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Identification of a putative kinase interacting domain in the durum wheat catalase 1 (TdCAT1) protein

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ABSTRACT

Catalases are crucial antioxidant enzymes that regulate plants responses to different biotic and abiotic stresses. It has been previously shown that the activities of durum wheat catalase proteins (TdCAT1) were stimulated in the presence of divalent cations Mn^{2+}, Mg^{2+}, Fe^{2+}, Zn^{2+}, and Ca^{2+}. In addition, TdCAT1s can interact with calmodulins in calcium-independent manner, and this interaction stimulates its catalytic activity in a calcium-dependent manner. Moreover, this activity is further enhanced by Mn^{2+} cations. The current study showed that wheat catalase presents different phosphorylation targets. Besides, we demonstrated that catalase is able to interact with Mitogen Activated Proteins kinases via a conserved domain. This interaction activates wheat catalase independently of its phosphorylation status but is more promoted by Mn^{2+}, Fe^{2+} and Ca^{2+} divalent cations. Interestingly, we have demonstrated that durum wheat catalase activity is differentially regulated by Mitogen Activated Proteins kinases and Calmodulins in the presence of calcium. Moreover, the V0 of the reaction increase gradually following the increasing quantities of Mn^{2+} divalent cations. Such results have never been described before and suggest i) complex regulatory mechanisms exerted on wheat catalase, ii) divalent cations (Mn^{2+}; Mg^{2+}; Ca^{2+} and Fe^{2+}) act as key cofactors in these regulatory mechanisms.

1. Introduction

Catalase is the first discovered antioxidant enzyme that functions mainly in the dismutation of excessive hydrogen peroxide (H_2O_2) into H_2O and O_2. This process requires a haem co-factor to alleviate stresses generated during developmental processes or by environmental stimuli [1–7] in all aerobic organisms [8]. In plants, catalase proteins are encoded by a multigenic family. Those genes were reported in many species with different numbers of genes. In fact, one gene was identified in different plants such as sweet potato [9], castor bean [10], *Triticum turgidum* ssp durum [11], cassava (*Manihot esculenta* Crantz; [12]), tomato [13], *Pyrus communis* L. [14] and *Triticum monococcum* [15]. It has been shown that catalase is phosphorylated in animals. In fact, human catalase was shown to be stimulated by different kinases, such as the non-receptor tyrosine kinase (c-Abl), the arginine tyrosine kinase phosphorylations on Y231 and Y386 residues [16], the PKCδ protein kinase phosphorylations and the p44/42 MAP kinase [17]. In human diseases such as

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thyroid disorders, tumor progression, and viral infection, catalase is essential for cellular defense [18]. However, the catalytic action of this enzyme can be altered by the existence of drugs and other molecules [19,20]. Many recent works have reported that the interaction between small molecules such as Erlotinib hydrochloride [18] and pyridine-derived Schiff base complexes [21] with catalase can change the structure and function of the catalase. The catalytic activity and structure of catalase have also been reported to change with the existence of isoxsuprine hydrochloride and levothyroxine [19].

In plants, it has been shown that CDPK8 specifically phosphorylates and activates CAT3 in response to ABA increase after abiotic stress application [22]. Besides, brassinosteroid-insensitive 1 (BRI1) associated receptor kinase 1, known as BAK1, activates and phosphorylates AtCAT1, AtCAT2 and AtCAT3 under high light conditions [23]. Whereas the AtSnRK2.4 regulates catalase levels and modulates its activity as well as the level of ascorbate in seedlings subjected to salt stress [24]. In rice, salt tolerance receptor-like cytoplasmic kinase 1 (STRK1) protein kinase is activated by phosphorylation of the OsCatC to regulate H_2O_2 homeostasis, thus playing a crucial role in improving salt tolerance [25].

Mitogen-activated protein kinase (MAPK) cascades are conserved signaling modules present in plants and animals, regulating plant responses to different biotic and abiotic stresses [26], also plant growth regulation and maturation and programmed cell death [27]. Each MAPK cascade operates, via phosphorylation, in a module structured by three sequentially acting kinases, as a minimum. The well-conserved MAPK (or MPK) is activated by a specific MAPK kinase (MKK also known as MAPKK). MAPKK kinases (MEKK or MAPKKK) are also phosphorylated by their upstream kinases, known as MAPKKK, which usually receive signals directly or indirectly from specific receptors [28]. MAPKs are well conserved among all eukaryotes and composed of 11 sub-domains (I–XI), present in all serine/threonine protein kinases [29]. MAPK activation occurs via double phosphorylation of Thr and Tyr residues at a conserved TxY motif (where x could be Asp or Glu), located between subdomains VII and VIII [26]. After phosphorylation, MAPKs are always translocated into the nucleus, where they activate a large number of targets by phosphorylation. MAPKs could also remain in the cytoplasm to phosphorylate distinct protein targets. Phosphorylation of target proteins occurs after interaction of MPK with its target through a short linear motif of 7–18 aa called the Kinase Interacting Domain (KID; (R/K)(1–2)-(X)(2–6)-Φ-x-Φ; where Φ denotes a hydrophobic residue) known also as D-motifs. Such motifs are usually found in basically disordered segments, potentially far away from target phosphorylation sites [30]. Kinase interacting domains are found in different targets such as negative regulators (phosphatases, MAPK phosphatases; [31]), WRKY transcription factors [32], activators (MAPKKs), scaffolding proteins and other substrates [33]. Phosphorylation of target proteins occurs on specific serine/threonine residues, close to a proline (S/T-P; [34]). In plants, MPK3, MPK4 and MPK6 were shown to play a crucial role in plant maturation, and cell proliferation [28,35,36] as well as in plant responses to biotic and abiotic stresses [37–39]. In wheat, the number and function of MAPKs remain poorly studied. In fact, it has been shown that TMPK3 interacts with its negative regulator TMKP1 via a conserved KID domain located at the C-terminal portion of the protein and this interaction stimulates the catalytic activity of TMKP1 in a dose-dependant manner [31].

Previous work dentified TdCAT1 from durum wheat (TdCAT1, accession number KP696753) after plant exposure to salt stress [11]. TdCAT1 presents different cation binding domains (Mn^{2+}, Fe^{2+}, Mg^{2+}, Cu^{2+}/Zn^{2+} and Ca^{2+}) at different portions of the protein which seems to be conserved among catalase proteins in plants [40]. On the other hand, TdCAT1 protein harbors a conserved putative inhibitory doamin and a Calmodulin binding domain (CaMBD) both located at the C-terminal portion of the protein [11,40] and interacts with CaM in Ca^{2+} independent manner [40]. Besides, CaM proteins stimulated the catalase activity of TdCAT1 in the presence of TdCaM1.3 in a Ca^{2+} dependent-manner [40]. Interestingly, the C-terminal portion of TdCAT1 also contains a putative consensus internal peroxisomal targeting signal (PTS1) composed of three crucial amino acids (QKL). This domain is responsible for a strong accumulation of TdCAT1 in the peroxisome and a poor accumulation in the cytoplasm [15]. Overexpression of TdCAT1 positively regulates yeast, Arabidopsis [11] and bacteria [41] responses to different abiotic stresses. In this study, we provide experimental evidence that durum wheat TdCAT1 physically interacts with wheat Mitogen Activated Protein Kinase 3 (TMPK3) and that this interaction stimulates the catalytic activity of TdCAT1. As far as we know, such results have not been described before in plant catalases.

2. Materials and methods

2.1. Bioinformatic analysis

The multiple sequence alignment and comparison of amino acid sequences were performed using the Clustal Omega Algorithm [42]. A dendrogram was constructed using 11 sequences by the Neighbor Joining method with bootstrapping (1000 replicates) using the same program. A Group-based Phosphorylation system (GPS: http://gps.biocuckoo.cn/) site was used to identify the potential MAPK target phosphorylation sites in wheat TdCAT1.

2.2. Proteins expression and purification

Cloning of genes, the expression and the purification of proteins were performed as previously described [41]. Each product was amplified by PCR with the Pfu Taq polymerase and using the appropriate primers, digested by the appropriate restriction enzymes, and cloned in-frame with a Histidine-tag into the pET28a expression vectors (Novagen, Madison, WI, USA) into *Eco*RI and *Xho*I restriction sites. The pHis_TdCAT1 and pHis_$TdCAT_{200}$ constructs that resulted were introduced into the BL21 *E. coli* strain (DE3) (Novagen). A single colony from each construction was grown overnight at 37 °C in LB medium with 100 g/mL Kanamycine and shaking at 220 rpm. After that, the culture was diluted 1:100 into fresh LB-Kanamycine medium and grown to an OD of 0.6 at 600 nm. After that, protein expression was prompted overnight at 37 °C with 1 mM isopropyl β-D-thiogalactopyranoside (IPTG). Bacterial cells were collected by

centrifugation at 4500 rpm for 10 min at 4 °C, followed by two cold water washes. The cells were then sonicated on ice in cold lysis buffer (Tris-HCl 100 mM pH 8; EDTA 1 mM; NaCl 120 mM; 1 mM DTT, 50 mM PMSF, and 0.5% Tween). The cells were then centrifuged at 4 °C for 45 min at 9000 rpm. pHis_TdCAT1 was recovered from inclusion bodies, whereas the deleted form was purified from the supernatant. GST_TMPK3 (accession no. AAC28850) was, all the same, expressed and purified as previously reported [31,39,40,43]. Recombinant proteins were extracted and purified by affinity chromatography on Glutathione Sepharose 4B beads (Amersham Biosciences) as previously described [44]. The Bradford method [45] was used to quantify proteins, however, SDS-PAGE electrophoresis was used to check the correct size of the recombinant proteins.

2.3. In vitro CAT activity assay

The CAT activity assays were achieved as previously described [40], using 160 μg of recombinant proteins mixed with 50 mM H_2O_2 in a phosphate buffer (75 mM) at pH 7.0 in the presence or absence of TMPK3 proteins. The enzyme substrate and buffer were incubated at 25 °C for 1 min before performing the reaction. After incubation, the reaction was stopped by adding 0.2 mL of 1 M HCl and CAT activity was measured spectrophotometrically at 240 nm using a specific absorption coefficient at 0.0392 cm^2 μmol^{-1} H_2O_2 in a final volume of 1 mL of substrate solution. One unit of catalase activity is defined as the quantity of enzyme catalyzing the decomposition of 1 μM H_2O_2 per minute. The determination of CAT activity was performed 4 times in each condition, and the mean values were used as the final results. Statistical analyses of the variations of the values of CAT activity were performed using the SPSS 16.0 statistical software program for Windows (SPSS, Inc., Chicago, IL).

2.4. Phosphatase treatments

To engender a dephosphorylated His-TdCAT1 (dpHis-TdCAT1) protein, 500 μg of the purified protein was treated with 1200 Units of λ−phosphatase (New England Biolabs) at 30 °C for 30 min in a buffer containing 50 mM HEPES, 10 mM NaCl, 2 mM DTT, and in presence of 1 mM Mn^{2+}. The reaction was stopped after 15 min of heating at 65 °C in the presence of 3 mM Na2EDTA.

2.5. GST-pull down assay

Before binding, Glutathione Sepharose 4B beads were washed with the appropriate Tris-HCl buffer (Tris-HCl 20 mM, NaCl 150 mM, pH 7.4, DTT 0.5 mM, EDTA 1 mM, 0.5% Triton, PMSF 1 mM), then the beads were equilibrated with the same buffer. The beads were then incubated with 12 μg of GST_TMPK3 or GST alone for 3 h at 4 °C before being washed three times to remove the unfixed proteins. The immobilized proteins were then incubated with 20 μg of recombinant His_TdCAT1 or His-$TdCAT_{200}$ proteins overnight at 4 °C. Proteins were dissociated from the beads after extensive washing in Tris-HCl 50 mM, glycerol 10%, pH 6.8, SDS 2%, bromophenol blue 0.1%, DTT 1 mM, and then separated by SDS-PAGE (10%). His_TdCAT1 and His-$TdCAT_{200}$ were lastly perceived by Western blot using the anti-Histidine antibody as pronounced by the manufacturer.

2.6. Statistical analysis

Data are reported as means ± S.E. The results were analyzed and compared by using a Student's t-test with differences considered significant at $P < 0.01$.

3. Results

3.1. TdCAT1 presents different MAPK phosphorylation targets

Recently, it has been shown that the TdCAT1 protein presented 4 Nitrosylation sites and 41 potential phosphorylation residues

Table 1
Different threshold of putative phosphorylation sites in TdCAT1 structure.

Threshold	Number of sites	Position	Residue	Sequence
High	3	9	S	PYKYRP**S**SSFNAPMW
		70	S	VVHARGA**S**AKGFFEV
		484	S	SLGQKLA**S**RLSSKPS
Medium	3	10	S	PYKYRPS**S**SFNAPMW
		351	T	RIFSYSE**T**QRHRLGA
		488	S	GQKLASRLS**S**KPSM
Low	6	11	S	YKYRPSSS**S**FNAPMWS
		177	S	WRILDLF**S**HHPESLH
		209	T	MDGSGVN**T**YTLVNRA
		246	T	EAVTVGG**T**NHSHATK
		437	S	QPGERYR**S**MDPARQE
		491	S	SRL**S**SKP**S**M

[41]. Interestingly, TdCAT1 presents 12 residues that could be targets of MAPK phosphorylation as revealed using Group-based Phosphorylation System (GPS: http://gps.biocuckoo.cn/) site. The results are shown in Table 1.

3.2. TdCAT1 interacts with TMPK3 via a conserved KID

The current study shows that the existence of a docking site in the sequence of interacting proteins is necessary for MAPKs binding to facilitate the phosphorylation of their targets. Those sites are called Kinase Interacting Domain (KID) or D-motifs, [(R/K)(1–2)-(X)(2–6)-Φ-x-Φ; where Φ denotes a hydrophobic residue]. KIDs are ubiquitous in different proteins isolated from yeast and mammals [46], and found in activator proteins (MAPKKs), negative regulators (protein phosphatases), as well as scaffolding proteins [47,48].

Sequence alignment analyses show that TdCAT1 contains a KID at its N-terminal part (232–242 aa), a conserved sequence of 11 aa (KSLLEEEAVTV) that can serve as a potential KID or D motif (Fig. 1). This domain is well conserved among other catalases identified in monocotyledons and dicotyledons (Fig. 2).

As subsequent step, we performed a pull-down assay to confirm the localization of this motif. For this purpose, we generated a recombinant, C-terminal-truncated form that lacks the whole KID ($TdCAT_{200}$) [40,41]. This truncated form with the full length was then used in GST pull down assays using GST_TMPK3 as bait, and then analyzed by western blotting using anti-histidine tag antibody. As reported by Fig. 3, His_TdCAT1 was not able to interact with beads (lane 1) nor with GST alone (lane 2). Interestingly, the deleted form His_TdCAT_{200} was not able to interact with beads (lane 3) nor with the recombinant GST_TMPK3 (lane 4) whereas the full length His_TdCAT1 was able to interact with GST_TMPK3 (lane 5) indicating that TdCAT1_TMPK3 interaction is specific and requires the whole binding domain located at the position 232–242. These results indicate that the D-motif might be required for the TdCAT1_TMPK3 interaction.

3.3. TdCAT1 activation by TMPK3 occurs independently of its phosphorylation status

Protein phosphorylation is an important post translational modification that enhances the catalytic activity of various proteins such as TMPK1 [31,49]. Moreover, we have previously shown that TdCAT1 phosphorylation is important for its activity, as TdCAT1 lost about 34% of its native activity after phosphatase treatment [41]. In a first step, we investigated whether the TdCAT1-TMPK3 interaction influences the catalase activity of TdCAT1. Thus, we carried out series of catalase activity assays in the presence of GST-TMPK3 recombinant proteins using 50 mM H_2O_2 as a substrate. Those reactions were performed in a phosphate buffer (75 mM) at pH 7.0 at 25 °C, as previously described [40].

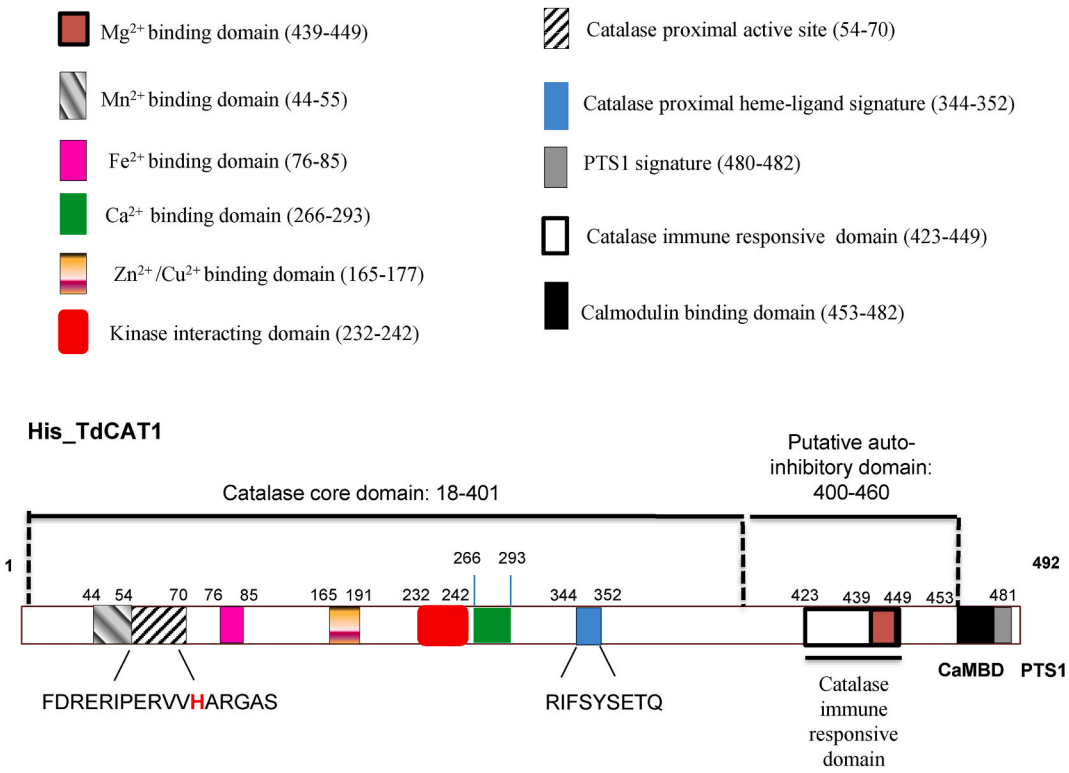

Fig. 1. A schematic representation of TdCAT1 structure showing the conserved amino acids and their positions, each color represents a distinct domain.

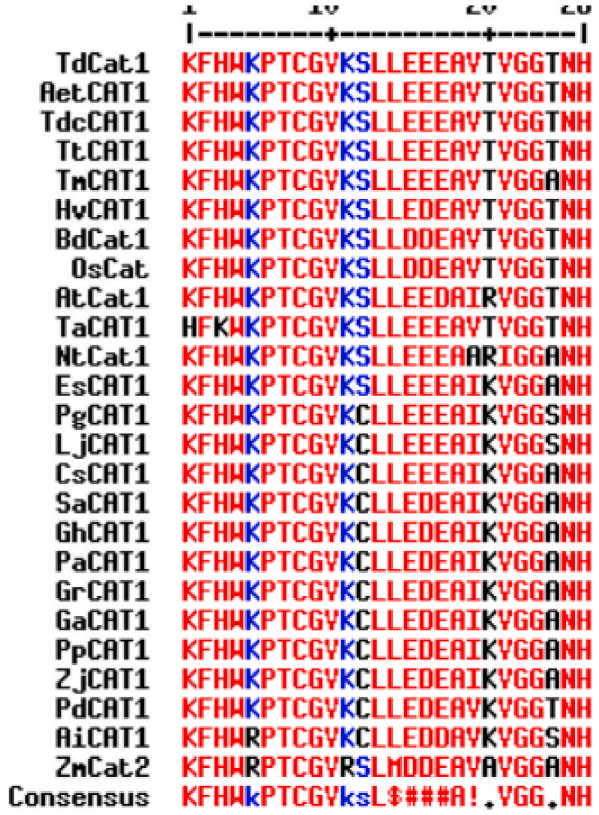

Fig. 2. Alignement of the 20 aa region containing the conserved KID interacting domain from catalase proteins in plants. This domain is well conserved among plant catalase such as (*Triticum turgidum* susp durm TdCAT1(AKC00864.1); *Triticum aestivum* TaCAT1 (NP_001392633.1); *Aoegilops tauchii* AetCAT1 (XP_020164896.1); *Triticum turgidum* TtCAT1 (OP434464); *Triticum monococcum* TmCAT1 (QBZ38484.1); *Hordeum vulgare* subsp *vulgare* (XP_044983038.1); *Brachypodium dictyoson* (XP_003558892.1); *Triticum diccocoid* TdcCAT1 (XP_037426584.1); *Arabidopsis thaliana* (OAO97606.1); *Panax ginseng* (ABY21704); *Soldanella alpine* (GenBank: CAB16749.1); *Arachis ipaensis* (XP_016167161.1); *Gossypium hirsutum* (P17598); *Lotus japonicus* (AAR84578.1); *Eleutherococcus senticosus* (AHA50082.1); *Gossypium raimondii* (XP_012464632.1).

As shown in Fig. 4a, catalase activity increases in the presence of TMPK3 in TMPK3 dose dependent manner compared with the catalase activity of TdCAT1 alone (first raw comparing with raws 4–7). This effect depends on the 3D structure of TMPK3 since using a heat trated TMPK3 in the reaction medium had no effect on that catalase activity of TdCAT1 (third raw). So we investigated whether the stimulatory effects of TMPK3 on TdCAT1 activity depends on TdCAT1 phosphorylation status, we performed catalase assays on either phosphorylated (His-TdCAT1) or dephosphorylated (dpHis-TdCAT1) recombinant proteins in the absence or presence of purified GST-TMPK3.

Interstingly, the presence of GST-TMPK3 enhances the activity of catalase for both phosphorylated and dephosphorylated proteins, but with an important activation for the phosphorylated TdCAT1 form (Fig. 4b). In fact, this stimulation was 20% higher for His-TdCAT1 compared to dpHis-TdCAT1. Such an effect was previously shown for other proteins as their incubation with TMPK3 increased their activities [31]. This effect depends on the 3D structure of the protein since the addition of heat denaturated TMPK3 protein had no effect on the stimulatory effect on the catalase activity of TdCAT1 (Fig. 4b).

3.4. TdCAT1 activation by TMPK3 depends on the putative kinase interacting domain and is promoted by Mn^{2+}, Fe^{2+} and Ca^{2+} divalent cations

In the absence of TMPK3 proteins in the medium, the catalase activities of both full length and deleted forms were measured. $TdCAT_{200}$ (containing the first 200 aa) had a low basal catalytic activity (4 μmol/min/mg of protein) as previously shown [40], while the non-truncated protein TdCAT1 presented a better catalytic activity (96.27 μmol/min/mg of protein) (Fig. 5a). Interstingly, addition of TMPK3 in the medium (with a molar ratio of 1:2) enhanced the activity of TdCAT1 but not the deleted form $TdCAT_{200}$ which lacks the putative KID. Those findings confirm that the stimulatory effect of TMPK3 on TdCAT1 catalase activity could be exerted through its binding to the conserved Kinase interacting domain (Fig. 5a). Moreover, some divalent cations like Mn^{2+}, Fe^{2+} and Ca^{2+} were reported to stimulate the catalase activity of TdCAT1 in vitro [40]. Therefore, we investigated the effect of TMPK3 on TdCAT1 activity in the presence of different metallic cations at a concentration of 2 mM. As shown in Fig. 5b, the catalytic activity of TdCAT1 was more pronounced in presence of TMPK3/Mn^{2+} cations (22% fold induction) compared with the presence of TMPK3 alone.

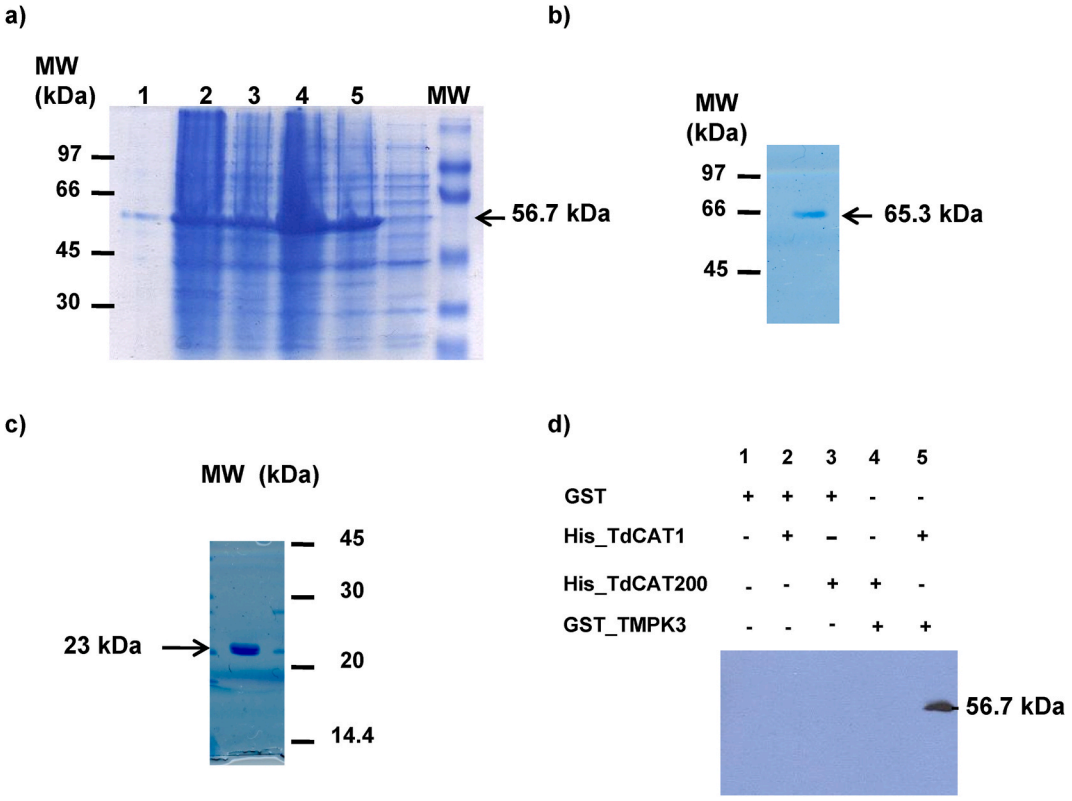

Fig. 3. TdCAT1 interacts with TMPK3 proteins in GST-Pull down assays. **(a)** Production and purification of His_TdCAT1 proteins, 1: Purification of the protein, 2: Production after 4 h incubation; 3: Production after 6 h incubation, 4: Production after 8 h incubation, 5: Production after 10 h incubation. **(b)** Purification of GST_TMK3 proteins. **(c)** Purification of TdCAT200. **(d)** GST-Pull down revealing a specific interaction between TMPK3 and TdCAT1 proteins via a conserved KID (232–242).

Interestingly, addition of TMPK3 in presence of $TdCAT_{200}$ did not affect the catalytic activity of the deleted form mainly in the presence of Mn^{2+} confirming that the effect of TMPK3 is mediated by the KID (Fig. 5b). The same effect was also observed in the presence of Fe^{2+} cations (data not shown).

In the presence of Ca^{2+} cations, the catalytic activity of TdCAT1 was enhanced but not that of the deleted form $TdCAT_{200}$ as previously shown [40]. The addition of TMPK3 to the medium enhances the activation of TdCAT1 activity but not $TdCAT_{200}$ confirming that the stimulatory effect of TMPK3 on TdCAT1 activity depends on the response of the KID (Fig. 5c). In the presence of Ca^{2+} cation, the stimulation of TdCAT1 activity seemed to be specific to Ca^{2+} since the addition of EGTA, a well known Ca^{2+} chelator, restored the activity of TdCAT1 in the presence of TMPK3. Moreover, the addition of Mg^{2+} cations stimulates also the catalase activity of TdCAT1 (Supplemental Figure 1) Cu^{2+}/Zn^{2+} cations had no effect on the catalase activity of either protein (data not shown). On the other hand, we have previously shown that the catalytic activity of the dephosphorylated catalase (dpTdCAT1) was not modified after incubation with cations [41]. The presence of divalent cations in a medium containing TMPK3 proteins did not modulate the catalytic activity of TdCAT1 after phosphatase treatment (Fig. 6). As a result, we can conclude that catalase phosphorylation is critical for cation stimulation of its activity.

3.5. Catalase activity of TdCAT1 is differentially regulated by TMPK3 and TdCaM1.3/Ca^{2+} complex

We have previously shown that TdCAT1 presents a calmodulin binding domain containing basic and hydrophobic amino acids at the C-Terminal portion of the protein (459–482 aa). TdCAT1 interacts with TdCaM1.3 in a calcium independent manner but stimulates its activity in a calcium dependent manner. This activity is further enhanced in the presence of Mn^{2+} cations [40]. Here, we investigated the effect of combining CaM/Ca^{2+} complexes and TMPK3 on the catalase activities of TdCAT1. Our results showed that TdCAT1 catalytic activity in the presence of a TdCAT1/TMPK3 molar ratio of 1:2, and increasing amounts of TdCaM1.3 proteins and 2 mM Ca^{2+} (with molar TdCAT1/TdCaM1.1 ratios ranging from 1:2 to 1:8), was stimulated (Fig. 7a). This effect seems to be Ca^{2+} dependent as addition of 5 mM EGTA restores the activity of TdCAT1 (Fig. 7a). Moreover, this stimulation was more pronounced with increasing Ca^{2+} concentrations (from 0.1 to 2 mM) in the reaction buffer (Fig. 7b). Besides, the addition of Mn^{2+} to the mixture, including TMKP3/TdCaM1.1/Ca^{2+}, resulted in the stimulation of TdCAT1 phosphatase activity. In fact, the V0 of the reaction raised gradually in the presence of increasing quantities of Mn^{2+} (Fig. 7c) reinforcing the relevance of Mn^{2+} ions in TdCAT1 activity

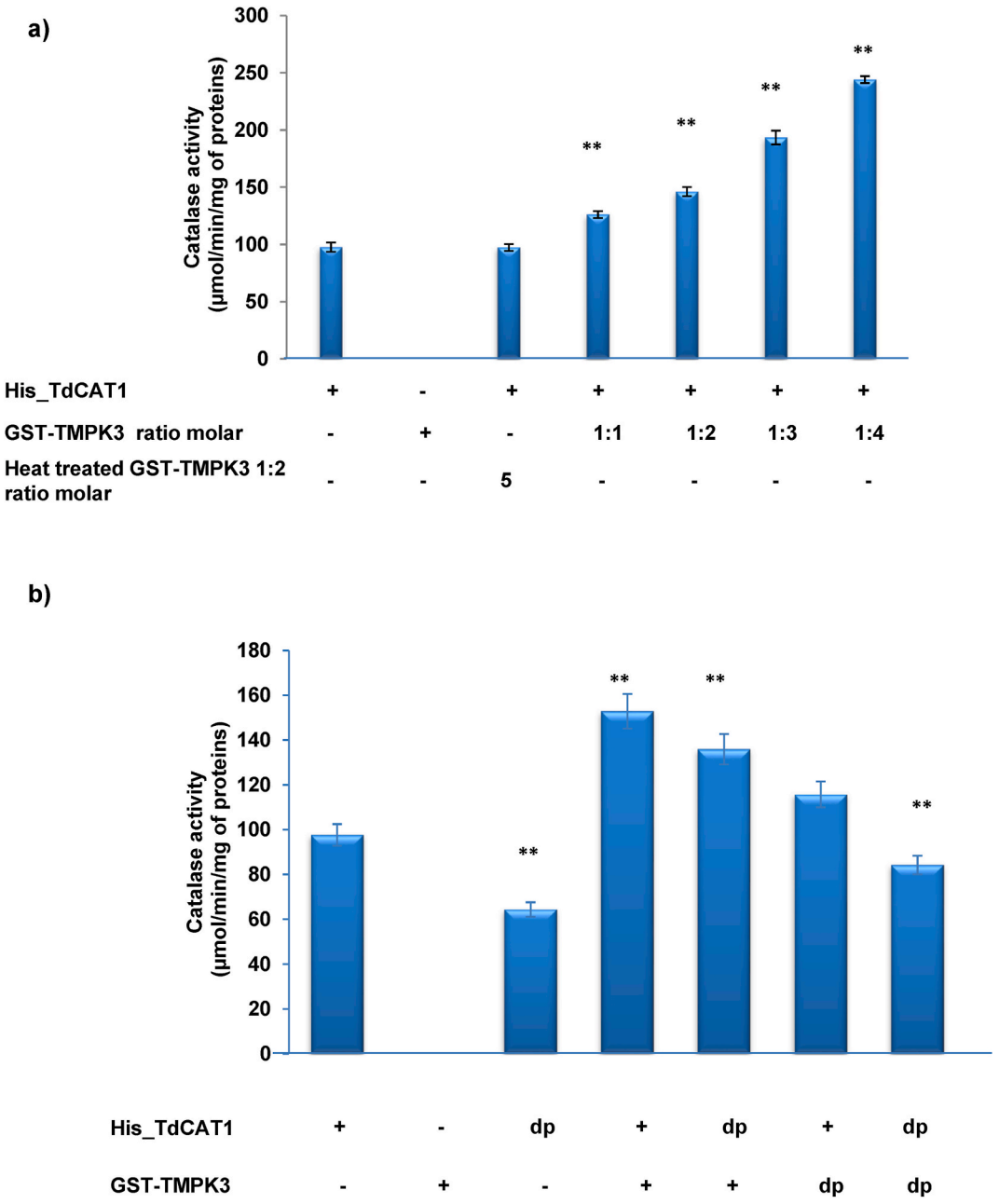

Fig. 4. Effect of TMPK3 on the Catalase activity of TdCAT1 in the presence of a fixed amount of TdCAT1 (160 µg) (a) catalase activity was performed in presence of increasing quantities of GST-TMPK3 protein ranging from 1:1 to 1:4 TdCAT1/TMPK3 ratio molar (b) Effect of proteins phosphorylation on the catalytic activity of TdCAT1, Catalase activity was performed in presence of phosphorylated or dephosphorylated TMPK3 (1:2 ratio molar) and TdCAT1; dp = dephosphorylated, (**) indicates value significantly different from the control. Statistical significance was assessed by applying the student t-test at $p < 0.01$.

stimulation. This induction started with 0.5 mM Mn^{2+} and increased gradually in the presence of increasing quantities of Mn^{2+}.

4. Discussion

Several studies describing how the binding of plant Catalases to different ligands [Salt Overly Sensitive 2 (SOS_2; [50]), CaM/Ca^{2+} complex [40,51] nucleoside diphosphate kinase 1 (NDK1; [52]), the phytohormone class called natriuretic (AtPNP-A; [53]) resulted in a pronounced modulation of their activities, were reported. Moreover, AtCAT1 catalase activity is regulated at the post-translational level [22]. Recently, some questions about the posttranslational modification of catalase (e.g. S-nitrosylation, phosphorylation,

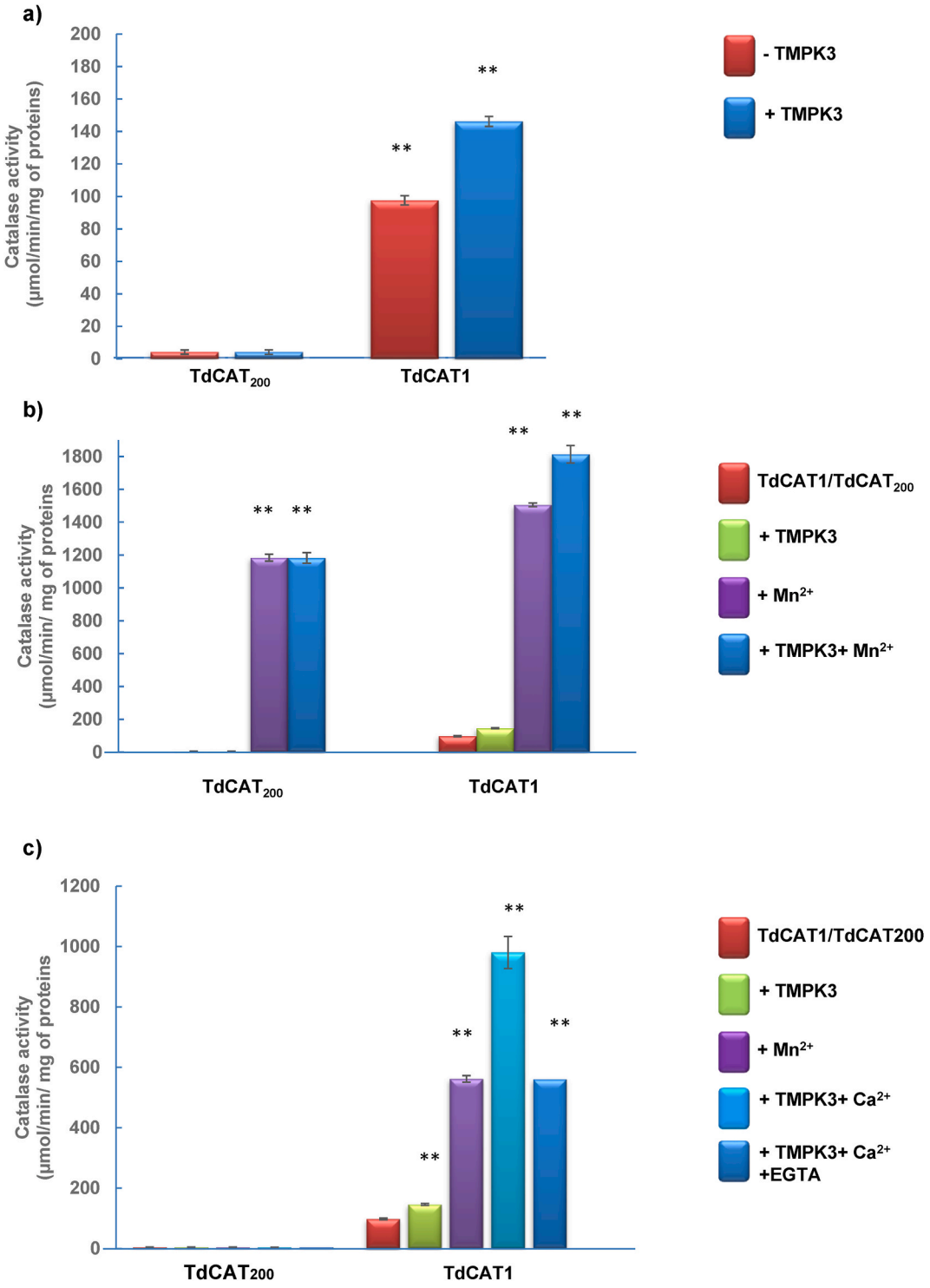

Fig. 5. Modulation of the catalase activity of the deleted form $TdCAT_{200}$ (160 µg) in presence of (a) TMPK3 (1:3 ratio molar) alone or in presence of (b) TMPK3/Mn^{2+} (2 mM) or (c) TMPK3/Ca^{2+} (2 mM). (**) indicates value significantly different from the control. Statistical significance was assessed by applying the student t-test at $p < 0.01$.

nitration, acylation, glycation, oxidation/carbonylation) and how this post-translational modification can contribute to catalase signaling specificity and physiological responses started to emerge [54]. Catalase phosphorylation acts neither by lowering nor by boosting the catalytic activity of the enzyme. Those modifications also depends on different factors such as species, growth satge,

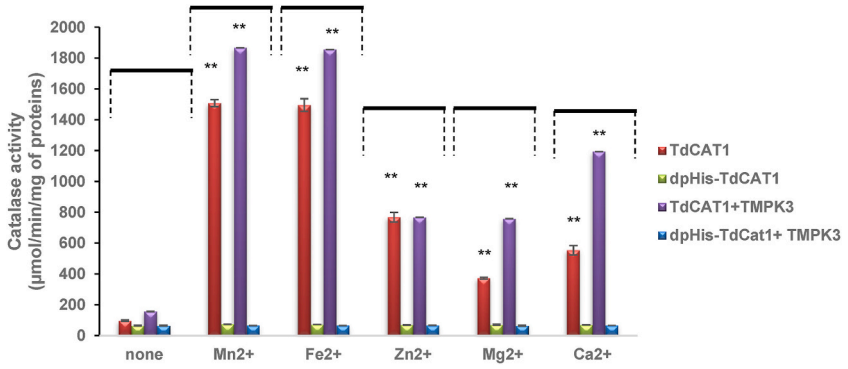

Fig. 6. Effect of TMPK3 on the catalytic activity of dephosphorylated TdCAT1 (160 µg; TdCAT1:TMPK3 ratio molar 1:3) in presence or absence of different divalent cations. (**) indicates value significantly different from the control. Statistical significance was assessed by applying the student t-test at $p < 0.01$.

developmental stage, and stress agents [25,55]. Phosphorylation is a well-studied mechanism in eukaryotes that is involved in many regulatory and complex processes. Catalase phosphorylation has been linked to regulatory situations in both animals and humans [17, 56]. Catalase regulation by phosphorylation has been reported in plants, for example, in *A. thaliana* under salt stress, (via SnRK2.4; [24]), lights (via BAK1 (brassinosteroid-insensitive 1 (BRI1); [23]) and drought stress conditions (via CDPK8; [22]). Moreover, catalase phosphorylation confers plant resistances to abiotic stress and blast disease in rice via OsCPK10 [57]. MAPKs signaling pathways are involved in regulating ROS levels by controlling different components such as RbohD, RbohF, PRX33, and PRX34 expression [58]. Moreover, MPK3 and MPK6 were shown to be able to phosphorylate the ERF6 transcription factor implicated in plants response to biotic stress [59–61]. Interestingly, the regulation of catalase activity by MAPKs has never been described so far. Thus, we investigated whether TdCAT1 could be a potentiel target of the isolated MAPK3 from wheat. Here, we confirm a possible phosphorylation of TdCAT1 by MAPK using bio-informatic analysis and in vitro catalase activity tests. Bioinformatic analysis show the existence of 41 putative phosphorylation sites in TdCAT1 sequence as previously shown [40]. Here we identified 12 putative phosphorylation sites that could be phosphorylated by MAPKs (Table 1). Among those putative sites, 2 residues have a proline amino acid at position −1; and are able to be phosphorylated by MPK3 as revealed by the scansite3 server (http://scansite3.mit.edu). We showed here that wheat TMPK3 activates the catalase activity of TdCAT1 in a TMPK3 dose dependent-manner (Fig. 4a). As far as we know, this is the first report to describe catalase's interaction with MAPK in vitro. In our assays, we did not have experimental evidence for TdCAT1 activation by TMPK3 via its phoshorylation. However, we have noticed that this activation occurs independent of its phosphorylation status. To verify our hypothesis, we performed some assays to check whether the binding of TMPK3 to TdCAT1 is crucial for its activation. Interestingly, this study shows that the existence of TMPK3 in the medium stimulates the TdCAT1 catalase activity independent of its phosphorylation statutus (Fig. 4b). The stimulation effect of wheat MPK3 observed on wheat catalase could be due to the TMPK3/TdCAT1 binding, which may result in a conformational change conferring higher catalytic activity.

The conserved Kinase Iinteracting Domain (KID or D-motif) at the C-terminal part of the TdCAT1 motif (232–232; Fig. 1) is the most likely binding site for TMPK3 in the TdCAT1 sequence. Indeed, GST pull down assays on the truncated form of $TdCAT_{200}$ revealed that the D-motif is required for TdCAT1/TMPK3 interaction, as previously reported for other proteins such as protein phosphatases identified in Arabidopsis (AtMKP1; [62]) and in durum wheat (TMPK1; [30]). Consequently, the deletion of the KID results in the abolition of the stimulatory effect of TMPK3 on TdCAT1 activity. Therefore, we think that the wheat kinase TMPK3 activates TdCAT1 through a direct interaction with the KID. The effect of TMPK3 was also seen in the existence of divalent cations (Ca^{2+}, Mn^{2+}, Mg^{2+}, Cu^{2+}, Zn^{2+} and Fe^{2+}) and/or the CaM/Ca^{2+} that were previously shown to enhance the catalase activity of TdCAT1 [39]. We showed here for the first time that the catalase activity of a plant catalase could be enhanced by MAPKs and cations (Fig. 6). Our assays showed that TdCAT1 is stimulated by TMPK3 in the presence of different divalent cations (Ca^{2+}; Mn^{2+}; Mg^{2+} and Fe^{2+}) but not in the presence of Cu^{2+} and Zn^{2+} (Fig. 3). On additional catalase assays, we found that the presence of TMPK3 and the CaM/Ca^{2+} resulted in a stimulation of TdCAT1 activity. Most interestingly, this stimulatory effect was further enhanced by adding the Mn^{2+} cation to this mixture but not the other cations. From our data, it can be concluded that TMPK3 and CaM/Ca^{2+} complex exert a novel regulatory effect on TdCAT1 activity. We can speculate that Mn^{2+} cations might modify the TdCAT1 conformation to promote or facilitate CaM and/or MPKs binding in a way that ultimately leads to the activation of the phosphatase activity. It is worthy to note that Mn^{2+} cations seem to have the most important effect on the catalytic activity of TdCAT1 since they cause the highest activation rate.

5. Conclusions

In **c**onclusion, our data provide experimental evidence of TdCAT1 activation by TMPK3 alone or in association with the CaM/Ca^{2+} complex, which suggests a complex regulatory mechanism exerted on TdCAT1. Most importantly, our results suggest that divalent cations (Mn^{2+}, Mg^{2+}, Ca^{2+} and Fe^{2+}) act as key cofactors in these regulatory mechanisms. As far as we know, this is the first report of a wheat CAT1 gene that describes its regulation by MAPK proteins in vitro. These results suggest that the studied kinase is implicated in modulating catalase activity to regulate ROS homeostasis required for plant responses to unfavorable conditions. Future works are

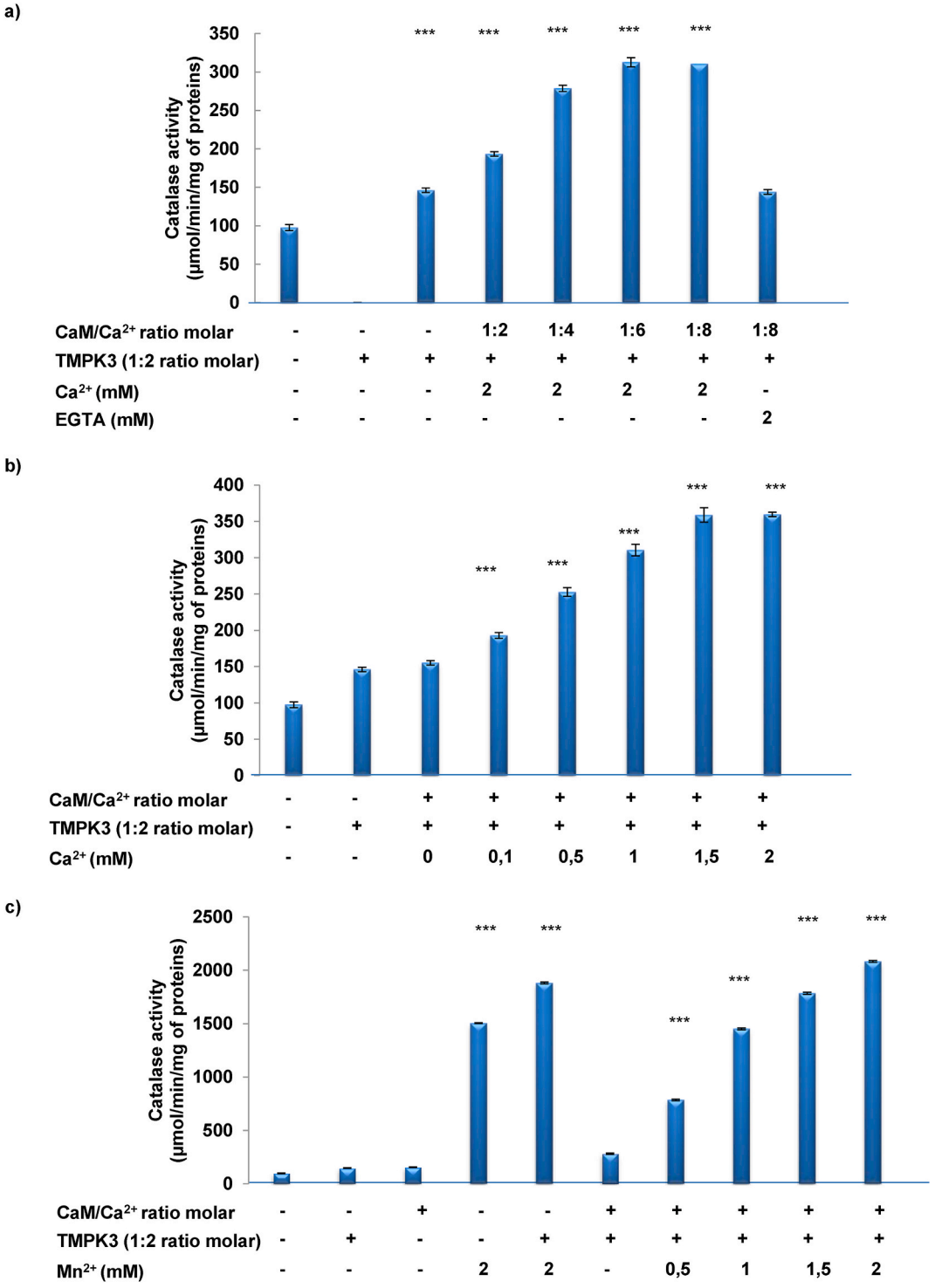

Fig. 7. The stimulatory effect of CaM/Ca^{2+} complex on TdCAT1 activity in presence of TMPK3 proteins, (a) CaM/Ca^{2+} complex stimulates the catalytic activity of TdCAT1 in dose dependant manner in presence of TMPK3 proteins (1:2 ratio molar), (b) stimulatory effect of CaM/Ca^{2+} complex on the catalytic activity of TdCAT1 in presence of TMPK3 proteins (1:2 ratio molar) and increasing quantities of Ca^{2+} (0,1 → 2 mM). (c) Stimulatory effect of Mn^{2+} cations on the catalase activity of TdCAT1 in presence of TMPK3, CaM (1:4 ratio molars, 2 mM Ca^{2+} and increasing quantities of Mn^{2+} (0.5 → 2 mM).

needed to 1) investigate the physiological significance of TdCAT1/TMPK3 interaction, especially during plant stress; 2) check whether such founding is conserved among other catalases isolated from other species and 3) catalytic TdCAT1 activity was also shown to be stimulated by TMPK3 proteins independently of its phosphorylation status. Thus, it would be interesting to investigate the role of catalase phosphorylation in plants by creating transgenic plants that overexpress different forms of catalase that have been mutated in the putative phosphorylation sites. Such research could aid in understanding the role of these stimulatory effects on plant catalases in the regulation of plant responses to abiotic and/or biotic stresses.

Author contribution statement

Mouna Ghorbel: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Najla Haddaji, Ahmed Alghamdi, Khalil Mseddi: Analyzed and interpreted the data; Wrote the paper.

Kaouther Feki, Sana Tounsi: Performed the experiments; Wrote the paper.

Mejda Chihaoui: Contributed analysis tools or data; Wrote the paper.

Faiçal Brini: Conceived and designed the experiments; Analyzed and interpreted the data; Wrote the paper.

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Data availability statement

Data are contained within the article or supplementary materials and can be made available on request.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.heliyon.2023.e18916.

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