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Effects of calcium concentration, calcium chelators, calcium channel-blockers on *Hsp70a* expression in *Chlamydomonas reinhardtii*

Tuba Sevgi*, Elif Demirkan

Biology Department, Faculty of Arts and Sciences, Bursa Uludag University, 16059 Bursa, Turkey.

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Corresponding Author
Tel.: +90 224 294 29 35
E-mail: tubaavci@uludag.edu.tr

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Abstract

In this study, calcium concentration, calcium chelators, and calcium channel blockers that could be effective in triggering the heat shock response in *Chlamydomonas reinhardtii* were investigated. For this purpose, continuously expressed and heat-inducible transformant *C. reinhardtii* strains were used, and heterologously expressed arylsulfatase activities were detected. After a short time of heat shock at 40°C, cultures were shifted to 23°C and different concentrations of calcium (0-1 M $CaCl_2$), EGTA (0-50 mM), BAPTA (0-2 mM), lanthanum (0-300 µM), gadolinium (0-350 µM), and verapamil (0-100 µM) applications were performed. To compare the arylsulfatase activity results at the transcript level, HSP70A expression level was analyzed. Arylsulfatase activity was increased with the increase of the calcium concentration, in the presence of calcium chelators, blockers, and parallel results were obtained in HSP70A expression level. These findings support that both extracellular and intracellular calcium influx is effective in the heat shock response of *C. reinhardtii.*

Introduction

Global warming showed itself in the form of heatwaves in recent years and endangers production efficiency. Each factor that affects production efficiency also affects the human population and welfare (Sung et al., 2003). It is predicted that heat waves will be stronger, long-lasting, and more frequent in the future (Meehl & Tebaldi, 2004). Therefore, it is imperative to understand the heat stress response and sensing mechanism behind generating more heat-tolerant crop plants by genetic engineering.

The heat stress response (HSR) has been studied in detail in bacteria, yeast, flies, and mammals, however, not all concepts have been explained and these may not apply to plant systems. The main reason for this is the plastids, which play a central role in the metabolism of plant cells and contain at least 1300 proteins. Plastids add an extra level of complexity to these eukaryotic cells. Therefore, it is clear that a simpler plant model organism is needed to study HSR (Schroda et al., 2015). *Chlamydomonas reinhardtii* is utilized as a plant model organism since the 1950s (Harris, 2001). Taking advantage of the relatively few players in the protein homeostasis network compared to plants, *C. reinhardtii* is a practical model to work for (Schroda & Vallon, 2009; Schulz-Raffelt et al., 2007).

Increased expression of heat shock protein (HSPs) genes has been used as a marker to study heat shock response in many experiments since the expression of HSPs is a conservative event that occurs in all organisms exposed to heat stress (Rütgers et al., 2017). The expression of genes encoding HSPs is induced when misfolded proteins accumulate in the cell. This was first demonstrated in *Escherichia coli* (Goff & Goldberg, 1985). Also in land plants and *Chlamydomonas* the accumulation of unfolded proteins was shown to trigger HSP gene expression (Kurepa et al., 2003; Sugio et al., 2009; Schmollinger et al., 2013).

Some studies have suggested that the expression of HSPs is due to increased fluidity in the plasma membrane (Gao et al., 2012; Saidi et al., 2009; Suri & Dhindsa, 2008; Wu et al., 2012). The primary heat sensors in the plasma membrane of plant cells were suggested to be calcium channels that open when membrane fluidity increases, thereby allowing the influx of extracellular calcium to trigger the HSR (Saidi et al., 2009, 2010). Saidi et al. (2011) suggested that heat stress in plants is sensed by the entry of extracellular Ca^{2+} through Ca^{2+} channels to the plasma membrane. Regarding this hypothesis, changes in temperature lead the membrane fluidity loss. Ca^{2+} influx activates a calmodulin-dependent kinase, cyclic adenosine monophosphate (cAMP) levels increase in heat stress, thus leading to the activation of cyclic nucleotide-gated calcium channels (CNGC). Then, the heat shock factor (HSF) was activated and mediated the expression of heat stress genes. Stimulation and expression of HSPs allow the cell to restore protein homeostasis while HSF is inactivated, and then the response is weakened. The role of calcium as a secondary messenger in heat shock signal transduction in *Chlamydomonas* has been controversial, insufficient therefore more studies are needed (Gong et al., 1998; Saidi et al., 2009; Gao et al., 2012; Zheng et al., 2012; Schroda et al., 2015).

The most powerful technique for evaluating the effect of a mutation in a promoter is to use a reporter gene whose expression can be detected and quantified a short time after transformation. A handful of reporter genes have been codon optimized in *Chlamydomonas* nuclear genome, including luciferase (Fuhrmann et al., 2004; Shao & Bock 2008), green fluorescent protein (Fuhrmann et al., 1999), xylanase (Rasala et al., 2012), and recently several additional fluorescents (Rasala et al., 2013). However, even with these optimized reporters, signal is low and expensive to detect. Instead heterologously expressed arylsulphatase (ARS), can be detected by simple and sensitive colorimetric assay. Also, almost completely secretes into the surrounding medium by cells lacking a cell wall and allows detectable enzymatic activity in culture media (Hostos et al., 1988; Ohresser et al., 1997). Therefore, ARS was utilized as a strong reporter for quantitative analysis of gene expression in this work.

In this study, calcium concentration, calcium channel blockers (lanthanum, gadolinium, and verapamil), and chelators (EGTA and BAPTA) were used to determine whether calcium affects triggering the heat shock response using inducible *HSP70A* promoter, continuously expressing *β2TUB* promoter and the *ARS* reporter.

Materials and Methods

Strains and culture conditions

C. reinhardtii strain cw15-302 *(arg7-)*, c124, and plasmids pCB412 (*ARG7* [Arginynosuccinate lyase] marker gene-containing plasmid), pJD55 (*β2TUB-ARS* containing plasmid), pCB803 (*HSP70A-ARS* containing plasmid) were kindly provided from M. Schroda (Technical University of Kaiserslautern, Germany) (Schroda et al., 2000), and *C. reinhardtii* nuclear transformation was performed using glass beads method (Kindle, 1990), and HSP70A III-1 and βTub II-32 transformants were used from our previous study as recombinant strains (Sevgi & Demirkan, 2021). *C. reinhardtii* recombinant strain HSP70A III-1 (containing *HSP70A* promoter and *ARS* reporter gene) and *C. reinhardtii* recombinant strain βTub II-32 (containing *β2TUB* promoter and *ARS* reporter gene) were grown in Tris Acetate Phosphate (TAP) medium under continuous light (14 µmol $E.m^{-2}s^{-1}$) on a rotatory shaker at 23°C and 150 rpm (Harris, 2001). Then in total, ~10^8 cells were centrifuged at 3500 rpm for 3 min and the pellet was transferred to 30 mL TAP medium in 100 mL flasks which were adjusted before to 40°C. The pellet was added to the medium, kept at 40°C for 5 min, and immediately shifted to 23°C for calcium applications.

Effect of calcium concentration, calcium ion channel blockers, and calcium chelators

Different concentrations of calcium ($CaCl_2$; 0 mM, 100 mM, 250 mM, 500 mM, 1M), EGTA (triethylene glycol diamine tetraacetic acid) (0 mM, 1 mM, 50 mM), BAPTA (1,2-bis [o-aminophenoxy] ethane-N,N,N',N'-tetraacetic acid) (0 mM, 1 mM, 2 mM), lanthanum (0 µM, 10 µM, 75 µM, 100 µM, 150 µM, 300 µM), gadolinium (0 µM, 100 µM, 150 µM, 250 µM, 300 µM, 350 µM), and verapamil (0 µM, 10 µM, 100 µM) (Sangwan et al., 2002) were used to investigate the effects on heat shock response. Thus, continuously expressed (βTub II-32) and heat-inducible (HSP70A III-1) recombinant *C.reinhardtii* strains were grown at 23˚C with continuous light (CL) until the cell density reached approximately ~10^6 cells/mL. After a short time heat shock, calcium, calcium chelator, and blocker applications were performed. For this purpose, a total of ~10^8 cells were centrifuged at 3500 rpm at room temperature for 3 min and the pellet was added to 30 ml TAP media at 40°C for 5 minutes then again ~10^8 cells were added to 30 mL TAP media containing calcium, calcium chelators, and ion channel blockers at different concentrations. The ARS activities were compared with the control (0mM).

Arylsulfatase enzyme activity assay

Arylsulfatase (ARS) activity was determined according to Ohresser et al. (1997). ARS is the α-naphthol concentration formed in 1 h at 37˚C according to the total amount of chlorophyll (µg α-naphtol/µg chlorophyll h). Chlorophyll concentration was detected according to Porra et al. (1989).

500 µl of culture (~10^8 cells/mL) were pelleted by centrifugation and the supernatant was used for ARS assay according to Ohresser et al. (1997). 500 µl of the reaction mixture contained 400 µl supernatant, 0.4 M glycine-NaOH buffer pH 9.0, 10 mM imidazole, and the

enzyme-substrate 0.3 mM 5-bromo-4 chloro-indolylsulphate (X-SO4). The samples were incubated for 1 h at 37°C with X-SO4 then the reaction was stopped with 500 µl of 4% SDS in 0.2 M Na acetate buffer pH 4.8 and 100 µl of 10 mg/mL tetrazotized-o-dianisidine (Sigma). This compound unites with the α-naphthol released in the reaction, an SDS soluble purple precipitate occurs. The ARS activity was measured at 540 nm within 2 min after the addition of tetrazotized-o-dianisidine, and the activity was expressed as µg naphthol per µg chlorophyll (chl) in the cell pellet per h at 37°C.

RNA extraction and gene expression analysis

HSP70A and *Chlamydomonas* β-subunit-like polypeptide (CβLP) expressions were measured by RT-PCR and compared with ARS activity results. For this purpose, 2×10^7 cells were collected for RNA extraction using the NucleoSpin RNA Plant Kit (Macherey-Nagel, Düren, Germany) by following the manufacturer's instructions. DNA contaminations were removed with Turbo DNase (RNase-free; Ambion, Massachusetts, USA). RT-PCR experiments were perfomed with using a OneTaq One-Step RT-PCR Kit (NEB, Massachusetts, USA) by StepOnePlus™ Real-Time PCR System (Applied Biosystems, Massachusetts, USA) (10 min 48°C, 10 min 95°C; 60 s were set at 65°C for a total of 40 cycles). Samples without a template or reverse transcriptase were always included as control. $HSP70A_{For}$GATCGAGCGCATGGTGC, $HSP70A_{Rev}$TCCATCGACTCCTTGTCCG, $C\beta LP_{For}$GCCACACCGAGTGGGTGTCGTGCG, $C\beta LP_{Rev}$CCTTGCCGCCCGAGGCGCACAGCG primers were used. The relative quantification of gene expressions was analyzed by the $2^{-\Delta\Delta Ct}$ method. The protocols of Livak & Schmittgen (2001) was used. *HSP70A* and *CβLP* expressions were measured by RT-PCR and compared with ARS activity results.

Statistical analysis

Data were expressed as mean and standard deviation (SD). Statistical analyses were performed using GraphPad Prism 9.2.0 (Demo Version; GraphPad, San Diego, CA) statistical package program. An independent sample t-test was applied to examine the effect of calcium, calcium chelators, and blockers at the transcript level. The significance was calculated using Student's t-test. A value of $p<0.05$ was considered statistically significant.

Results and Discussion

In this study, the effect of calcium, which is thought to be effective in membrane fluidity and heat shock response, on *HSP70A* expression using *C. reinhardtii* recombinant strains (HSP70A III-1 and βTub II-32) was investigated by using strong promoter (*HSP70A* and *βTub*) and reporter (*ARS*) genes.

Many studies have been performed on perception and response of heat stress in *Chlamydomonas*, plants and other organisms (Morimoto, 1998; Schroda et al., 2000; Schmollinger et al., 2013). Temperature changes may alter the fluidity of biological membranes, which may impair the barrier function of membranes between intercellular compartments and the activity of integral membrane proteins (Saidi et al., 2011). For *Chlamydomonas*, it is still being investigated whether heat shock can be perceived by changes in membrane fluidity, unlike land plants. Some studies in plant cells support that the primary heat sensors in the plasma membrane are calcium channels (Gao et al., 2012; Liu et al., 2003; Suri & Dhindsa, 2008; Saidi et al., 2009; Wu et al., 2012). Results from some studies indicated that calcium accumulating in the cytosol of heat-stressed cells was only derived from extracellular stores (Sangwan et al., 2002; Saidi et al., 2009; Wu et al., 2012), other studies also pointed to intracellular calcium stores as a source (Gong et al., 1998; Zheng et al., 2012). In land plants, the influx of extracellular calcium was found to be substantial as the HSR was diminished when calcium chelators EGTA or BAPTA, or the calcium ion channel blockers lanthanum, gadolinium or verapamil were applied prior to HS (Heat Shock) (Link et al., 2002; Sangwan et al., 2002; Liu et al., 2003; Suri & Dhindsa, 2008; Saidi et al., 2009; Gao et al., 2012; Wu et al., 2012).

As the first step, the possible effect of extracellular calcium on the heat shock response was analyzed in recombinant strains of *C. reinhardtii* (HSP70A III-1 and β_2TUB II-32). Secondly, different concentrations of calcium chelators EGTA and BAPTA and the calcium ion channel blockers lanthanum, gadolinium or verapamil were applied to recombinant *C. reinhardtii* strains. The highest ARS activity was obtained in transformant HSP70A III-1 as 1338 µg-αnaphthol/µg chl at 100 mM calcium and the enzyme increased 4.5 times compared to the control. A sharp decrease was observed in ARS activity at concentrations between 100 mM and 150 mM while a slight increase was observed at concentrations between 150 mM-500 mM. This indicates that HSR may have been triggered by opening calcium channels in the cell membrane and allowing calcium flow into the cell. Therefore, it seems that calcium played a role as a second messenger. In the positive control strain β_2TUB II-32 ARS activity remained constant as expected (Figure 1). Wu et al. (2012) reported that HS-triggered rapid increases in Ca^{2+} in cytosol is important in mediating downstream HS-related gene expression for the acquisition of thermotolerance in rice. Saidi et al. (2009) suggest that early sensing of mild temperature increments occurs at the plasma membrane of plant cells independently from cytosolic protein unfolding and the heat signal is translated into an effective HSR by way of a specific membrane-regulated Ca^{2+} influx, leading to thermotolerance.

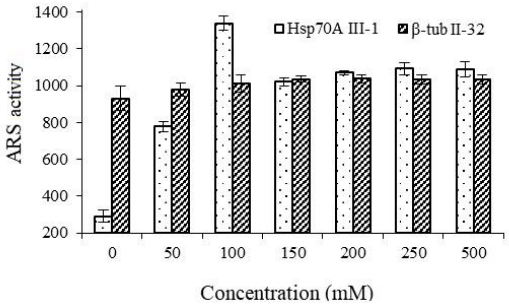

Figure 1. The ARS enzyme activities (µg α-naphtol/µg chlorophyll h) of recombinant *C. reinhardtii* strains (HSP70A III-1 and β_2TUB II-32) at different $CaCl_2$ concentrations (0-500 mM). The data presented are mean± SD of three independent experiments. SD, Standard Deviation.

In the studies with Ca chelators and blockers, it was observed that as the EGTA concentration increased, the ARS enzyme activity is also increased in the HSP70A III-1 transformant (Figure 2). When the EGTA concentration reached 50 mM, the amount of ARS increased approximately 5 times compared to control (0mM) (Figure 2a). In BAPTA, this increase is 4 times (Figure 2b). According to ARS activity results, it can be said that the heat stress response was triggered in the presence of calcium chelators, EGTA, and BAPTA. The induction of ARS activity in the presence of EGTA and BAPTA may be due to the insufficient concentration of calcium chelators or the excessive calcium flow released from the intracellular calcium stores. Schmollinger et al. (2013) obtained inconsistent role of extracellular calcium in mediating the HSR with *Chlamydomonas*, while washed cells treated with calcium chelator BAPTA displayed a delayed and less pronounced induction of *HSP* gene expression and reduced thermotolerance under HS, washed cells supplemented with EGTA behaved like controls. Zheng et al. (2012) found that the intracellular calcium (Ca^{2+}) increased rapidly after HS in the Ca^{2+}/calmodulin HS signal transduction pathway. Our results support both the role of extracellular and intracellular calcium utilization in heat stress induction.

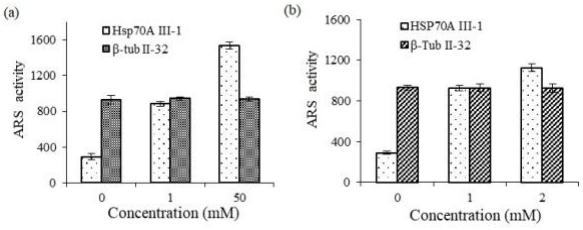

Figure 2. The ARS enzyme activities (µg α-naphtol/µg chlorophyll h) of recombinant *C. reinhardtii* strains (HSP70A III-1 and β_2TUB II-32) at different EGTA (0-50 mM) (a), and BAPTA (0-2 mM) concentrations (b). The data presented are mean± SD of three independent experiments.

The effect of different concentrations of calcium channel blockers (lanthanum, gadolinium, and verapamil) on ARS enzyme activity was also investigated. In the presence of lanthanum, ARS activity increased approximately 4-fold for HSP70A III-1 at 75µM concentration (Figure 3a). At 100 µM lanthanum concentration, the activity decreased considerably. When the gadolinium concentration was examined, it was determined that the ARS activity increased 4 times at 150 µM in the HSP70A III-1 transformant (Figure 3b). In the presence of different concentrations of another calcium channel blocker, verapamil, the ARS enzyme activity in the HSP70A III-1 transformant increased 2.5 times at 10 µM compared to the control, while this increase was 4.9 times at 100 µM (Figure 3c). As expected, ARS activity was stable in the transformant β_2TUB II-32 at all concentrations of lockers. It has been reported that the blockers inhibit HS detection/signal transmission (Lancaster & Batchelor, 2000; Saoudi et al., 2004). In our study, it was determined that the response was triggered in the presence of calcium blockers. However, decreases in ARS activity or stability at high concentrations of lanthanum and gadolinium suggest that they act as calcium blockers. It has been stated that the extracellular calcium influx in plants is mediated by the opening of specific calcium channels as a result of increased membrane fluidity at high temperatures (Saidi et al., 2009).

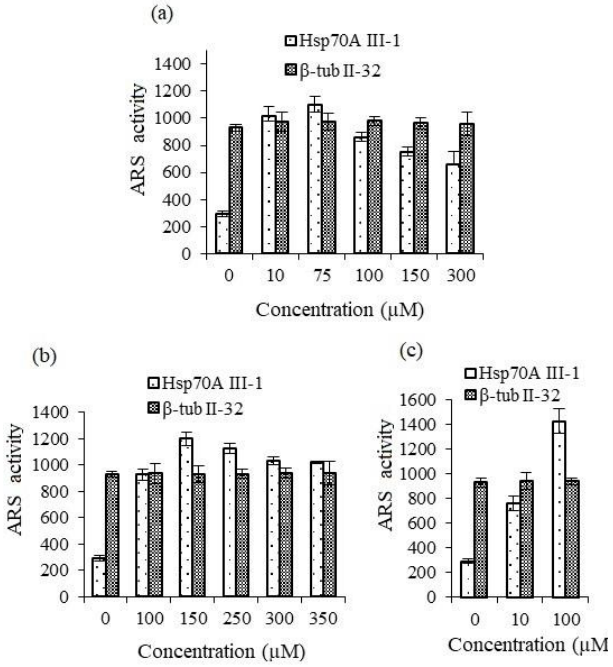

Figure 3. The ARS enzyme activities (µg α-naphtol/µg chlorophyll h) of recombinant *C. reinhardtii* strains (HSP70A III-1 and β_2TUB II-32) at different lanthanum (0-300 µM) (a), gadolinium (0-350 µM) (b), verapamil concentrations (0, 10, 100 µM) (c). The data presented are mean± SD of three independent experiments.

All results were evaluated comparatively with RT-PCR. As a result, transcript expressions were in parallel with ARS activities in the samples exposed to different concentrations of EGTA, BAPTA, lanthanum, gadolinium, and verapamil (Figure 4).

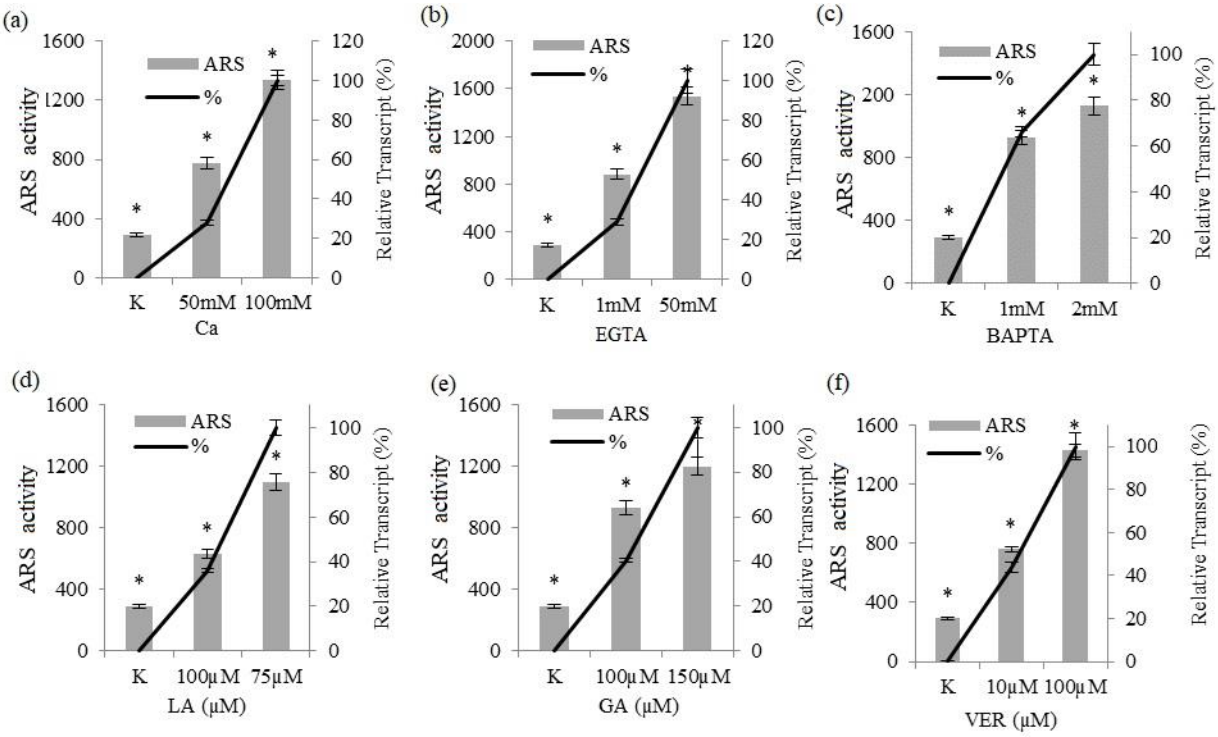

Figure 4. The ARS enzyme activities (µg α-naphtol/µg chlorophyll h) and relative HSP70A expression levels of recombinant C. reinhardtii strain HSP70A III-1 at different calcium concentrations (a), exposure to different concentrations of EGTA (b), and BAPTA (c), lanthanum (d), gadolinium (e), and verapamil (f) as determined by qPCR. The results show the mean of the data from three qPCR replicates and two biological replicates. The highest expression level of the respective transcript was set to 100%. Student's t-test was used for statistical analysis of the samples. *, P< 0.05

Conclusion

This study was aimed to investigate the effect of calcium concentration, calcium channel blockers, and chelators triggering the heat shock response. Our results demonstrated increase in calcium concentration, the presence of calcium chelators and blockers induced the increase in ARS activity. *ARS* induction was also confirmed by qRT-PCR analysis. The increase in *HSP70A* transcript expression levels support that the selective *ARS* reporter gene used in the study is favorable. Overall, our results suggest that both extracellular and intracellular calcium influx was shown to be effective in heat shock response in *C. reinhardtii.* However, further studies investigating the molecular mechanisms are required.

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Author Contributions

TS: Data Curation, Formal Analysis, Investigation, Methodology, Writing Original Draft, Funding, Project Administration, Resources. **ED:** Conceptualization, Supervision, Writing, Review and Editing

Conflict of Interest

The author(s) declare that they have no known competing financial or non-financial, professional, or personal conflicts that could have appeared to influence the work reported in this paper.

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