Insect Biochemistry and Molecular Biology 92 (2018) 40–44

Contents lists available at ScienceDirect

Insect Biochemistry and Molecular Biology

journal homepage: www.elsevier.com/locate/ibmb

Enhanced heat tolerance in transgenic silkworm via overexpression of *Pyrococcus furiosus* superoxide reductase

Liang Jiang [1], Chunlin Huang [1], Bingbing Wang, Huizhen Guo, Qiang Sun, Fei Xia, Guowen Xu, Qingyou Xia*

State Key Laboratory of Silkworm Genome Biology, Southwest University, Chongqing 400715, PR China

ARTICLE INFO

Article history:
Received 7 April 2017
Received in revised form
27 October 2017
Accepted 20 November 2017
Available online 21 November 2017

Keywords:
Pyrococcus furiosus superoxide reductase (PfSOR)
Reactive oxygen species (ROS)
Overexpression
Silkworm
Transgenic
Heat tolerance

ABSTRACT

Heat shock causes a serious harm to organisms by accelerating the production of reactive oxygen species (ROS). *Pyrococcus furiosus* superoxide reductase (PfSOR) is an enzyme that efficiently detoxifies ROS. In order to generate a silkworm strain with high heat tolerance for sericulture, we synthesized an artificial DNA sequence encoding PfSOR based on the codon bias of *Bombyx mori*. PfSOR was successfully overexpressed in transgenic silkworm (named A4SOR) and BmE cells, as determined by RT-PCR and western blot analyses. An SOR activity assay confirmed that the expressed enzyme was functional in A4SOR. After exposure to a temperature of 35 °C for 44 h, the mortality rate was about 30% lower in transgenic A4SOR than in non-transgenic silkworms. Moreover, transgene expression had no apparent effect on economic characteristics of silkworms. The heat tolerance of silkworm was thus enhanced by expressing an archaeal SOR; this can be useful for sericulture in regions where the average temperature exceeds the optimal environmental temperature for *B. mori* of 25 °C.

1. Introduction

Insects are poikilotherms and are therefore unable to regulate body temperature, making them susceptible to changes in environmental conditions. The optimal environmental temperature of *Bombyx mori*, the only fully domesticated insect, is 25 °C (Jiang et al., 2017); high temperature shock can lead to various diseases and poor cocoon yield in sericulture (Li et al., 2012). In most parts of China, summer temperatures exceed 30 °C. To avoid the economic losses caused by high temperature in sericulture, silkworms feed less in the summertime. To date, no effective strategies have been established to prevent heat shock in sericulture.

Heat stress accelerates the production of reactive oxygen species (ROS) such as superoxide (O_2^-), singlet oxygen ($^1O_2$), hydroxyl radical (·OH), and hydrogen peroxide (H_2O_2) (Geng et al., 2016; Suzuki et al., 2012). Excessive ROS causes oxidative stress, which in turn leads to DNA damage, lipid peroxidation, protein degradation, enzyme inactivation, and cell death (Apel and Hirt, 2004; Geng et al., 2016; Im et al., 2009; Kai et al., 2012). On the other hand, ROS are the products of aerobic metabolism and function as signaling molecules that regulate multiple biological processes, including growth, development, and stress responses (Baxter et al., 2014; Farmer and Davoine, 2007; Geng et al., 2016). Thus, maintaining an appropriate balance between ROS production and clearance is important for normal organismal functioning.

O_2^- is the primary product of oxygen reduction, which is normally metabolized by superoxide dismutase (SOD) to produce H_2O_2 and O_2. H_2O_2 triggers additional signals, while O_2 can stimulate additional ROS production (Geng et al., 2016; Im et al., 2009). Overexpression of SOD increases oxidative stress tolerance (Gupta et al., 1993; Im et al., 2009; Van Breusegem et al., 1999), including heat tolerance in cultured tobacco cells (Vacca et al., 2004) and transgenic potato (Kim et al., 2010). However, since SOD production is regulated by the transcriptional or posttranscriptional mechanisms of the host, the functional range of temperatures for SOD activity is limited (Foyer and Noctor, 2005; Grene, 2002; Im et al., 2009).

Superoxide reductase (SOR) of the anaerobic hyperthermophilic archaeon *Pyrococcus furiosus* has a detoxification mechanism that differs from that of SOD, which can reduce O_2^- to H_2O_2 without producing O_2 (Geng et al., 2016; Im et al., 2009; Jenney et al., 1999). Exogenous PfSOR is not regulated by host transcriptional (or post-

* Corresponding author.
E-mail address: xiaqy@swu.edu.cn (Q. Xia).
[1] These two authors contribute equally to this work.

https://doi.org/10.1016/j.ibmb.2017.11.010
0965-1748/

transcriptional) mechanisms (Geng et al., 2016; Im et al., 2009), and enzymatic activity is extremely stable within the temperature range of 4 °C–100 °C (Grunden et al., 2005; Jenney et al., 1999). The lack of O_2 production by PfSOR prevents further ROS generation (Jenney and Adams, 2001; Jenney et al., 1999; Weinberg et al., 2004); in addition, SOR complexes with ferrocyanide decrease O_2^- to H_2O without producing detectable H_2O_2 (Im et al., 2009; Kovacs and Brines, 2007; Molina-Heredia et al., 2006). PfSOR overexpression has been shown to enhance heat tolerance in tobacco cells (Im et al., 2005), *Arabidopsis* (Im et al., 2009), and *Cornus canadensis* (Geng et al., 2016). However, there are no reports to date of increasing stress tolerance by expressing archaeal genes in insects. In the present study, we overexpressed PfSOR in silkworm in order to increase its heat tolerance.

2. Materials and methods

2.1. Silkworm strains and cells

The Dazao (DZ) silkworm strain and BmE cell line were maintained at the Gene Resource Library of Domesticated Silkworm and State Key Laboratory of Silkworm Genome Biology (Southwest University, China), respectively.

2.2. Vector construction

Using the PfSOR gene sequence (accession no. AE010234), we synthesized an artificial DNA sequence (referred to as SOR-as) based on the codon bias of *B. mori* (Fig. 1). The sequence encoded the PfSOR protein with an N-terminal Flag tag. The enhancer hr3 (Wang et al., 2013), A4 promoter (A4P), and SV40 termination signal (Jiang et al., 2012a, 2013), which were conserved in our laboratory, were inserted along with SOR-as into the transgenic vector *piggyBac* [3 × p3 dsRed af] with the reporter gene 3 × P3-dsRed-sv40 to generate the transgenic vector pb-A4SOR (Fig. 2A). We added a His tag to the C terminus of SOR-as, and then constructed vector 1180-hr3-A4P-Flag-SOR-His-SV40 (named SOR-FH) using the basic vector 1180.

2.3. Transgenic silkworm generation and insertion site analysis

Non-diapausing DZ embryos were microinjected with pb-A4SOR and transgenic silkworms were screened as previously described (Jiang et al., 2012a, 2012b). One transgenic line, A4SOR, was obtained. Genomic DNA was extracted from A4SOR for inverse PCR analysis using the transposon-specific primers pBacL and pBacR (Jiang et al., 2012a, 2013).

ATGGACTACAAGGACGATGACGATAAGATCTCAGAAAC
AATCAGATCTGGTGACTGGAAGGGAGAAAAACACGTG
CCTGTTATTGAATACGAAAGAGAAGGTGAACTGGTCAA
GGTGAAAGTTCAAGTCGGAAAGGAAATACCTCACCCAA
ACACAACTGAACACCACATCAGATACATTGAACTGTACT
TCTTGCCGGAAGGAGAAAATTTCGTGTACCAGGTTGGC
AGAGTCGAATTCACTGCTCACGGCGAAAGCGTGAACGG
TCCGAATACCTCAGACGTTTACACAGAACCCATCGCTTA
CTTCGTCTTGAAGACTAAAAAGAAAGGCAAACTCTACG
CCCTGTCATACTGCAACATTCACGGTTTGTGGGAAAATG
AAGTGACCCTCGAA**TAA**

Fig. 1. Artificial DNA sequence SOR-as. Black and bold, ATG start and TAA stop codons; gray underlined sequence, Flag tag; black sequences, optimized coding sequence of *P. furiosus* SOR with silkworm codon bias.

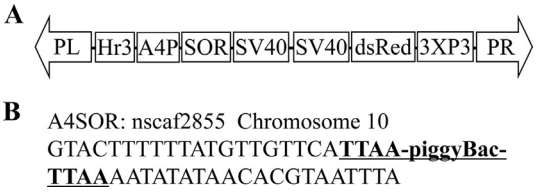

Fig. 2. Generation of transgenic silkworm. (A) Schematic illustration of the pb-A4SOR vector. PL and PR, left and right terminal inverted repeats, respectively, of transgenic vector *piggyBac* [3 × p3 dsRed af]; 3 × P3-dsRed-sv40, reporter gene; Hr3, enhance from BmNPV virus; A4P, silkworm A4 promoter; SOR, SOR-as sequence; SV40, polyadenylation signal. (B) Insertion site of transgenic silkworm A4SOR.

2.4. Analysis of SOR expression patterns

Total RNA was extracted from A4SOR and non-transgenic DZ (4- and 8-day-old eggs, hatched silkworms, first instar molts, second instar larvae, second instar molts, third instar larvae, third instar molts, fourth instar larvae, fourth instar molts, fifth instar larvae, wandering silkworm, and pupae). RT-PCR analysis was carried out with the SOR-qRT primer set (F: 5′-GAAAGTTCAAGTCGGAAAGGA-3′, R: 5′-GCAGTATGACAGGGCGTAGAGT-3′). TIF-4A primers were used as a control (Guo et al., 2016). RNA and protein were extracted from the midgut and integument of A4SOR and DZ from day 3 fifth instar larvae. The RNA was used for RT-PCR with primers SOR-qRT and TIF-4A, and the protein was used for western blotting with anti-Flag and -tubulin (control) antibodies. BmE cells were transfected with SOR-FH or 1180 (control) vector. RNA was extracted 24 h later for RT-PCR analysis using primers SOR-qRT and TIF-4A, and protein was extracted 48 h post-transfection for western bolt analysis using anti-Flag, -His, and -tubulin antibodies.

2.5. Determination of SOR activity

Midgut proteins were extracted from 10 A4SOR and non-transgenic DZ larvae on day 3 of the fifth instar. The extract was centrifuged for 30 min at 12,000 rpm and 4 °C, and the supernatant was used for analysis of SOD/SOR activity with the water-soluble tetrazolium (WST) SOD Assay kit (Im et al., 2005, 2009). The protein sample was incubated at 80 °C for 15 min, and heat-denatured protein was removed by centrifugation for 30 min at 12,000 rpm and 4 °C; the supernatant was also used for SOD/SOR activity analysis. One unit of SOD/SOR activity was defined as the amount of enzyme inhibiting the rate of WST-8 reduction by 50% (Im et al., 2005, 2009). The assay was performed three times.

2.6. Analysis of heat tolerance and economic characteristics

Newly exuviated fifth instar larvae of A4SOR and DZ were fed at 35 °C for 44 h, and then returned to 25 °C for feeding until the wandering stage. Each line was analyzed three times and each repeat consisted of 50 larvae. Three non-treated repeats also included 50 larvae each. Mortality up to the wandering stage was recorded. The cocoon shell rate was determined according to published methods (Jiang et al., 2012a, 2013).

2.7. Starvation treatment

The fifth instar larvae of A4SOR and DZ were fed with mulberry leaves at 0–72 h (day 1- day 3), which were starved with no food supply after 72 h (day 4). The control silkworms were normally fed. Each line had three repeats and each repeat contained 50 larvae. The survival was determined daily until the day 11.