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An acidified thermostabilizing mini-peptide derived from the carboxyl extension of the larger isoform of the plant Rubisco activase

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ABSTRACT

Thermostable fusion peptide partners are valuable in engineering thermostability in proteins. We evaluated the Arabidopsis counterpart (AtRAce) and an acidified derivative (mRAce) of the conserved carboxyl extension (RAce) of plant Rubisco activase (RCA) for their thermostabilizing properties in *Escherichia coli* and *Saccharomyces cerevisiae* using a protein fusion strategy. We used AtRAce and mRAce as fusion tails for the thermolabile protein RCA2 from *Arabidopsis thaliana* and *Nicotiana tabacum*. The homologous fusion of AtRAce with Arabidopsis RCA2 and the heterologous fusion of AtRAce with tobacco RCA2 increased the thermostability of both proteins. The acidified derivative mRAce conferred greater thermostability upon both proteins as compared with AtRAce. Moreover, mRAce enhanced the thermostability of other two thermolabile proteins from *Jatropha curcas*: the cytosolic ascorbate peroxidase 1 (JcAPX1) and the TATA-box binding protein isoform 1 (JcTBP1). We further report – for the first time – that JcTBP1 mediates heat tolerance *in vivo* in yeast. Thus, our study identifies a C-terminal acidic mini-peptide – the acidified derivative mRAce – with potential uses in improving the thermostability of heat-labile proteins and their associated heat tolerance in host organisms.

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1. Introduction

Protein thermostability determines the activity, and long-term usage or storage of a protein at high temperatures (Böttcher and Bornscheuer, 2010; Joo et al., 2011; Park et al., 2014; Wijma et al., 2013; Yu and Huang, 2014). Heat stable proteins from thermophiles and hyperthermophiles (organisms adapted to high temperatures) are well characterized in terms of their folding properties and thermodynamic stability (Luke et al., 2007). Protein engineering for thermostability is crucial for industrial and biomedical uses of recombinant proteins (Böttcher and Bornscheuer, 2010; Joo et al., 2011; Park et al., 2014; Wijma et al., 2013), and constructing heat-tolerant organisms (Kurek et al., 2007; Mordukhova et al., 2008). Protein engineering methods for increased thermostability can be grouped into two categories: site-directed/random mutagenesis for a specific target protein (Wijma et al., 2013), and addition of a thermostabilizing fusion peptide to the proteins of interest (Huang et al., 2006; Luke et al., 2011; Park et al., 2002, 2004; Zhang et al., 2015). Of the two methods, the fusion strategy is simple, has universal applicability, and does not require protein structural information (Huang et al., 2006; Luke et al., 2011; Park et al., 2002, 2004; Zhang et al., 2015). Nevertheless, fusion strategy-mediated protein thermostabilization depends on an effectual fusion partner that is certainly of lasting demands (Park et al., 2002, 2004; Zhang et al., 2015).

Ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco) activase (RCA) is a nuclear-encoded chloroplast protein required for the light activation of Rubisco *in vivo*, and is critical to plant growth and productivity (Portis et al., 2008; Yin et al., 2014). However, RCA is thermolabile (Crafts-Brandner et al., 1997; Salvucci et al., 2001), and the heat-deteriorated RCA is incapable of maintaining Rubisco in its active form. The lack of active Rubisco causes low net photosynthesis (Pn), which inhibits growth and lowers yield in plants (Crafts-Brandner and Salvucci, 2000; Salvucci and Crafts-Brandner, 2004). Many plant species consist of two isoforms of RCA, α and β (Carmo-Silva and Salvucci, 2013) that only differ in 20–30 amino acid residues at the carboxy terminal (Werneke et al., 1989). These isoforms may arise due to alternate splicing as in spinach and

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Arabidopsis (Werneke et al., 1989), or may be the product of two separate genes as in cotton (Salvucci et al., 2003). The larger form, RCA1, is more thermostable than the smaller form, RCA2 (Crafts-Brandner et al., 1997). Heat induced large isoform [RCA (L)] helps in photosynthetic acclimation to moderate heat stress while the two smaller isoforms [RCA (S)] maintain Rubisco activity under normal conditions in rice plants (Wang et al., 2010). The large isoform of RCA contributes to high-temperature acclimation of plants (Wang et al., 2010) and differential heat-adaptability in different genotypes (Weston et al., 2007). Thus, the short carboxyl extension in the large isoform of RCA may contribute to the thermostability of Rubisco activase, making it an ideal target for engineering protein thermostability through fusion strategy.

The cytosolic ascorbate peroxidase 1 (APX1) plays a central role in H_2O_2 scavenging and is involved in numerous developmental processes in Arabidopsis (Davletova et al., 2005; Koussevitzky et al., 2008) and cotton (Li et al., 2007). Moreover, APX1 is heat-labile in Arabidopsis (Panchuk et al., 2002), and *Jatropha curcas* (Zhang et al., 2015), indicating that it is responsible for plant heat-sensitivity. Eukaryotic TATA-box binding protein (TBP) is a universal transcription factor required for initiation by all three nuclear RNA polymerases (White and Jackson, 1992). It consists of a highly conserved large C-terminal domain responsible for DNA binding and basal transcription and an extremely variable N-terminal domain possibly involved in activated transcription (Davidson, 2003). Only a single TBP exists in yeasts and animals while most plants comprise two isoforms encoded by separate genes that probably perform nonoverlapping functions (Vogel et al., 1993; Zhu et al., 2002). The maize TBP, unlike animal TBPs, is functionally interchangeable with the yeast homolog for conferring yeast cell viability (Vogel et al., 1993). TBP is universally thermolabile and even moderate heat treatment can completely abolish its activity for initiating the basal transcription *in vitro* (Iwataki et al., 1997; Nakajima et al., 1988; Zhu et al., 2002). Thus, TBP is a rate-limiting factor for basal transcriptional activity (Iwataki et al., 1997), and can down-regulate the expression of some vital genes, leading to the decreased tolerance and viability of organisms under heat stress. Therefore, RCA, APX, and TBP are good candidates for studying thermostability engineering to improve the important agronomic traits of plants under heat stress conditions.

In this study, we evaluated the use of the Arabidopsis counterpart (AtRAce) and an acidified derivative (mRAce) of the conserved carboxyl extension (RAce) of plant RCA1 as a fusion tail for thermostabilization in recombinant *Escherichia coli* and *Saccharomyces cerevisiae* cells. We tested these fusion tails to confer thermostabilization on RCA2 in Arabidopsis and tobacco, cytosolic ascorbate peroxidase 1 (JcAPX1) and TATA-box binding protein isoform 1 (JcTBP1) of *J. curcas*. Our results showed that mRAce indeed enhanced the thermostability of these heat labile proteins. Moreover, our data provides the first report of JcTBP1-mediated heat tolerance in yeast.

2. Materials and methods

2.1. Gene cloning

Seedlings of plant species (Arabidopsis, tobacco, *J. curcas*) were harvested for total RNA extraction by Trizol reagents (Invitrogen, USA). Aliquots of RNA samples were reversely transcribed as the PCR templates by M-MLV RTase (Promega, USA). Gene amplification was conducted in a gradient thermocycler (Eppendorf, Germany) by DreamTaq DNA polymerase (Thermo Scientific, USA), using the corresponding cDNA templates and primers (Table 1). Briefly, the genes encoding both isoforms of Arabidopsis RCA (AtRCA1, AtRCA2), and the smaller isoform of tobacco RCA (NtRCA2) without chloroplast transit peptides were amplified by primer pairs AtRCA1-5Nd/AtRCA1-3Xh, AtRCA1-5Nd/AtRCA2-3Xh, and NtRCA2-5Nd/ NtRCA2-3Xh, respectively. The gene encoding JcTBP1 was amplified with primer pair JcTBP1-5Nd/JcTBP1-3Xh. The purified PCR products of genes *AtRCA1*, *AtRCA2*, *NtRCA2* and *JcTBP1* were digested with *Nde*I and *Xho*I (Thermo Scientific, USA), and subcloned into the prokaryotic expression vector pET32a(+) (Novagen, USA) to generate plasmids pET(AtRCA1), pET(AtRCA2), pET(NtRCA2) and pET(JcTBP1), respectively, which were further verified by sequencing.

2.2. Construction of expression vectors

The short DNA fragment encoding the C-terminal extension (AtRAce, 438-474 aa) of AtRCA1 was amplified with the primer pair AtRAce-5Sa/AtRCA1-3Xh from the template plasmid pET(AtRCA1). Based on the consensus sequence of the short carboxyl extension of RCA1 among most plant species, we designed an acidified mini-peptide termed mRAce. The coding DNA for this mini-peptide was obtained by PCR synthesis with four long primers mRAce-F1Sa, mRAce-F2, mRAce-R2 and mRAce-R1Xh (Table 1). The AtRAce or mRAce DNA fragment was double digested by *Sal*I and *Xho*I, then inserted into the plasmids pET(AtRCA2), pET(NtRCA2), pET(JcAPX1) (Zhang et al., 2015), and pET(JcTBP1) at the *Xho*I site downstream of the gene coding sequence, to create their corresponding fusion expression vectors such as pET(AtRCA2-mRAce). Furthermore, the JcTBP1 and JcTBP1-mRAce DNA fragments from plasmids pET(JcTBP1), pET(JcTBP1-mRAce) treated with *Nde*I/*Xho*I dual digestion and Klenow blunting, were ligated into the *BamH*I/*Xba*I-digested & Klenow-blunted yeast plasmid pYES2 (Invitrogen, USA), to achieve the yeast expression vectors pYES2(JcTBP1), pYES2(JcTBP1-mRAce), respectively.

2.3. Thermostability analysis of the recombinant proteins expressed in E. coli by SDS-PAGE

E. coli strain BL21(DE3) (Novagen, USA) containing any constructed prokaryotic expression vector was grown to an OD_{600} of 0.6 in LB medium (100 mg/L ampicillin) at 37 °C, then induced overnight at 25 °C by 0.5 mM IPTG (Sigma, USA). Bacterial cells were harvested from a 14 ml culture by centrifugation, resuspended in 4 ml of 100 mM PBS buffer (pH 7.4) (additionally containing 2 mM ascorbic acid for JcAPX1 and its fusion protein), and lysed by ultrasonification.

Aliquots (each 16 μl) of the soluble fraction of bacterial cell lysates were simultaneously heat-treated for a certain amount of time (*e.g.*, 15 min for RCA2 and its fusion proteins) under a series of gradient temperatures automatically appointed within a range (*e.g.,* 30–50 °C for RCA2 and its fusion proteins) in a gradient thermocycler (Eppendorf, Germany), and then each was fractioned into the supernatant (S) and pellet (P) for 12% SDS-PAGE. Subsequently, the solubility of the recombinant proteins on the gels at each point of heat treatments were estimated by the program "Quantity One" (Bio-Rad, USA) and used for thermostability appraisal.

2.4. In gel activity staining of the recombinant JcAPX1 and its fusion proteins

Recombinant expression of JcAPX1 and its fusion proteins in *E. coli*, sample heat treatments, native-PAGE, and in gel APX activity staining were conducted as mentioned above and described previously (Mittler and Zilinskas, 1993; Zhang et al., 2015). The stained gels were documented using a gel imager (Bio-Rad, USA).

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