</i>	M0RMV9	M0RV12
<i>Oryza sativa</i>	Q7XN85	P93431
<i>Ostreococcus lucimarinus</i>	A4RW51	A4RW20
<i>Phaseolus vulgaris</i>	V7BY25	O64981
<i>Physcomitrella patens</i>	A9TYZ9	A9TBPO
<i>Ricinus communis</i>	B9RGR9	B9SDY7
<i>Solanum lycopersicum</i>	K4BLT6	K4D489
<i>Spinacia oleracea</i>	A0A0K9QK09	P10871
<i>Triticum aestivum</i>	W5BC60	Q9M4V3
<i>Vigna radiata</i>	XP_014501031.1	O98997
<i>Zea mays</i>	A0A144KR40	C0P5X1

Table 8.1: List of species and accession numbers for Rca2 and Rca sequences from plants and green algae used for the phylogenetic analysis.

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