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A rice SOUL family heme-binding protein REAC1 enhances the antioxidative capacity of *C. elegans* through modulation of ROS-related gene expression

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The development and identification of beneficial components from crop resources are vital for individuals, especially the elderly, as they are capable of facilitating health. Red rice is widely consumed and possesses potential therapeutic effects to some extent, but it is important to discover the specific roles of each component when consumed purposefully. In this context, the REAC1/rHBP2, a red rice heme-binding protein (HBP) from the SOUL family, was revealed that possesses a role in boosting the antioxidative capacity of *C. elegans* that consume this protein. The Arabidopsis plants overexpressing *REAC1* presented more tolerance to oxidative stress related to the wild-type plants. Furthermore, REAC1 derived from engineered bacteria exhibited clear activities of heme-binding and hydroxy radical inhibition in vitro. While no adverse effects were observed in the nematodes that were treated with REAC1, they exhibited enhanced motility and improved survival under oxidative conditions simulated by treatment with 5 mM H_2O_2 compared to the control group. Additionally, the levels of endogenous reactive oxygen species (ROS) were significantly reduced, and the expression of redox-related genes, such as *SOD-3* and *CAT-1*, was evidently upregulated in the treated nematodes. Taken together, these results suggest that the red rice heme-binding protein REAC1 plays a critical role in the enhancement of the antioxidative capacity of *C. elegans* through ROS-related regulation, thereby offering a promising approach for individuals to combat oxidative stress.

Keywords Heme-binding protein, Antioxidative stress, ROS, Rice, *C. elegans*

It was predicted that the world's population would reach approximately 9.7 billion in next three decades, and the aging population in some countries could grow at an even greater rate, as people longevity-extending due to the economical improvement and scientific advancement[1–4]. Rapid increasing of Aging may result in a high burden in the context of severe climate, environmental changes and chronic diseases[5–7]. Therefore, providing more stable crop production and facilitating the health of the aging population via various pathways or strategies is critical[3,4,8,9].

Aging is characterized by a decline in tissue and organ function over time, while excessive oxidative stress and damage largely contribute to this progression, and the latter can even lead to severe age-related diseases[10–12]. Reactive oxygen species (ROS) are a group of molecules with high activity that are derived from the partial reduction of molecular oxygen in cells. As a specific byproduct of metabolism, the endogenous redox mechanism partially depends on ROS-related regulation[11,13]. Usually excessive levels of these molecules, can severely impact on cellular health, resulting in oxidative stress and subsequent damage.

For example, ROS trigger nucleic acid fragmentation, enzyme deactivation, polysaccharide depolymerization, lipid peroxidation and other destructive biochemical processes, ultimately leading to aging and death[14]. To some extent, persistent or long-term oxidative stresses facilitate the initiation and progression of aging-related diseases, such as Parkinson's disease (PD)[15,16], Alzheimer's disease (AD)[6,12] and malignant tumors[17,18], which are increasingly becoming significant health concerns in aging societies. If there is lack of effective treatment, preventing such diseases is difficult during aging. PD is a neurodegenerative disease characterized by the loss of

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dopamine-producing neurons in the midbrain, which leads to incapacitating symptoms, including bradykinesia and muscular rigidity[6,19,20]. Oxidative stress is a critical factor responsible for the initiation and progression of PD, which is due to patients with PD having high oxidative stress and lower antioxidant activity, as evidenced by the characterization of the biomarker activities of the superoxide dismutase (SOD) and catalase (CTL) enzymes[20]. By contrast, in AD patients, biomarkers of oxidative stress have also been documented. The finding that oxidative stress occurs early in AD supports its significant role in AD pathogenesis[6,21,22]. Therefore, inhibiting excessive oxidation in individuals as they age via effective strategies or treatments is critical for preventing aging-related diseases in the population.

For individuals, the most important basis for maintaining physiological health depends on foods or nutrients containing essential compounds or factors. For instance, the EGCG (Epigallocatechin gallate), a main active ingredient of green tea, takes on valuable capacity to extend *Caenorhabditis elegans* (*C. elegans*) longevity under stress; and the phytochemical compounds in *A. zerumbet* have been demonstrated possesses beneficial effects on the lifespan of *C. elegans*[4,23]. By contrast, the iron and iron-containing prosthetic groups are involved in diverse fundamental processes that constitute or support each kind of life. Hemes are macrocycles that contain iron; specifically, the iron atom forms a complex with the protoporphyrin ring[24,25]. In humans, more than 60% of iron is utilized as the heme moiety of hemoglobin[26–28]. In higher plants, heme biosynthesis mainly occurs in plastids, and both mitochondria and plastids are involved during the last few steps[25]. Once synthesized, the hemes are distributed to different plant organelles, where they can be bound covalently and noncovalently to proteins[25,29]. Heme-binding protein (HBP) plays significant roles in diverse processes, including respiration, photosynthesis, and defense against different stresses[30–32]. However, despite their necessity and critical role, free hemes efficiently induce cytotoxic damage in cells because of their inherent peroxidase reactivity and capacity to produce ROS.

HBP family members, such as the albumin, hemopexin and SOUL families, vary greatly in terms of their regulation in the context of different species[27]. SOULs may represent a novel HBP family with specific sequence conservation and biological properties; in vertebrates, SOULs are involved in circadian rhythm/light perception, programmed cell death, and necrosis induction[33]. Research on SOUL/HBP proteins in *Arabidopsis* revealed that these proteins have organelle-specific activities, such as defense against abiotic stress and plant pathogens[34–36]. Rice is a primary source of sustenance for a large portion of the global population. However, many beneficial components remain to be explored. In this study, we found that a red rice heme-binding protein (HBP) of the SOUL family, REAC1/rHBP2, enhances the antioxidative capacity of *C. elegans*. It modulates the expression of ROS and ROS-related genes in the nematode, suggesting that the protein may be a potential regulator of resistance to oxidative stress in individuals.

Materials and methods

Plants, *C. elegans* and bacterial strains

The wild-type (Wt) *Arabidopsis thaliana* Col-0[37] and related transformants were used for characterizing the role of target gene in plants; the initial gene expression and cloning plant materials were derived from the Dahonggu cultivation variety in Enshi of Hubei province; the *C. elegans* Bristol N2, *ced-9* dominant expression mutant MT4470 and *Escherichia coli* var strain OP50[38] were used in the study to evaluate the activity of the protein. The prokaryotic expression vector pCold I (simply referred as pC) purchased from Takara-Bio (Dalian, China) and overexpression vector pCX-SN were utilized for vector construction. *E.coli* expression strain BL21 (DE3) harboring the related vectors were used in this study.

Vector construction

The 651 bp full-length ORF sequence of *REAC1* was amplified by primer-specific PCR, and the primer sequences include Forward, ATGGGGATGGTGCTGGGCAAGAT and Reverse, TCACTCGACGGGGACCAT. Then the *REAC1* CDS fragment was inserted into the plant transformation vector pCX-SN[39] leading to the gene was driven by the CaMV35S (Cauliflower Mosaic Virus 35 S) promoter. The construct was introduced into *A. tumefaciens EHA105*, which was then used for the Arabidopsis transformation through the floral dip method described in previous reports[37,40]. Homozygous T3 progeny transformants were screened out and used in the further research.

For *REAC1* prokaryotic expression, two restriction sites (BamHI and EcoRI) were designed in the primer sequences (Supp. Table 2) for the recombinant plasmid pC-*REAC1* construction, leading to the gene under the control of *cspA* promoter (*PcspA*). The PCR system was as follows: 1 µL of template DNA, 5 µL of GoTaq G2 Green Master Mix (Promega (Beijing) Biotech Co., Ltd.), 1 µL of the primer mixture, supplemented with ddH_2O to a total volume of 10 µL. The reaction procedure was consisted of an initial denaturation at 95 °C for 5 min, followed by denaturation at 94 °C for 30 s, annealing at 54 °C for 30 s, extension at 72 °C for 45 s, 30 cycles. All the vectors were subjected to sequencing again in Sangon Biotech Co., Ltd (Shanghai, China).

Protein expression

The pC-*REAC1* and empty vector pC were respectively transformed into *E. coli* BL21 (DE3) cells and screened on the 100 µg/mL Luria-Bertani (LB)-Ampicillin (Amp) medium. The initial liquid culture from single colony was inoculated at a 1:100 dilution into fresh LB-Amp medium and grown for about 4 h at 37 °C to reach an optical cell density (OD) of approximately 0.6 at A600. After 1.0 mM IPTG (isopropylthio-β-D-galactoside) was added, the samples were induced at 16 °C for up to 4 h. Cells were collected by centrifugation at 5000 rpm for 10 min and washed twice with washed buffer (20 mM NaCl, 50 mM Tris-HCl, pH 7.3). The bacterial precipitates were resuspended in 5 mL of the homogenization buffer (3–4 mL/g wet cells) were homogenized by ultrasonication. After centrifugation (12000 rpm, 15 min), the supernatant was used as a crude extract for further isolation by His-tag affinity Ni NAT agarose resin (Takara-Bio, Kyoto, Japan)[41]. Protein samples were subjected

to SDS-PAGE, demineralization and stored with 50% glycerol at-80 °C or used for further assays. Before further assays, the proteins were assessed the concentration by Bradford method[42].

In vitro heme-binding activity

Affinity-elution assays were conducted[32,36] to analyze the heme-binding characteristics of the target protein. The process involved multiple washing and elution steps to confirm the accuracy of the results. Chloroprotoporphyrin IX iron (III) (hemin chloride, HeCD) was utilized as the substrate for binding with the REAC1 protein. Following the affinity of the Ni-NAT resin to the target protein REAC1, which was tagged with a 6×His fusion, excess resin was removed. Subsequently, 200 µL of a 1% HeCD solution was added to the samples for additional binding. After 10-minute incubation on ice, the samples were washed three times with 800 µL of washing buffer (Wh) to thoroughly eliminate any non-specific affinity or excess HeCD in the system. The washed samples were then treated with elution buffer to extract the specific affinity HeCD into new tubes. Each washed or eluted solution was collected in separate tubes to observe the actual impact of the washing or elution process. For spectral analysis using ultraviolet-visible spectrophotometer, a 1% HeCD heme solution was used either without the target protein (Free sample) or with the target protein REAC1 (Binding sample).

Hydroxyl radical scavenging activity

To evaluate the reduction activity of REAC1, a range of protein sample and vitamin C solutions with mass concentrations from 0.05 to 1.00 mg/mL in ddH_2O were prepared. Next, 200 µL of sample solutions at varying mass concentrations were combined with 0.2 mL of a 6 mM of ferrous sulfate solution and a salicylate-ethanol solution within the reaction system. This mixture was then supplemented with 0.2 mL 6 mM of hydrogen peroxide solution, and the absorbance of the mixtures was measured at a wavelength of 510 nm using UV-VIS (ultraviolet-visible) spectrophotometer. The hydroxyl radical scavenging rate or inhibition level was calculated based on the formula: (1-(A1-A2)/A0)*100%, where A0 and A1 represent the absorbance of the blank control and the sample, respectively; while A2 is referred to the absorbance value of the sample without the salicylate-ethanol solution[43].

C. elegans culture and treatment

The performance of *C. elegans* was referred previous report[38,44,45]. *C. elegans* N2 were maintained at 20 °C on nematode growth medium (NGM) agar plates seeded with E.coli var strain OP50. To achieve synchronous nematodes, gravid nematode adults were treated using a bleaching solution (NaOH (5 M), NaClO (13%); 1:1) followed by three washing steps in liquid NGM. The remaining eggs were allowed to hatch either on fresh NGM agar plates seeded with OP50 for three days (synchronous L4 larvae) or in 1.5 ml S-medium for 12 h (synchronous L1 larvae). For the subsequent treatments, the larvae were fed on the suspensions containing heat inactivated bacterial mixture, such as OP50/BL21-H or OP50/BL21-C, and each suspension supplemented FUDR (50 µM) to prevent nematode spawning. In the suspensions OP50 vs. BL21- cells were controlled such as a ratio of 1:1 or 1:3, but the whole concentration of the bacteria was referred to 1.0×10^9 cfu/mL. After a three-day treatment period, the nematodes matured into adults for further assays.

Testing the body size of nematodes

To investigate the impact of the protein treatment on nematode growth, the body size and stage-specific morphological characteristics were tested[38]. At the specified time intervals, nematodes from each group were transferred in 10 µL of medium onto a microscope slide and combined with 10 µL of levamisole (20 mM). Photographs of individual nematodes were taken and analysed to measure their length using the ImageJ (NIH, Bethesda, MD, USA). For each treatment, ≥ 15 worms were used and the experiment was conducted three times at least.

H_2O_2 and heat tolerance assay

For the H_2O_2 tolerance assay, nematodes from each group were respectively collected using M9 buffer and then underwent further testing. The nematodes were exposed to 5 mM H_2O_2 in a 24-well plate, and their survival was monitored at 20 min intervals using a dissecting microscopy. If treated nematodes did not respond to poking with a metal wire, they were judged to be dead. Consequently, the survival rate was determined by calculating the ratio of surviving nematode in the population. For heat stress assay, the previous report[4]was referred with a little modification. The synchronous nematodes were gathered and cultured for 72 h before being placed in a 35 °C incubator for 12 h. This time was noted as the starting point, and the count of nematodes was documented every 2 h until all of them in some group perished. For the assays, each treatment ($n \geq 20$) was carried out in triplicate.

Motility

To assess motility, L4 stage *C. elegans* for each treatment were cultured in S liquid medium respectively supplemented various bacterial suspension mixture under 20 °C shock conditions for three days. Afterward, the nematodes were collected in a dish with M9 solution, and their body bends were counted every 10-minute for three consecutive observations under light microscopy. Each group of nematodes was replicated at least three times ($n \geq 20$).

Endogenous ROS level

A method for quantifying relative fluorescence was referred[45] to measure the endogenous level of reactive oxygen species (ROS) in nematodes. The nematodes from various groups were fed on different bacterial lawns for 24 h and then collected with M9 buffer. They were washed three times to remove, bacteria and then

were resuspended in M9 buffer. A 50 µL aliquot of the suspension was mixed with H2DCF-DA (50 µL) in a 48-well plate, leading to a final concentration of 0.1 mM for the ROS-specific fluorescent probe H2DCF-DA (2', 7'-dichlorodihydrofluorescein diacetate). Two control wells, such as that containing nematodes from each bacterial mixture lawn without H2DCF-DA or containing H2DCF-DA without nematodes were set up, respectively. The samples were incubated at 25 °C for 1 h, after which the nematodes were then transferred to slides, mounted with glycerol, and examined using a fluorescence microscope. ROS signal was then captured imaged and quantified using the fluorescent microscope (Eclipse Ni, Nikon; Tokyo, Japan; ImageJ, NIH; Bethesda, USA). The fluorescence levels were calculated by subtracting the initial readings from the final readings for each well. Three independent experiments were conducted and for each replicate, ≥ 15 nematodes were analyzed.

Total RNA extraction and qRT-PCR

Total RNA from nematodes that were fed different bacterial mixtures were washed and collected in M9 buffer, followed by isolation using Trizol reagent (Invitrogen). The RNA underwent RT-PCR using the PrimeScript™ FAST RT reagent Kit with gDNA Eraser (TaKaRa, Dalian China) according to the manufacturer's instructions. For the first strand cDNA synthesis, a mixture containing each essential reagent such as Random 6-mers, dNTPs and RNA was prepared. The cDNA was evaluated subsequently directly by analyzed *Actin* expression using agarose gel electrophoresis. Then quantitative RT-PCR (qRT-PCR) using the TB Green Premix Ex Taq (Tli RNaseH Plus) (2× Conc.) kit (TaKaRa-bio, Dalian, China) was carried out on the StepOnePlus real-time PCR system (Applied Biosystems)[37]. Reactions were initiated at 95 °C for 3 min, followed by 40 cycles of 95 °C for 15 s, 58 °C for 15 s and 72 °C for 30 s, followed by melt curve analysis. Relative expression levels were calculated using the $2 - \Delta\Delta CT$ method, with *Actin* serveed as the internal reference for normalizing gene expression data. The gene-specific primers were shown in Supp. Table 3.

Nucleotide or gene sequence

The referred gene can be accessed by using the species name and accession number in Genbank. The genes of *C. elegans* described in this study can be retrieved on line from the NCBI https://www.ncbi.nlm.nih.gov/ or https://wormbase.org/.

Data statistics and analysis

Statistical analyses were based on the data given as mean ± SD using PASW Statistics (SPSSInc.; Chicago, USA). Statistical significance was determined by one-way ANOVA. Differences were considered to be significant at a level of $P < 0.05$ ("*") or $P < 0.01$("**").

Results

REAC1 is involved in the regulation of oxidative stress in plants

Gene expression is coordinated with cell growth and development and external factors, such as light, heat and other environmental stresses[7,46,47]. To explore genetic regulation in response to photoperiod in red rice, a gene that predictably encodes a heme-binding protein, was identified and referred to here as *REAC1* (or *rHBP2*). The REAC1/rHBP2 consists of 216 amino acid (aa) residues, shares approximately 58% identity with the AtHBP2 (AT2G37970, SOUL2)[33], whereas all residues of the REAC1 are identical to the *Oryza glaberrima* homolog XP_052150752.1, a protein with unknown function up to today (Supp. Fig. 1). Under a regular photoperiod (Light) or completely dark (Dark) conditions, except for that the chlorophyll levels of the rice seedlings sharply differed, there appeared little other difference in the seedlings during our testing periods (Supp. Fig. 2A). However, overall, *REAC1* transcripts began to accumulate greatly on the fourth day of rice seedling growth under both dark and light conditions (Fig. 1A and initial details shown in the Supp. Fig. 2B). To further explore the role, the *Arabidopsis thaliana* was stably transformed with a vector[39] encoding *REAC1* driven by the CAMV35S promoter, and two independent lines of the transformants, OeH-#1 and -#3 with relatively high expression levels of *REAC1* were screened and used for further research (Supp. Fig. 3A). Given in the plant size, there were few differences between the two transformants and wild-type (Wt) plants grown on MS agar media (Supp. Fig. 3B). However, the transformants presented increased tolerance to H_2O_2 stress. As shown in Fig. 1B, when 6-day-old seedlings of similar size were subjected to a 10 mM H_2O_2, few differences were detected between the Wt and transformants in the initial three days. However, on days five and seven, the Wt plants were more sensitive to stress, as shown by the greater number of wilted or stressed leaves (Fig. 1C) and lower chlorophyll level (Fig. 1D) relative to the other two lines. These findings indicate that *REAC1* improves oxidative stress tolerance in Arabidopsis.

REAC1 protein activity in vitro

Structure or configuration provides insights into the function on a molecular level[48]. Molecular modeling indicated that there are secondary motifs including at least five helices, ten strands and fourteen β-turns in REAC1 (Supp. Fig. 4A), which constitute the complicated structure, and the ten strands likely form a relatively closed barrel in REAC1 (Fig. 2A). For HBPs, the residue His, Cys, and Tyr are potential binding sites for hemes[32]. In REAC1, there are one Cys, two His, and seven Tyr residues, respectively, and relatively, the sites of Tyr (Y) residues took on higher conservation within the homologs (Supp. Fig. 1). The Tyr residues surround the barrel almost exactly inside or outside, which might be the sites for heme binding (Supp. Fig. 4B). To assess the underlying role, the complete ORF sequence of *REAC1*was used to construct a recombinant expression vector in the context of pCold I (here simply referred to as pC). pC-*REAC1* and the control vector pC were separately transformed into *E. coli* BL21 (DE3) cells (the engineered strain named BL21-H and the control strain harboring the empty vector pC named BL21-C) (Supp. Fig. 5A). *REAC1* was successfully expressed in the prokaryotic cells (Fig. 2B), leading to a greater tolerance to H_2O_2 for the bacteria than those without the additional protein.

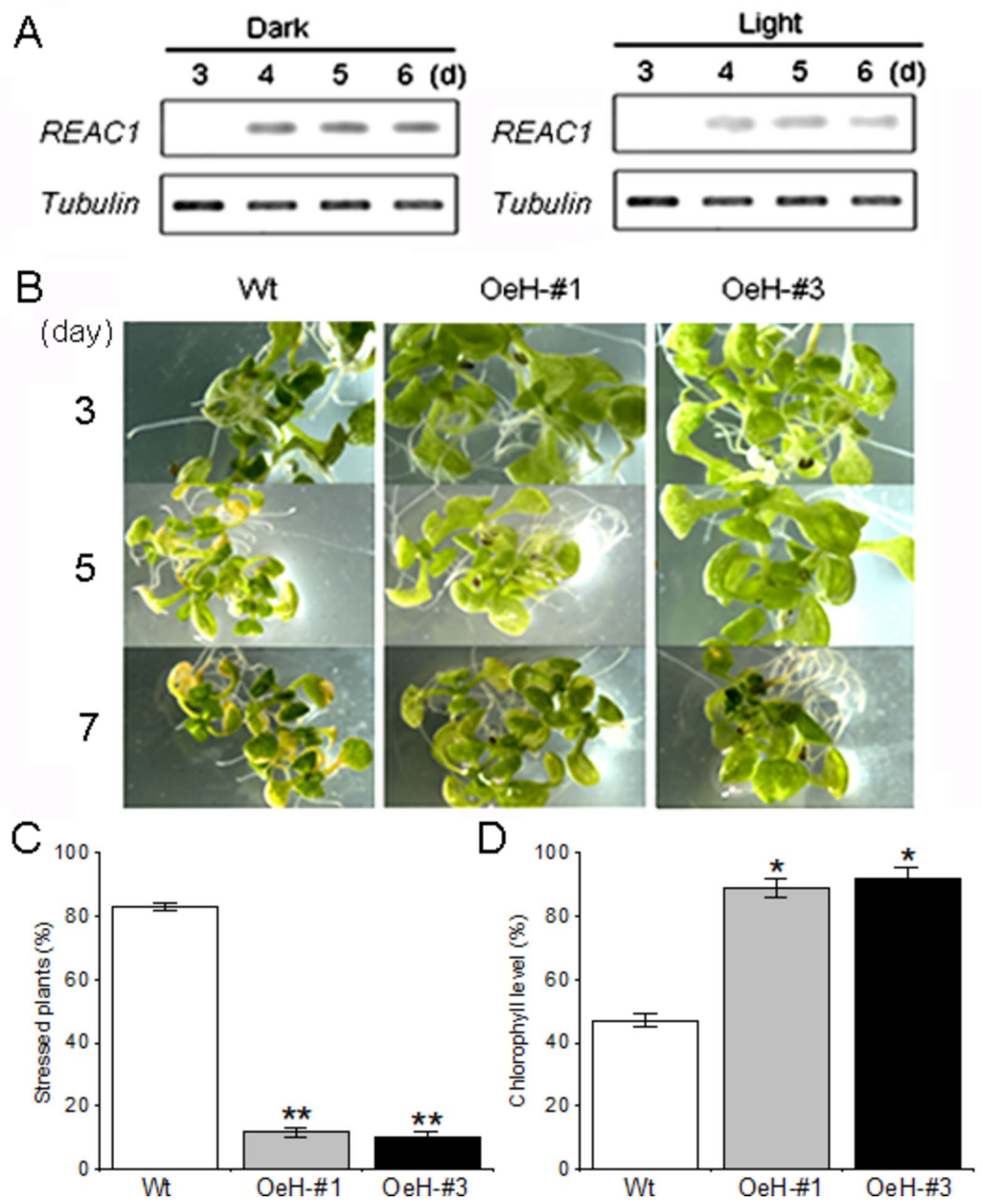
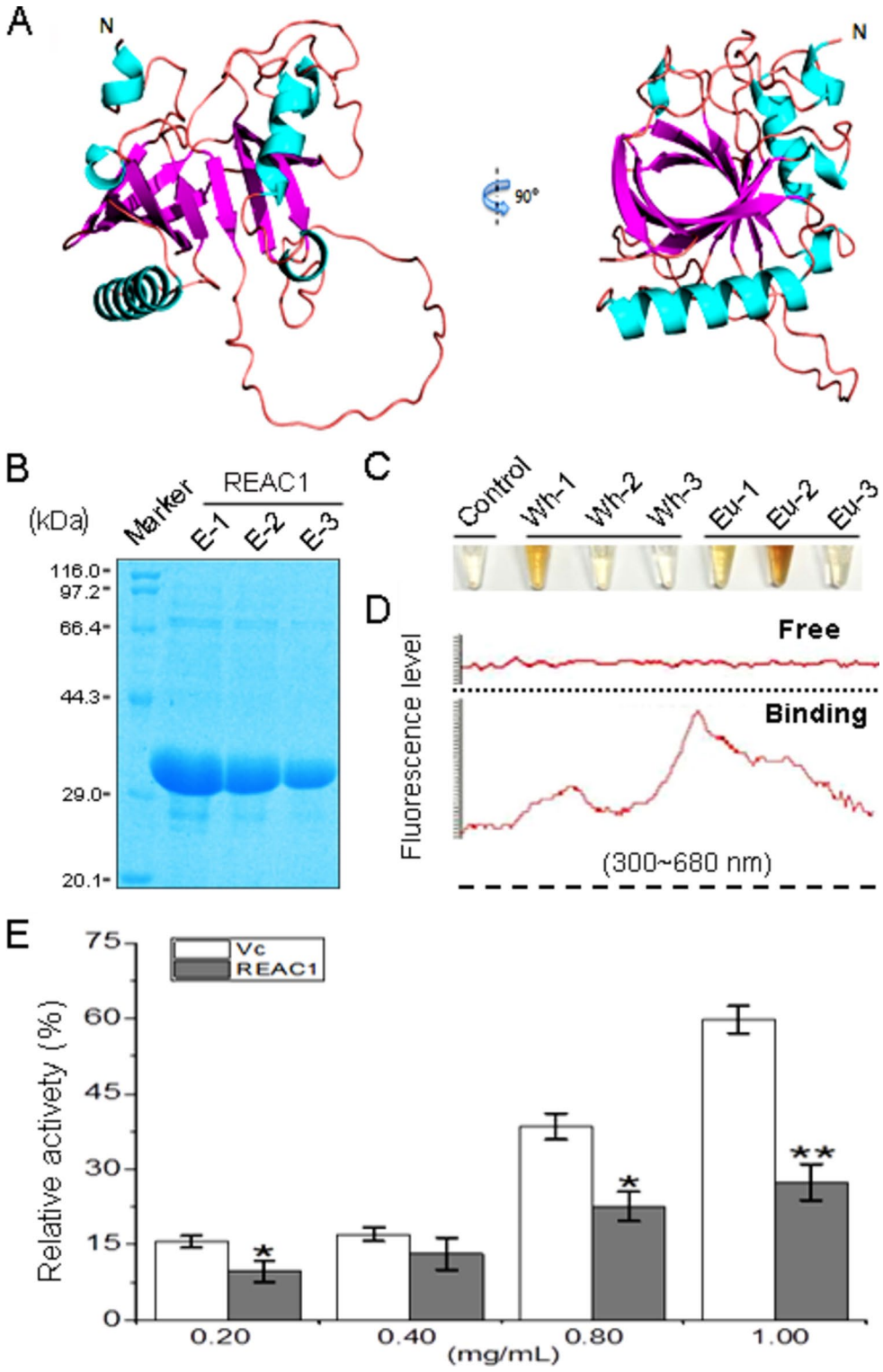


Fig. 1. Overexpression of *REAC1* enhanced tolerance to H_2O_2 in Arabidopsis. (**A**) On the whole, the transcriptional expression pattern of *REAC1* showed similar under dark or normal photoperiod conditons. *REAC1* transcripts increased sharply at the four day and then kept similar level to six day in the testing period. *Tubulin* served as a control in the semi-qRT-PCR assay. (**B**) The two transformants OeH-#1 and -#3 were more resistant to 10 mM H_2O_2 than the Wt plants, as relatively there were much less stressed plants (**C**) and higher chlorophyll content in the two transformants (**D**).

Because it was found that the related plaque diameter was larger than the other plaques, as shown in Supp. Fig. 5B.

In vitro, the isolated protein REAC1 had a high affinity for hemin chloride, (HeCD), as shown in Fig. 2C. After removing almost all redundant or nonspecific affinity HeCD, the HeCD that took on specific affinity by the REAC1 protein could be collected in the second tube via elution buffer, suggesting that REAC1 would likely bind hemes. The results from the spectrum analysis were consistent with the results of the affinity assays, as there were different curves without (Free) or with REAC1 (Binding) supplemented with HeCD buffer (Fig. 2D). Furthermore, radical inhibiting assay was carried out, and several substrates were tried, however, relatively it seems that just the effect of hydroxy radical inhibiting was clear. For instance, as shown in the Fig. 2E, with a concentration of 0.60 mg/mL, the inhibition ability of REAC1 reached approximately 22%. Although the inhibition ability was lower than that of the control Vc (Vitamine C), the protein REAC1 retained oxidation resistance in vitro.



REAC1 improves nematode movement

Long-term oxidative stress leads to severe impairment of health and aging[10,11]. Exploring the potential effects of protein or antioxidants supplementation as a modulator among consumed foods or compounds is interesting[5,44]. For this purpose, *C. elegans* N2 was treated with a bacterial mixture of OP50 and the engineered bacteria expressing REAC1 (OP50/BL21-H), whereas control nematodes were treated with OP50/BL21-C. The ratio of OP50 to BL21-H or BL21-C in the mixture suspensions was controlled by testing the bacterial concentration. Notably, the motility of nematodes treated with REAC1 was significantly improved. For example, when the ratio was 1:1, the mean number of body bends per unit of time (15 s) increased by 24.0% over that that of the control group on the sixth day (Fig. 3A). For individuals, motility represents the critical status of health or physiological conditions[49]. While reproduction refers to a specific period of physiology throughout the lifespan, it should be a valuable reflection of individual health. It was notable that the groups treated with REAC1 produced many more

◀**Fig. 2**. REAC1 protein structure modeling and its activity. (**A**) Adavance structure modeling for the REAC1 using Swiss-model, the fully automated protein structure homology-modelling server in https://swissmodel.expasy.org/. The primary optical templates for REAC1 modeling was identified as AlphaFold DB model of Q8LQ41_ORYSJ (gene: Q8LQ41_ORYSJ, (Rice)). The REAC1 protein N terminal was simply marked, and the helices, sheets and turns in the molecules were highlighted in different colour. (**B**) Isolation of the protein REAC1 using a Ni-NAT affinity method. "E-1 to 3" indicates three accumulations of the engineering protein using elution buffer separately, and the molecular weights are referred to the protein marker on the gel left. (**C**) Washing or eluting the affinity sample indicates the heme binding of REAC1. After Ni-NAT affinity to the target protein REAC1, samples were subjected to heme solution chloroprotoporphyrin IX iron(III) (hemin chloride, HeCD), then equal volumes (800 μL) of washing buffer (Wh) or elution buffer (Eu) were used to completely remove non-specific affinity or redundant HeCD or elute specific affinity HeCD. Wh-3 indicated that there were little redundant HeCD in the samples, while Eu-2 showed that almost all of the HeCD that had been specificly binded by the REAC1 were collected in the second tube in comparison with the other two tubes. (**D**) Absorbance (Abs) analyses specific binding to the chloroprotoporphyrin IX iron(III) (hemin chloride, HeCD) by REAC1. The curves between the HeCD samples without (free) or with (binding) of target protein REAC1 showed clear differences under the same spectrum conditions. (**E**) Antioxidative activity of REAC1 was assessed through hydroxy radical scavenging assay. Vitamine C (Vc), served as a control at varying concentrations (0.05 ~ 1.00 mg/mL). Each assay at different concentrations was repeated three times.

nematodes than did the control group during the same periods, suggesting that intake of the engineered proteins facilitated or enhanced the reproductive capacity of the nematodes (Fig. 3B). These physiological alterations suggested that nematodes that were fed REAC1 provided with enhanced motility.

REAC1 enhances nematode antioxidative capacity

Environmental stressors like excessive oxidative stress can boost aging and disease development[6,12,13,20]. To evaluate the antioxidative effects of REAC1 on animals, nematodes treated with a bacterial mixture suspension for up to three days were collected and subjected to 5 mM H_2O_2 treatment to simulate oxidative stress conditions. During the testing period, nematodes fed REAC1 were more resistant to H_2O_2-induced oxidation than those in the control group. The survival rate of the *C. elegans* N2 treated with REAC1 was approximately 2.0 and 4.5 times greater than that of the control group at 60 and 90 min, respectively. Even at 240 min, a significant percentage (around 20%) of the nematodes in the treated groups remained alive, whereas all the nematodes in the control group had perished (Fig. 3, N2). Severe oxidative stresses even result in cell death[50,51]. *Ced-9* inhibits cell death and is homologous to the Bcl2 family of mammalian antiapoptotic genes[52,53]. We also used the nematode mutant MT4770, which has *ced-9* dominant expression, to evaluate the REAC1 intake effect. In contrast, MT4770 also exhibited improved tolerance to H_2O_2 when fed REAC1, and it seemed to have greater resistance to H_2O_2 than to N2. Except for the H_2O_2 treatment, heat stress assays were also carried out to characterize nematode tolerance. As shown in the Supp. Fig. 6, during the testing period the nematodes treated with REAC1 displayed a clear boosting tolerance compared to the control group. Hence, it suggested that REAC1 enhances nematode antioxidant capacity.

REAC1 protein reduces nematode ROS levels

Endogenous ROS can cause cellular damage, contributing to aging and senescence[54]. The enhanced ability of nematodes to withstand external oxidative stress is likely linked to their internal oxidative status. The presence of ROS serves as a direct indicator of the body's oxidative condition. To examine the effect of the REAC1 protein on ROS, nematodes were exposed to H2DCF-DA. The results depicted in Fig. 5A demonstrated that relatively nematodes treated with the REAC1 protein presented clearly lower ROS levels, achieving just approximately 24.2% of that in the control group. However, there was little variation in worm body length relative to that in the control group (Fig. 5B). An in-silico enzymatic or chemical digest of REAC1 was performed using the MS-Digest web tool, and several derived peptides took on potential oxidation site modification (Supp. Table 1). Therefore, consumption of the REAC1 protein may induce alterations in the ROS regulatory network within nematodes, thereby reducing excessive ROS accumulation and exerting protective effects against oxidative stress.

Significant alteration in the expression levels of ROS-related genes

The ROS-related regulation in nematodes is known to be controlled by several genes, such as *MEV-1* and *CAT-1*, which are involved in oxidative stress and lifespan regulation in *C. elegans*[55,56]. Furthermore, the *SOD-3*, *CTL-1*, and *SEK-1* genes encode superoxide dismutase, catalase, and bispecific mitogen-activated protein kinase, respectively, and they contribute to antioxidative stress regulation[57–59]. To investigate the potential mechanism induced by the REAC1 protein, the expression of genes associated with antioxidant stress or ROS-related regulation in *C. elegans* was explored via qRT-PCR. The results revealed a notable alteration in the expression of antioxidant-related genes in nematodes after consuming the REAC1 protein. As shown in the Fig. 6, compared with those in the control group, the relative expression levels of *CAT-1*, *SOD-3*, *SEK-1* and *GST-7* clearly increased, respectively, in the treatment group. By contrast, *MEV-1* and *CTL-1* were downregulated. Given in that *MEV-1* plays negative role in oxidative stress and *CTL-1* takes on activity downstream of *SOD-3*, the changed expression of *MEV-1* and *CTL-1* was reasonable to understand. Therefore, it is suggested that REAC1 intake indeed benefits *C. elegans* because of the alteration in expression of the genes, which are involved in regulating cellular oxidative defense.

(D) Absorbance (Abs) analyses specific binding to the chloroprotoporphyrin IX iron(III) (hemin chloride, HeCD) by REAC1. The curves between the HeCD samples without (free) or with (binding) of target protein REAC1 showed clear differences under the same spectrum conditions.

(E) Antioxidative activity of REAC1 was assessed through hydroxy radical scavenging assay. Vitamine C (Vc), served as a control at varying concentrations (0.05~1.00 mg/mL). Each assay at different concentrations was repeated three times.

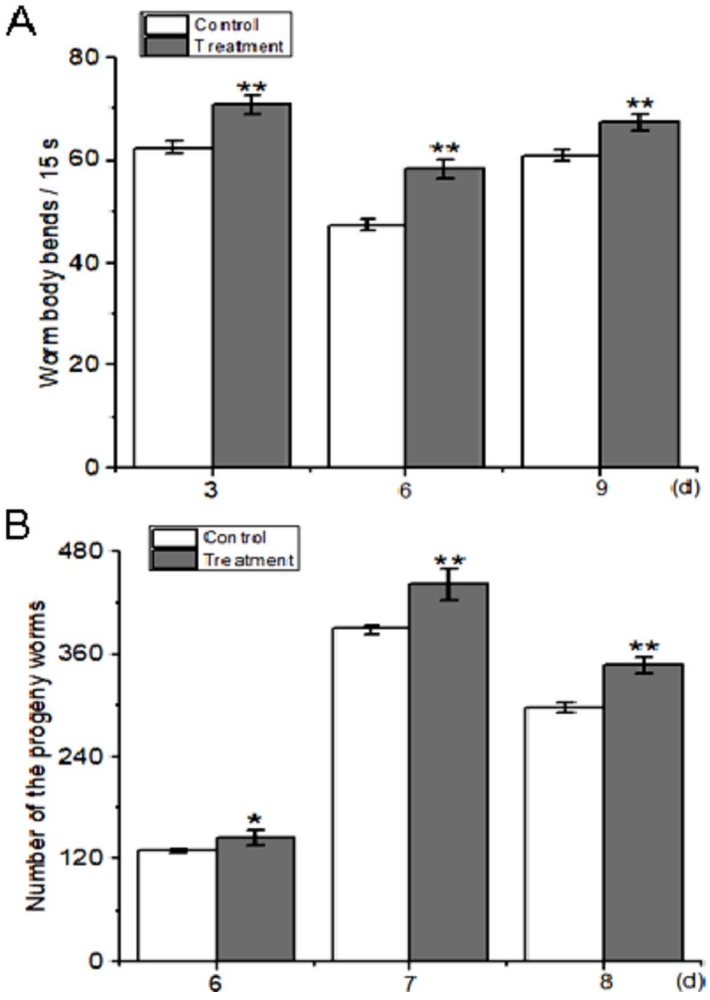


Fig. 3. Improvement of nematode motility and reproductive ability. (**A**) The mobility of nematodes treated with the engineered proteins REAC1 was significantly improved. L3 stage C. elegans were cultured in S liquid medium for 3 days for each treatment, after which the nematodes were collected and their body bends were observed at 15 s intervals each day. For each sample $n \geq 20$, and three replicates were conducted. (**B**) The reproduction capacity of nematodes treated with the engineered proteins REAC1 was significantly higher than the control group. L3 stage C. elegans were cultured in S liquid medium for 3 days for each treatment, after which the nematodes were tested for the reproduction capacity by scoring the progeny in the following days. For each sample $n \geq 15$, and three replicates were conducted.

Discussion

Red rice is well known for its health benefits and culinary uses due to its rich content of advantageous components[60,61]. However, most crucial factors from this species require further exploration via novel mechanisms. In this study, the red rice SOUL family heme-binding protein RECA1 was fed to *C. elegans* and demonstrated antioxidative stress properties, as the treated nematodes exhibited significantly improved motility and increased survival under 5 mM H_2O_2 conditions (Figs. 3 and 4). These alterations are closely linked to the physiological status of nematodes, highlighting the substantial benefit provided by the protein. The clear decline in endogenous ROS levels and the alteration of several genes involved in ROS-related regulation (Figs. 5 and 6) may explain the observed improvement in their responses.

The first SOUL protein was identified from chicken retina and pineal gland, and it was expressed response to light signaling[62]. Previous study indicated that the Arabidopsis SOUL family protein SOUL2 is cytosolic and in vitro binds to heme as well as other porphyrins[34]. During seedling growth, *REAC1* starts to be continuously expressed at a specific stage (Fig. 1A), suggesting that this gene might have an essential role in rice. Apparently, the overexpression of *REAC1* had little effect on seedling growth in our assay. Still, the Arabidopsis transformants were more tolerant to treatment with H_2O_2, suggesting that this gene is involved in oxidative stress-related regulation in vivo (Fig. 1B–D). Free heme easily induces cytotoxic damage due to their inherent peroxidase reactivity and capacity to produce ROS in plant cells[25]. REAC1 protein displayed a high affinity for the heme-like compound hemin chloride (HeCD), which might be due to the conservation of Tyr (Y) and His and their locus in the protein. REAC1 protein displayed radical scavenging activity in vitro (Fig. 2E). Therefore, it can be speculated that REAC1 takes on the ability to bind heme, which may account for its role in resistance to H_2O_2 damage in plants.

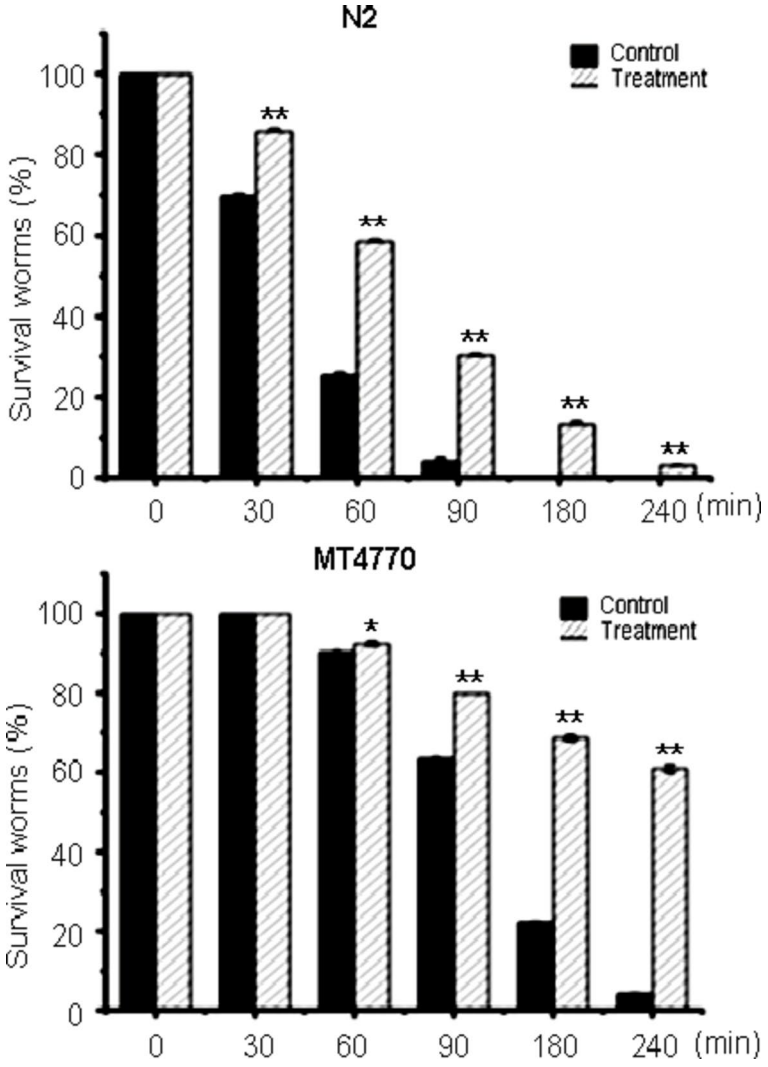


Fig. 4. REAC1 increased the nematode antioxidative capacity. Intaking the REAC1 improves nematode wild type N2 (**A**) and mutant *ced-9* (MT4770, **B**) motility. The collected nematodes were exposed to 5 mM H_2O_2 in a 48-well plate, and their survival was scored intervals under a dissecting microscopy. For each sample $n \geq 20$, and three replicates were conducted.

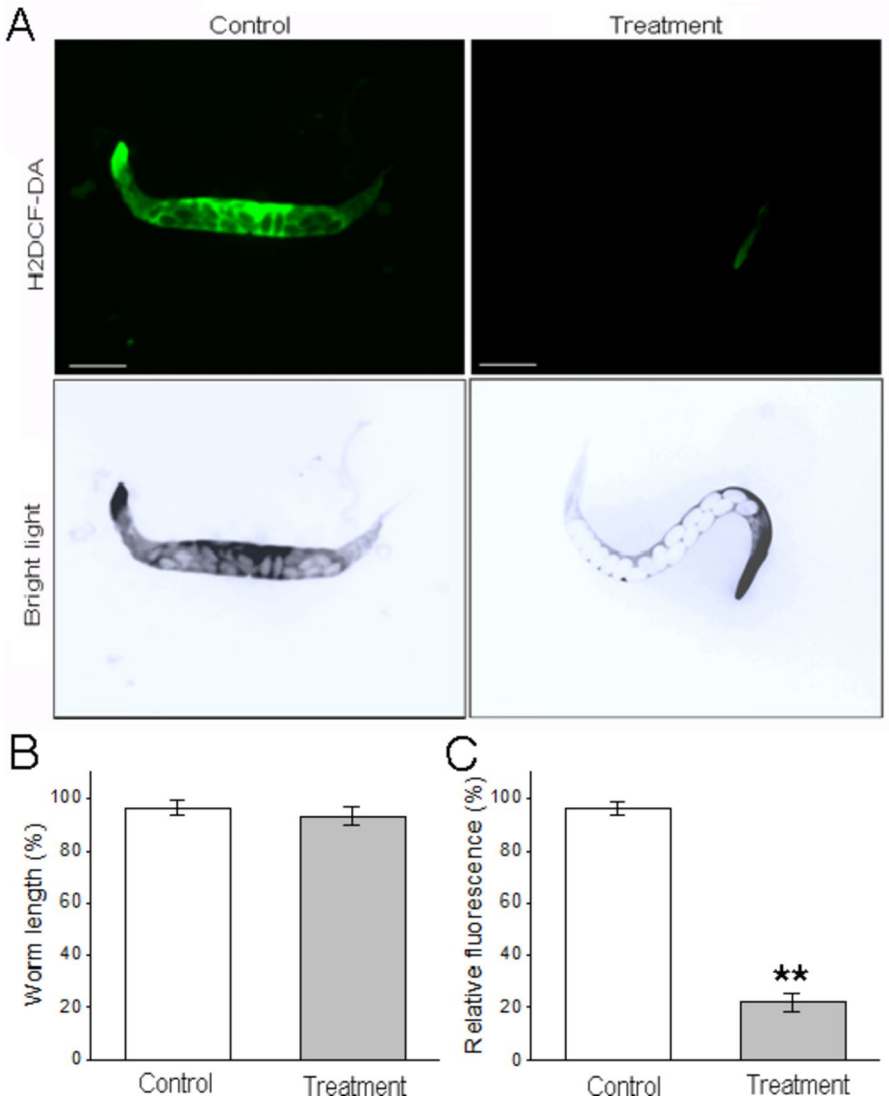


Fig. 5. Endogenous ROS of nematodes that intake REAC1 was clearly inhibited. The fluorescence level indicated by H_2DCF-DA in the control group (Control) were signifcantly higher than that in the REAC1 treated group (Treatment) (**A** and **C**), while the worm size showed no difference between the two goups (**B**). Scale: 20 μm.

For unique individual, aging is easily accelerated by long-term excessive oxidative stress, which may even leads to chronic diseases or a decline in or complete loss of motility[3,10–12]. To cope with the problems, effective strategies might be going to arise from the creation of novel crop varieties or innovative resources, which may greatly benefits people health[3,4,23]. The consumption of REAC1 is strongly associated with improvements in the motility and antioxidative stress capacity of nematodes, highlighting the critical role of this protein in laboratory settings. For individuals, most chances, including competition with others and defense from other stresses, are conferred by motility[6,49]. By contrast, aging and aging-related chronic diseases easily lead to a decrease in motility. The improved motility observed in the treated nematodes suggests profound physiological alterations due to protein intake. Reproduction refers to a fresh stage of life for each organism. We also found that the reproductive posterities of nematodes treated with REAC1 increased (Fig. 3A), suggesting that physiological changes indeed occurred in the nematodes.

Environmental stressors can lead to excessive production of ROS, which can be harmful to cellular macromolecules[5,6,14]. Antioxidant supplementation is helpful for health because it inhibits ROS damage[5,9]. In vivo, H_2O_2 can be generated as a byproduct of oxidative protein folding in the endoplasmic reticulum and is homolytically cleaved in the presence of redox-active Fe(II) iron (Fenton reaction) to form highly reactive hydroxyl radicals[14,63]. Nematodes that treated with REAC1 exhibited increased tolerance to oxidative environments (Fig. 4A), such as the exposure to 5 mM H_2O_2. The findings indicate that the consumption of REAC1 has a beneficial impact on nematodes. The specific Cys and Met residues in the REAC1 protein might facilitate antioxidative properties in vitro, as the sulfhydryl (R-SH) groups in the residues posssess antioxidative stress activity and generally interacts with radical species via hydrogen donation from the SH group[64]. However,

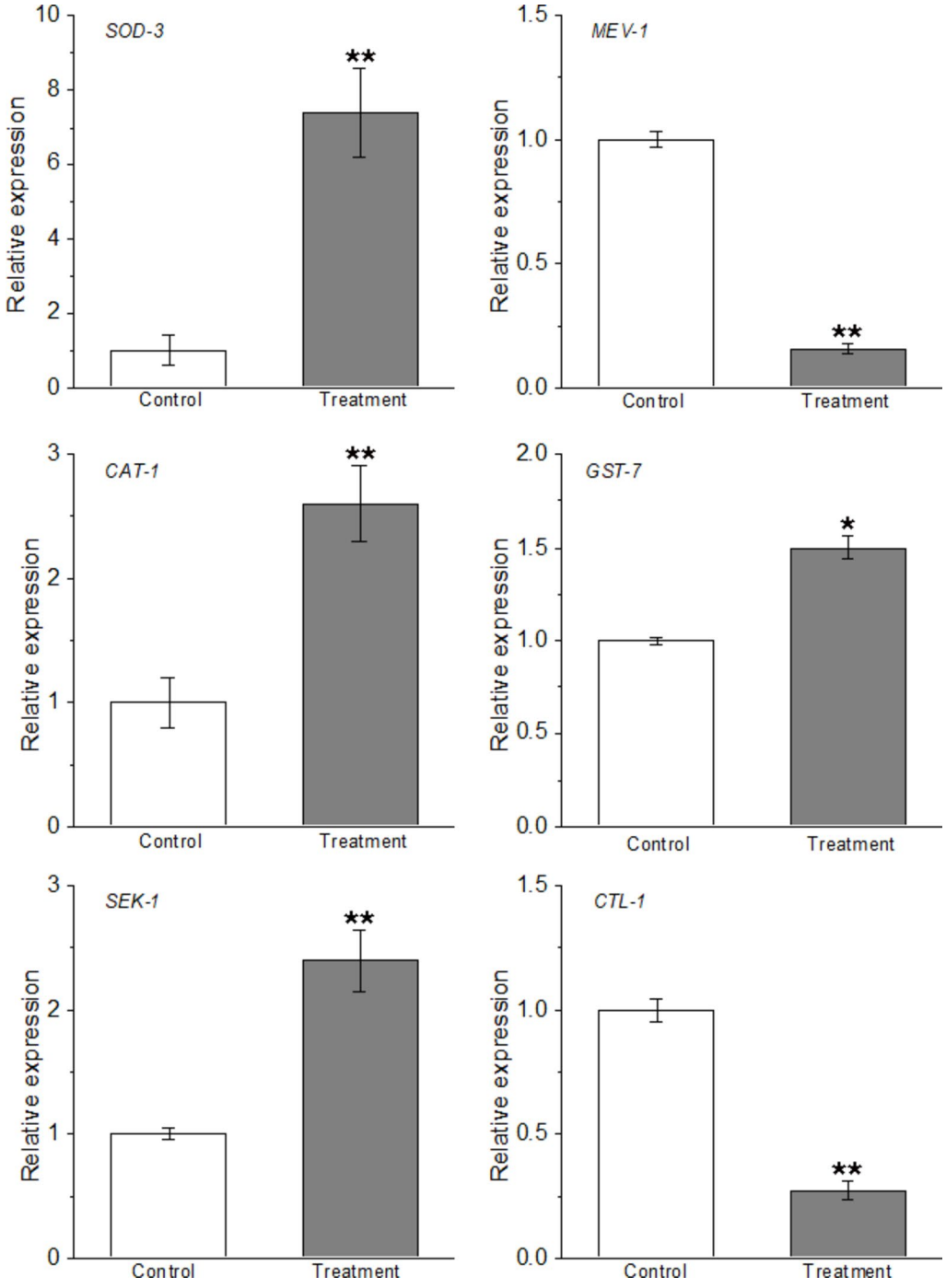


Fig. 6. Relative expression of the redox regulation-related genes in nematodes. The transcript levels of *SOD-3*, *CAT-1*, *SEK-1* and others in the nematodes treated with REAC1 were clearly changed in comparison to the control group as evidenced by qRT-PCR. The specific primer pairs corresponding to the genes listed in the supplementary table. *Actin* was used an endogenous reference.

the greater tolerance of the nematodes to H_2O_2 did not result from only special residues. There should be multiple regulatory effects or alterations in the treated nematodes. Polypeptides resulting from the digestion of engineered protein are likely involved in the regulation of ROS. The alteration in endogenous ROS levels in these treated nematodes provided additional evidence of their physiological conditions.

Proteins are the essential building blocks of most animal tissues. While protein intake has no nutritional value unless proteases and peptidases hydrolyze proteins to aa, dipeptides, or tripeptides in the lumen of the small intestine[65]. The result from in silico enzymatic or chemical digest of protein provides more details for the displayed benefits in *C. elegans*. Based on the web server tool MS-Digest, (https://prospector.ucsf.edu/prospector/cgi-bin/msform.cgi?form=msdigest)[66], it was notable that several peptides derived the REAC1 contain

potential oxidation sites due to specific residues in the protein (Supp. Tabel 1). In this context, it is reasonable to understand the enhancement of antioxidative capacity in nematodes treated by the REAC1.

The expression of several genes associated with oxidation and aging in the treated nematodes, such as *SOD-3*, *CAT-1*, *SEK-1* and *GST-7* differed from that in the control group (Fig. 6). SODs are usually relatively unstable and can be dismutated to H_2O_2 and molecular oxygen in the very short term by superoxide dismutases (SODs)[14]. CAT-1 is the homolog of the mammalian vesicular monoamine transporter VMAT2, and it plays a key role in dopamine release and packaging in *C. elegans*[56]. The upregulated *SOD-3* and *CAT-1* were consistent with the reduced ROS levels in the treated nematodes. The expression of genes such as *SEK-1*, which enables MAP kinase kinase activity and protein serine kinase activity and is involved in defenses and other diseases, such as PD, is increased[59], potentially implying that REAC1 or its byproduct of digestion inspires several pathways involved in defense or PD. In contrast, *MEV-1* contributes to succinate dehydrogenase (ubiquinone) activity and negatively regulates the lifespan and oxidative stress response of *C. elegans*. The mutation *mev-1* leads to high sensitivity to oxidative stress[67]. While the *CTL-1* is predicted to be involved in the hydrogen peroxide catabolic process and *CTL-1* functions in the ROS detoxification pathway, but downstream of *SOD-3*[57]. In the treated group, *MEV-1* and *CTL-1* were relatively downregulated. The expression of the genes is primarily influenced by the protein REAC1, particularly regarding its amino acid residues and unique advanced structures. To further understand the roles of REAC1 requires fresh assay, however, it is clear that consumption of REAC1 helps nematodes tolerance to oxidative stress.

Individuals always encounter various and challenging environmental conditions throughout their lives[68]. When cells undergo excessive oxidation or disruption of redox regulation, aging and even diseases might be accelerated. Over time, chronic diseases associated with aging and oxidative imbalances pose major challenges for individuals and can significantly hinder economic and social advancement[6,16]. Therefore, maintaining the cellular oxidation equilibrium to delay aging and enhance overall well-being is crucial for individuals. In addition, physiological functions are subject to various factors, such as exercise capacity, metabolism and adipose tissue levels. Accumulating evidence has indicated that adequate and appropriate supplementation with beneficial proteins is essential for maintaining health during aging[5,9]. The red rice SOUL family heme-binding protein REAC1 is crucial for combating oxidative stress, indicating that the protein may hold potential for practical applications in the future.

Conclusions

In the context of aging accelerating in global population, it is crucial to create novel crop varieties or find health benefit elements from various resources. The protein REAC1, a heme-binding protein from red rice SOUL family, demonstrated significant antioxidative stress activity in vitro. The transformants overexpressing *REAC1* are more tolerance to oxidative stress. When the nematode *C. elegans* was fed REAC1, it showed clearly enhanced motility and improved survival under oxidative stress conditions compared to the control group. Results also showed that the nematode endogenous levels of ROS were reduced, and that the expression of redox-related genes, including *SOD-3* and *CAT-1*, was upregulated after consumption of the engineered protein. Taken together, these findings indicate that REAC1 is involved in combating oxidative stress and can enhances the antioxidative capacity of *C. elegans*. Additionally, the protein might possess valuable application in crop improvement or individual health benefit in the future.

Data availability

Data is provided within the manuscript or supplementary information files.

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Author contributions

Q.F. conceived and designed the study and reviewed the manuscript. Q.F., H.H., C.Z., H.W., and F.W. performed experiments and analyzed data. Q.F. and C.Z. performed repeated experiments for antioxidative stress activity of the gene, Q.F. wrote and provided a review of the manuscript.

Declarations

Competing interests

The authors declare no competing interests.

Additional information

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