The Pharma Innovation Journal 2022; 11(7): 2071-2075

www.ThePharmaJournal.com

The Pharma Innovation

ISSN (E): 2277-7695
ISSN (P): 2349-8242
NAAS Rating: 5.23
TPI 2022; 11(7): 2071-2075

www.thepharmajournal.com
Received: 15-05-2022
Accepted: 24-06-2022

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Site-directed mutagenesis at position 396 to enhance the thermo-stability of RuBisCO activase

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DOI: https://doi.org/10.22271/tpi.2022.v11.i7z.14395

Abstract
Rubisco activase (Rca) is a catalytic chaperone that remodels Rubisco's active site, facilitates inhibitor release, and restores catalytic competence. Changes in the chloroplast environment generated by changes in light levels entering the leaf, including redox status and the adenosine diphosphate (ADP)/adenosine triphosphate (ATP) ratio, influences activity and its effect on Rubisco activation and photosynthesis. Three Rca protein isoforms are encoded by the *Triticum aestivum* (wheat) genome: 1β (42.7 kDa), 2β (42.2 kDa), and 2α (46.0 kDa). One site-directed mutant were designed to elucidate if differences in the amino acid sequences between Rca of wheat and thermotolerant cultivar. The effect of IPTG concentration was checked, and it was found that 0.9mM is the best suited for the induction of mutant protein.

Keywords: Heat stress, RuBisCO, RuBisCO activase, site-directed mutagenesis, wheat

Introduction
Wheat is a cereal crop that provides energy and nutrition to the world's population. According to Reynolds *et al*., 2011 [20], wheat accounts for about one-fifth of the world's total calorie consumption. Wheat is best suited to temperate regions (Gill *et al*., 2004) [6]; however, it can also be grown in semi-arid areas. The increasing demand for wheat predicts that the world's grain production will need to increase by around 2.4% to meet the population's needs in 2050. (Ray *et al*., 2013) [19]. Global wheat productivity has only increased by 0.9% annually despite the increasing demand for wheat. Constantly increasing optimum temperature is considered one of the most detrimental stress. Earth's air temperature is expected to increase by 0.2 °C per decade; this will cause temperatures to increase by 1.8- 4.0 °C by the end of 2100 from the current level (IPCC, 2007). The effects of heat stress on the plants can cause changes in different physiological, morphological and biochemical properties. In many wheat-growing regions of India, a drop in yield and seed set has been observed due to terminal heat stress. It is predicted that global climate change may aggravate the problem of heat stress (Kumar *et al*., 2012).
Rca is a nuclear-encoded, cytosol-synthesised protein (Salvucci *et al*., 1985) [22] and a member of the AAA+ family of ATPases (Spreitzer and Salvucci, 2002) [30]. In plants, two isoforms may be present: The long Rca (α) isoform of 43- 47 kDa and the short (β) Rca isoform of 41-42 kDa (Salvucci *et al*., 1987) [23]. The (α) isoform has an extended C-terminus containing redox-sensitive Cysteine residues (Portis *et al*. 2008) [18]. The long and short Rca isoforms from spinach can stimulate the RuBisCO activation *in vitro*. However, their sensitivity to ATP and ADP varies (Shen *et al*., 1991) [27]. The Rca encoding genes can vary from one species to another, and in some species, the Rca transcript may undergo alternative splicing to produce different isoforms of Rca (Carmo-Silva *et al*., 2014) [2].
The RuBisCO and Rca interaction mechanism, which removes the inhibitory sugar-phosphate from the RuBisCO active sites, is still unclear. Rca performs a dual function; it catalyses RuBisCO activation and ATP hydrolysis. During the interaction between both the proteins, hydrolysis of ATP promotes movement of the C-terminal sensor-2 domain of Rca (Henderson *et al*., 2011, Parry *et al*., 2008) [7, 17]. The sensor-2 domain has an arginine residue which interacts with the nucleotide bound to the domain and explains the coupling of ATP hydrolysis and Rca sensor-2 region movement. This would explain the movement of the Rca sensor-2 region and the movement of the N-terminal domain of Rubisco. The movement in the RuBisCO N-terminal region could break the interaction between the bound sugar-phosphate

and Glu-60, Lys-334 in loop 6, helping release sugar-phosphate from the catalytic site.

Salvucci and Crafts-Brandner (2004) [21] have characterised the Rca enzymes from the heat-tolerant plants and identified their differences. It has been suggested that the differences in thermotolerance of Rca can be used the increase the thermotolerance of crop species. But, such modifications are a difficult task as this could be a complicated task as, Rca exhibits species specificity (Wang *et al*., 1992) [31]. There has been some success in developing chimeric RCA; In Arabidopsis plants, expressing a thermally stable chimeric Rca has shown increased growth at elevated temperatures and an increase in the photosynthetic rate (Kumar *et al.*, 2009) [10]. In transgenic Arabidopsis, a chimeric RCA was created from tobacco (Nicotiana tabacum) to ensure the gene's functional activity and protein interactions but contained the Arabidopsis Rubisco recognition domain (Kumar *et al.*, 2009) [10].

Kurek *et al*. (2007) [12] used gene shuffling techniques to generate Arabidopsis RCA variants that provide better thermal stability. This technology shows an alternate route to engineering crop plants by manipulating Rca with increased productivity at higher temperatures. Lazar and Goodman (2000) [14] reported that in certain crop plants, heat stress affects alternative splicing resulting in unique products. It has the ability to increase the thermal stability of the enzyme by changing the sequence composition (DeRidder *et al.*, 2012) [5]. It has been hypothesised that Rubisco and Rca co-evolved in that amino acid changes occurring in one of the proteins through mutation or selection pressure resulted in complementary changes (Wang *et al*., 1992) [31]. To develop more thermo-tolerant Rca forms, the interactions between the two enzymes are likely to be critical for parallel preservation.

Materials and methods

Cloning of Rca into an expression vector

Total RNA was isolated from leaves of HS-treated wheat cv. HD2985 using the Trizol method. The Bioanalyzer (Agilent, USA) and a 1.2 % agarose gel were used to evaluate the quality of the obtained total RNA. Total RNA with an OD 260 to 280 ratios of 2.0 was used to make cDNA using the Fermentas Revert Aid H minus cDNA synthesis kit (Thermo Fisher Scientific, USA) according to the manufacturer's instructions. The transcript-specific primers, cDNA template (60 ng/lL), and high fidelity Pfu enzyme (5 U/µL) were used in RT-PCR according to conventional techniques, and the amplified product was verified on a 1% agarose gel. A gel elution kit was used to elute the amplicon (Promega, USA). The restriction enzymes SbfI/XmnI and SbfI were used to restrict the pMal-c5x expression vector (New England Biolabs Pvt. Ltd., U.K.) and the eluted product, respectively. The gel-eluted restricted products were ligated with T4 DNA ligase at 16 °C before being transformed into the E. coli BL21 strain. The final cloning of Rca in the pMAL-c5x expression vector was validated by colony PCR and restriction digestion.

Primer Designing and production of mutant Rca

The PCR template for modified pMAL-TaRca1 expression plasmids was a pMAL-C5x vector containing the coding region for the mature wheat TaRca1gene. SDM primers were designed using the TaRca1 sequence and the NEBasechanger (https://nebasechanger.neb.com/) website (Table 1). To make one mutant, the target residue was changed, and the appropriate primer sequences were utilised. DNA sequencing of the complete Rca coding area for expression construct confirmed the SDM alterations. Plasmids were subcloned into E. coli strain BL21(DE3) for expression.

Table 1: Sequence of Mutagenic primer

Primer (F) Glutamine Lysine	**CATGCTGGTC aag GAGCAGGAGA**	**69 °C**
Primer (R)	TGGCCGTACTCCATGAGC	68 °C

Optimising the conditions for recombinant protein expression

The overnight grew BL21 containing pMal-c5x-Rca culture was inoculated in 5 mL fresh Luria Broth (L.B.) medium supplemented with ampicillin (100 µg/mL) and incubated at 37 °C for 2–3 h (OD600 - ~ 0.6); 200 µL of culture was separated into different tubes and induced with different concentrations of Isopropyl b-D-1-thiogalactopyranoside ranging from (0.3–1.5 mM) for 1 h Moreover, bacterial cells were separated by centrifugation at 14,000 rpm for 5 minutes, and the pellets were re-dissolved in 1 mL SDS loading buffer. Based on the production of the fusion protein (MBP–Rca) by electrophoretic separation of 10 µg of extracted protein, the optimal IPTG concentration was validated on a 10% SDS-PAGE gel (Laemmli 1970). The optimal IPTG concentration for induction and expression was found to be 0.9 mM (at 37 °C).

Results and Discussion

Cloning of TaRca1 into a pMAL-c5x expression vector

To clone the Rca gene in the pMAL-C5x expression vector, forward and reverse ORF-specific primers were constructed. An amplification of ~1.3 kb was achieved. The amplicon was gel eluted before being mobilised into the pMAL-C5x expression vector (New England Biolabs Ltd., U.K.) according to the manufacturer's instructions. On the Luria Broth plate, a large number of positive transformants were found (confirmed by colony PCR, restriction analysis, and Sanger's sequencing) (Result already published).

Amplification of Mutagenic Rca

pMAL-c5x-Rca plasmid was used as a template for the RT-PCR amplification. We used a mut396 primer for the amplification of TaRca1. The amplified product was checked on 1% agarose gel, and we observed an amplicon of ~7.4 kb (Fig. 1). This amplified product is mixed with KLD (Kinase, Ligase, DpnI) reaction mixture as per the manufacturer's instruction (Q5® Site-Directed Mutagenesis Kit, NewEngland Biolabs) and transformed on an ampicillin plate (Fig. 2). Positive transformed cells were used for the production of mutagenic protein.

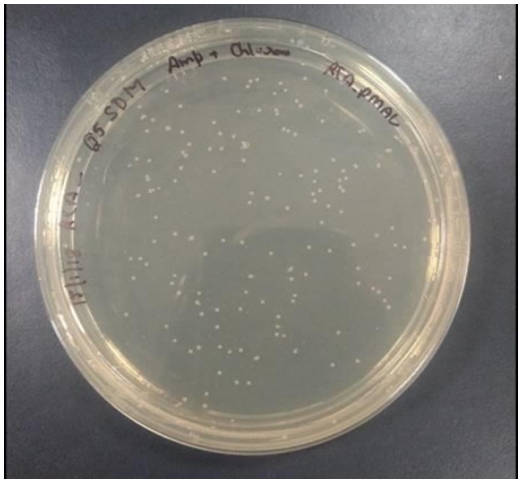

Fig 1: Amplification of Rca cloned in pMAL-c5x vector using mutagenic primer

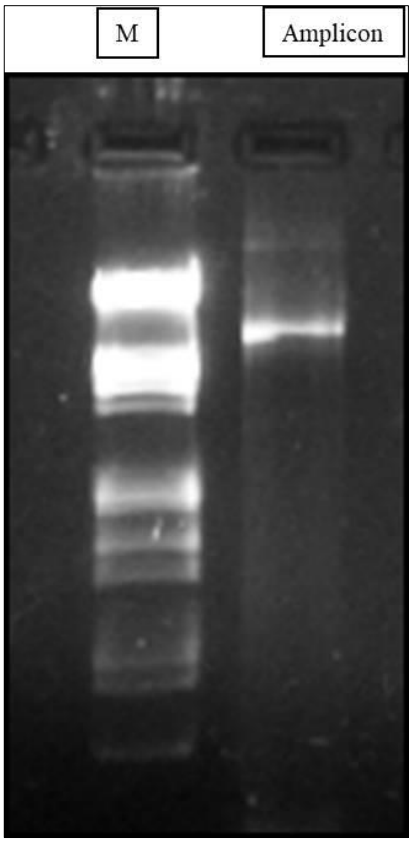

Fig 2: Colonies of BL21 (E. coli) on L.A. plate containing ampicillin

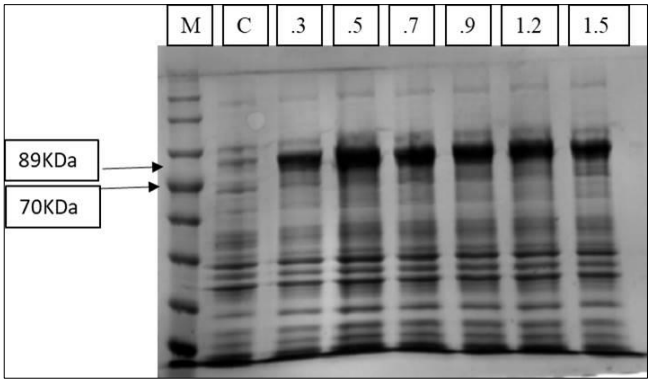

Fig 3: Condition optimisation of IPTG for optimum production of mutant Rca protein

Production of the mutant protein

Positively transformed cells were picked to produce the target protein (pMAL-c5x-Rca). We standardised the condition for optimum production of the fusion protein. The condition was to find the optimum IPTG (Isopropyl ß-D-1-thiogalactopyranoside) concentration. The fusion protein was harvested after sonication and resolved electrphotecially on 10% SDS-PAGE (Fig. 3). The fusion protein showed a band corresponding to ~88 kDa. We optimised the IPTG (Isopropyl ß-D-1-thiogalactopyranoside) concentration, and we found that 0.9mM is the production was maximum.

Discussion

The P-Loop is the ubiquitous structural domain of the AAA+ protein family to bind the Phosphate-containing substrate (Kinoshita *et al*., 1999) [9]. Shen *et al*. (1994) [28] reported that the hydrolysis of purine nucleotide induces conformational changes, a core function of this protein; this mechanism is also applicable to Rca. The three-dimensional structures of P-loop proteins show that the conserved Glycine residues (G-X-X-X-X-G-K-S/T) allow the hydrogen bond between the amino acids and two terminal phosphate groups of nucleotide (Smith and Rayment, 1996) [29]. Many functional amino acid residues at other locations have been found in some proteins that may have contributed increased thermostability of Rca (Shen and Ogren 1992) [24]. Shen *et al*. (1991) [27] reported that single amino acid substitution Ala/Arg-107Lys increases the Rca activity of 41-kDa isoform in spinach (*Spinacia oleracea*) when Gln-109Glu, the Rca activity in 45-kDa isoform was found similar to the wild-type Rca. Similarly, in Arabidopsis, single amino acid substitution Gln-111Glu/Asp in Phosphate binding loop increases the activation of RuBisCO by Rca (Kallis *et al*., 2000) [8], indicating that acidic amino acid at this location can enhance the activity of Rca. The presence of acidic amino acids (aspartate and glutamate) in the Walker B motif prepares water molecules for nucleophilic cleavage of γ-phosphate of ATP (Ogura *et al*., 2004) [16]. The Walker B motif is closely associated with the Sensor 1 region, which is involved in a hydrogen-bonding network that assists in placing a water molecule required in γ-phosphate cleavage (Chen *et al*., 1993) [3]. Heat-tolerant activase enzymes from plants grown in warm regions, such as creosote bush, jojoba (*Simmondsia chinensis*), tobacco, and cotton, have been characterised (Salvucci and Crafts-Brandner, 2004) [21], but the enzymes have not yet been expressed in transgenic heat-

sensitive hosts.

Our result shows that the induction of TaRca1 increases. The Rca isoforms in wild rice species and cultivated rice and wheat Rca1β have Isoleucine at position 159, whereas, in wheat Rca2β, methionine is present. A single amino acid substitution Met-159Ile in the Rca2β isoform increases the optimum temperature, whereas the efficiency is maintained by Rubisco activation by Rca (Degen *et al*., 2020) [4]. Similarly, In Arabidopsis, the rate of ATP hydrolysis increases at elevated temperatures by mutant Rca protein with one single residue substitution (Kurek *et al*., 2007) [12]. Among the three Rca isoforms in wheat, Rca1β activates RuBisCO at a slower rate, but at a higher temperature, it has higher RuBisCO activation and high ATPase activity.

Two mutant Rca2β proteins with 8 and 11 residue substitutions for amino acids found in the warm adapted species showed increased thermostability to 5–7 °C in wheat (Scafaro *et al*., 2019) [25]. Yang *et al*. (2020) [32] showed that acetylation of two conserved lysine residues 126 and 164 of Rca at high-temperature decreases the ATPase activity and RuBisCO activation. Kumar *et al*. (2009) [10] created a chimeric activase by replacing a Rca domain consisting of 267–334 amino acid residues in tobacco with the Arabidopsis Rca domain. Li *et al*. (2005) examined the ability of two tobacco activase mutants to trigger the activation of wild-type and mutant Chlamydomonas Rubiscos. The mutant activase L314V only activated D94K Rubisco, whereas mutant activase D311K activated wild-type and mutant P89R RuBisCO but not D94K RuBisCO. Scafaro *et al*. (2019) [26] reported that a point mutation of Lys428Arg altered the ADP inhibition in wheat. This may be due to substitution at the C-terminal increases ATP-dependent catalytic velocity and ATP substrate affinity. The chimeric activase retained the thermostability of tobacco and activated the RuBisCO of Arabidopsis as efficiently as the Arabidopsis isoform. The increased thermostability of mutant Rca may be due to increased hydrophobicity as substituting amino acid with an uncharged R group or non-polar amino acid residue may increase the hydropathy index (Kyte and Doolittle, 1982) [13]. Matsuura *et al*. (2015) [15] observed that increased hydropathy index results from substituting the amino acids increase the thermotolerance in CutA1 protein from E. coli.

Summary and Conclusion

Wheat is a temperate region crop but is also grown in the subtropical region. An increase in temperature during anthesis and grain filling will lead to a reduction in economic yield. The plant can tolerate heat by responding to physical changes within its body and creating signals for regulating its metabolism. Rca plays an essential role in regulating the activity of RuBisCO. Rca maintains the activation state of RuBisCO, and a decrease in the activity and amount of Rca leads to decreased photosynthesis under heat stress. The wheat Rca protein sequence was used to identify variation among the Rca sequences of heat-tolerant Rca. We identified position 396, where modification was done. We also saw the effect of different IPTG concentrations on the production of mutant Rca.

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