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ThPOD3, a truncated polypeptide from *Tamarix hispida*, conferred drought tolerance in *Escherichia coli*

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Abstract The *ThPOD1* gene encodes a peroxidase and was isolated from a *Tamarix hispida* NaCl-stress root cDNA library. We found that *ThPOD1* expression could be induced by abiotic stresses such as cold, salt, drought and exogenous abscisic acid. These findings suggested that *ThPOD1* might be involved in the plant response to environmental stresses and ABA treatment. To elucidate the function of this gene, recombinant plasmids expressing full-length *ThPOD1* as well as *ThPOD2* (aa 41-337), and *ThPOD3* (aa 73-337) truncated polypeptides were constructed. SDS–PAGE and Western blot analyses of the fusion proteins revealed that the molecular weights of ThPOD1, ThPOD2 and ThPOD3 were ~57, ~50 and ~47 kDa, respectively. Stress assays of *E. coli* treated with the recombinant plasmids indicated that ThPOD3 could improve resistance to drought stress. This finding could potentially be used to improve plant tolerance to drought stress via gene transfer.

Keywords Peroxidase · qRT-PCR · Prokaryotic expression · Abiotic stress · *Tamarix hispida*

Introduction

Plant peroxidases (class III peroxidases) are heme-containing enzymes that are present in all land plants, and are members of a large multigenic family [1, 2]. Probably due to the high number of isoforms and heterogeneous regulation of expression, plant peroxidases are involved in a broad range of physiological processes such as initiation of seed germination [3], cellular growth and cell wall loosening [4], cell wall cross-linking [5], lignification and suberization [6], and senescence [7]. Moreover, peroxidases fulfill important functions in stress-related processes such as plant-pathogen interactions, defense against abiotic stress and wound healing [8–10]. To date, many reports on the purification, molecular cloning and physiological function of POD isoforms have indicated that POD isoforms are critical in plants [11–15]. POD genes have been extensively studied in crops and herbage plants due to their importance in defense/stress responses; however, in woody plants, their functions are not well known.

A gene encoding peroxidase (named *ThPOD1*) was isolated from a *Tamarix hispida* NaCl-stress root cDNA library [16]. After searching in GenBank database of NCBI, ThPOD1 protein containing four conserved domains such as secretory peroxidase, plant peroxidase, ascorbate peroxidase and peroxidase were found out (Fig. 2e). Liu et al. [17] provided experimental evidences that a truncated polypeptide could directly express and confer function in the host *E.coli*. In this study, we constructed the recombinant plasmids expressing the full-length of *ThPOD1*, two truncated *ThPOD2* encoding of the plant peroxidase functional region, and *ThPOD3* encoding of the ascorbate peroxidase functional region. These polypeptides were identified by SDS–PAGE and Western blot. By Stress assay and comparing the survival ratios of the constructed recombinants, ThPOD3 conferred the highest stress tolerance. The result could potentially be used to improve plant tolerance to drought stress using gene-transfer technology.

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Materials and methods

Plant treatment

Tamarix hispida seedlings were planted in a mixture of turfy peat and sand (2:1 v/v) in a greenhouse with 75% relative humidity and a constant temperature of 24°C.

In order to detect induction of *ThPOD1* expression under various conditions, 3-month-old *T. hispida* seedlings were treated with 4°C, 400 mM NaCl, 20% polyethylene glycol (PEG), and 100 μM ABA. Leaf and root tissues of the seedlings were harvested at 0, 6, 24, 48 and 72 h after each treatment and immediately stored in liquid nitrogen.

RNA isolation and quantitative real time (qRT) PCR assay

Total RNA was extracted from leaf and root tissues of *T. hispida* plants using a cetyl trimethyl ammonium bromide (CTAB) method [18]. The extracted RNA was then treated with RNase-free DNase (TaKaRa) to remove DNA contamination. Total RNA (1 μg) then reverse-transcribed with a reverse transcriptase kit (MBI Fermentas) in a volume of 20 μl. PCR primers were designed to amplify target cDNA fragments (Table 1). The *β*-actin (EG971352), α-tublin (EH050602), *β*-tublin (EH052343) and 18s rRNA (EF416283) genes were used as internal references to verify successful reverse transcription and to calibrate the cDNA template. Quantitative real-time RT-PCR was repeated in six replicates for each sample using SYBR-Green PCR Master Mix (MJ Research) on a DNA Engine Opticon™2 instrument (MJ Research) following the manufacturer's recommendations.

Construction of full-length *ThPOD1* and truncated clones

ThPOD1 encoding of the mature peptide, *ThPOD2* encoding of the plant peroxidase functional region, and *ThPOD3* encoding of the ascorbate peroxidase functional region were amplified with specific primers (Table 2). The forward primer contained an engineered *BamH* I site (underlined) and the reverse primer incorporated an engineered *Xho* I site (underlined). The PCR cycle profile was: 94°C for 3 min; followed by 29 cycles of 94°C for 30 s, 56°C for 30 s and at 72°C for 60 s, with a final extension at 72°C for 7 min. The purified PCR products and pET32a(+) plasmid (Novagen, USA) were digested with *BamH* I and *Xho* I, and then purified and ligated together. *E. coli* JM109 was transformed with the recombinant vector by heat shock and cultured at 37°C on LB agar with ampicillin (100 μg ml^{-1}) for selection of transformed clones. The recombinant plasmids were respectively named pET32a (+)–*ThPOD1*, pET32a(+)–*ThPOD2*, and pET32a(+)–*ThPOD3*. The cloned genes were confirmed by restriction digestion and DNA sequencing.

Induction and expression of recombinant proteins

When *E. coli* Rosetta gami (Novagen, USA) cultures reached the exponential growth phase, isopropyl-*β*-D-thiogalactopyranoside (IPTG) was added to a final concentration of 0.1 mM in order to induce expression of the inserted gene. In order to confirm the expression of inserted genes in *E. coli* Rosetta gami cells, SDS–PAGE was carried out using the method of Sambrook and Russell [19]. The *E. coli* Rosetta gami transformed with the pET32a vector was used as a control group.

Table 1 The primer sequences used in internal references genes for qRT-PCR

Accession number	Gene name	Forward primer nucleotide sequence (5′-3′)	Reverse primer nucleotide sequence (5′-3′)
(EG971352)	*β*-actin	AAACAATGGCTGATGCTG	ACAATACCGTGCTCAATAGG
(EH050602)	α-tublin	CACCCACCGTTGTTCCAG	ACCGTCGTCATCTTCACC
(EH052343)	*β*-tublin	GGAAGCCATAGAAAGACC	CAACAAATGTGGGATGCT
(EF416283)	18s rRNA	GTAGTTGGACCTTGGGGTGG	CATTACTCCGATCCCGAAAGCC

Table 2 The primer sequences used in constructing prokaryotic expression vector

Gene name	Forward primer nucleotide sequence (5′-3′)	Reverse primer nucleotide sequence (5′-3′)
ThPOD1	ATCG<u>GGATCC</u>CAAGCACAAAAAAGCCTACCGCCG	CGAT<u>CTCGAG</u>TGTATTCTGACCGCCTTCCATACTC
ThPOD2	ATCG<u>GGATCC</u>GGGTTGTCCTGGTCGTTTTACAGT	CGAT<u>CTCGAG</u>TGGCATTTCTGGCAAAACAATTTGC
ThPOD3	ATCG<u>GGATCC</u>GCCGGATTACTTCGCCTTCATTTC	CGAT<u>CTCGAG</u>TGGCATTTCTGGCAAAACAATTTGC

Purification of ThPOD1 and polyclonal antibody preparation

The recombinant protein ThPOD1 was purified using His-Bind Column Kits as described by the manufacturer (Novagen, Madison, WI). The protein concentration was analyzed using the Bradford technique, and total bacterial protein was resolved on 12% SDS–PAGE. The ThPOD1 fusion protein was cut out of the gel, ground into powder, and lysed in physiological salt solution. The solution was mixed with Freund's complete adjuvant at a ThPOD1 concentration of 1–2 mg/ml, and an adult rabbit was subcutaneously injected with 0.5 ml every week for a total of 4 weeks. About 35 days later, the rabbit was sacrificed and the polyclonal antibody was obtained from the total blood.

Western blotting characterization

Western blots were performed as described [20], with some modifications. The anti-ThPOD1 polyclonal antibody was used to recognize ThPOD2 and ThPOD3. Immunoreactive bands were visualized by the ECL method (Amersham Life Sciences, Buckinghamshire, UK).

Abiotic stress tolerance assay in transformed *E. coli* cells

In order to evaluate the protective abilities of ThPOD1, ThPOD2 and ThPOD3 fusion proteins, the effects of salt (0.6% w/v NaCl) and drought (20% w/v PEG6000) on the growth of transformed *E. coli* Rosetta gami cells with pET32a (empty vector) and pET32a–ThPOD1 (recombinant plasmid), pET32a–ThPOD2 (recombinant plasmid) and pET32a–ThPOD3 (recombinant plasmid) were examined. After cultures were adjusted to OD_{600} 0.6, IPTG was added to a final concentration of 0.1 mM in order to induce expression of the inserted genes. After incubation for 12 h at 20°C, the *E. coli* cells were added respectively to LB liquid culture containing NaCl, PEG and ABA. The original OD_{600} values of all *E. coli* groups were adjusted to the same value, and then culture of recombinant *E. coli* and control strains continued at 37°C. OD values were recorded every hour. The experiments were repeated six times, and the mean and standard deviation were calculated.

Determination of *E. coli* survival ratio under abiotic conditions

Transformed and control *E. coli* were diluted serially as above and spread onto LB plates containing 0.6% NaCl and

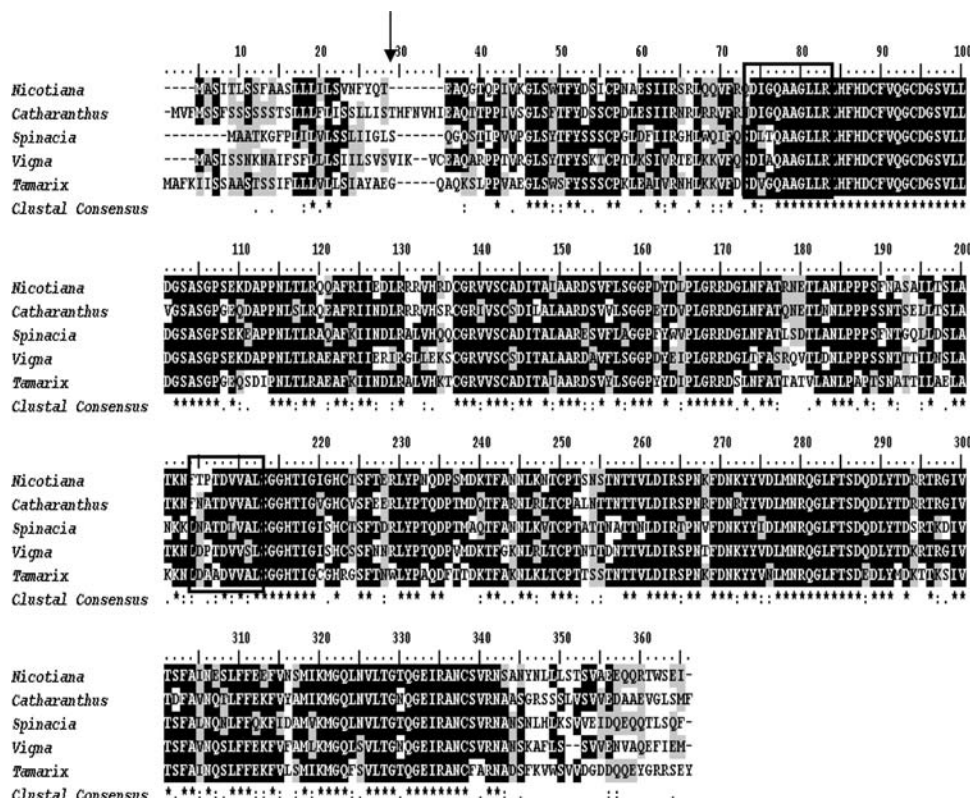

Fig. 1 Alignment of the deduced amino acid sequence of the product of *ThPOD1* from *T. hispida* with those of POD genes from other plants. Conserved residues such as those of the POD active site and distal and proximal heme binding sites are indicated with *black boxes*. *Arrows* indicate the signal cleavage site, sequence data were obtained from NCBI database and aligned using ClustalX and GeneDoc. (GenBank Accession Nos. of PODs are as follows: gi AAK52084 for Nicotiana tabacum, gi CAJ84723 for *Catharanthus roseus*, gi CAA71490 for *Spinacia oleracea*, gi BAA01950 for Vigna

20% PEG. After the plates were incubated for 1–2 days at 37°C, the number of colonies on each plate was recorded. The survival ratio of the transformants under abiotic conditions was calculated according to the following formula:

$$\text{Survival ratio} = (\text{mean number of colonies on stressed plate}/\text{mean number of colonies on LB plate}) \times 100\%.$$

Experiments were repeated six times with three replicate plates each time. Mean survival ratios and standard deviations were calculated.

Results and discussion

cDNA cloning and sequence analysis of putative *ThPOD1*

A 1,376 bp gene (*ThPOD1*) was isolated from a *T. hispida* NaCl-stress root cDNA library [16]. This gene was highly homologous to peroxidase genes in other plant species. The predicted molecular mass and pI of the *ThPOD1* product are 39.3 kDa and 6.59, respectively. The encoded protein contains an N-terminal signaling peptide that could guide the protein to vacuoles or to a secretory pathway. The molecular mass and theoretical pI of the deduced mature peptide are 36.28 kDa and 6.65, respectively.

Sequence alignment was performed with homologues in other plant species using the NCBI database (http://www.ncbi.nlm.nih.gov/sites/gquery). The analysis indicated that *ThPOD1* is highly homologous to other plant PODs such as Nicotiana tabacum (GenBank accession no. AAK52084; 64% homology), *Catharanthus roseus* (GenBank accession no. CAJ84723; 65%), *Spinacia oleracea* (GenBank accession no. CAA71490; 66%), and Vigna (GenBank accession no. BAA01950; 67%). As presented in Fig. 1, the essential active center (aa 74-84: AglLRLhFHDC), the distal and proximal heme binding site (aa 204-213: VVALSGGHTI), the two putative calcium binding sites (212, 265) and the eight cysteine residues necessary for the formation of four disulfide bridges (51–132, 84–89, 138–333, 218–245) conserved in class III peroxidases were found to be highly conserved in all species tested. These regions are common in plant PODs and are reported to be important for catalysis and protein folding [21]. These findings showed

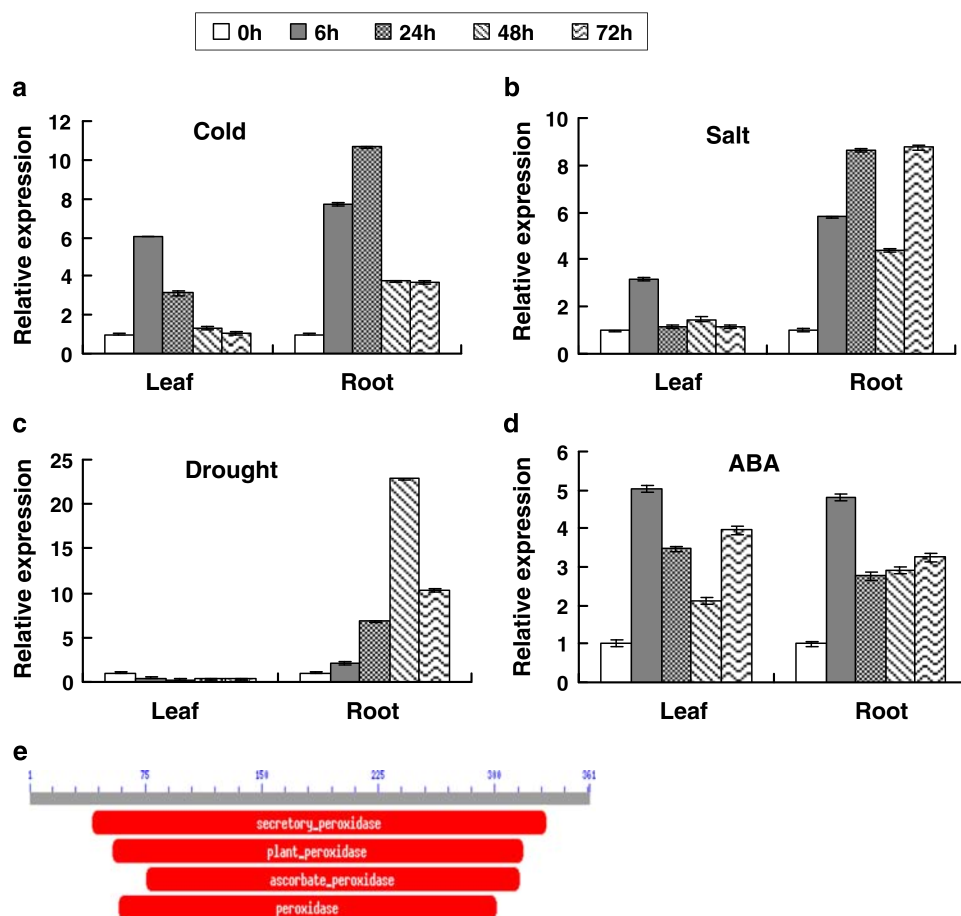

Fig. 2 *ThPOD1* gene expression levels under different abiotic stresses assayed using qRT-PCR. **a** Low temperature stress (4°C); **b** salt stress (400 mM NaCl); **c** drought stress (20% PEG6000); **d** ABA stress (100 μM). qRT-PCR data was normalized using *T. hispida* β-actin, α-tublin, β-tublin genes, 18s rRNA and results are shown relative to 0 h. Histogram shows the standard deviation from six replicates performed for each time point. **e** Conserved domains of ThPOD1 protein obtained via NCBI searches

that *ThPOD1* belongs to the POD family. However, the characteristics and function of *ThPOD1* remained to be elucidated.

Expression patterns of *ThPOD1* under different abiotic stress conditions

The steady-state mRNA levels of *ThPOD1* under the stress of cold, high salt, drought stress and ABA treatment were assayed with qRT-PCR (Fig. 2a–d). Under cold treatment (4°C), the mRNA level of *ThPOD1* in leaf tissues increased dramatically at 6 h and then gradually decreased; in root tissues, the expression of *ThPOD1* rapidly increased at 6 h and reached a peak at 24 h, then declined (Fig. 2a). Under high-salt conditions, leaf expression of *ThPOD1* was similar to with cold treatment, while in roots, *ThPOD1* was up-regulated 6 h after it was treated with high-salt, and at 24 and 72 h, expression was over eightfold higher than in the control sample (Fig. 2b). During drought stress, *ThPOD1* was down-regulated in leaves, but in roots, expression was obviously increased after 6 h and reached a maximum at 48 h, after which it began to decrease (Fig. 2c). Interestingly, expression of *ThPOD1* was also induced by ABA treatment, and large amounts of *ThPOD1*-mRNA were found in both leaves and roots (Fig. 2d). However, in sunflowers, it has been reported that the transcription of the homologous gene was suppressed by ABA [22]. Also, in rice leaves, the transcription level of a JA-inducible POX gene was reported to be reduced after treatment with ABA [23]. Many abiotic stress-inducible genes are controlled by ABA but some are not, indicating that both ABA-dependent and -independent regulatory pathways are involved in stress-induced gene expression [24, 25]. These results suggest that *ThPOD1* responds to ABA signals and is involved in ABA-dependent signal pathways.

Further in silico analysis indicated that secretory peroxidase, plant peroxidase, ascorbate peroxidase and peroxidase, show homology to amino acids 41-337 in *ThPOD1* (Fig. 2e). It is therefore likely that these domains play an important role in the response to abiotic stress. To further investigate the function of these domains, we expressed the full length gene and truncated polypeptides.

SDS–PAGE and Western blot analysis of recombinant polypeptide

Full-length cDNA and fragments (encoding functional regions of plant peroxidase and ascorbate peroxidase) were introduced into the expression vector pET32a (+) in order to allow expression as a Trx fusion protein in *E. coli*. SDS–PAGE analysis indicated that the molecular weights of the recombinant proteins agreed with the predicted molecular weights as shown in Fig. 3a (lanes 4, 5 and 6 arrowed).

To determine whether the recombinant proteins were successfully translated, immunoblotting was conducted using anti-ThPOD1 polyclone antibodies. ThPOD1, ThPOD2 and ThPOD3 were detected at band sizes of ~57, ~50, and ~47 kDa, respectively, which are the expected molecular weights of the translated products of *ThPOD1*, *ThPOD2* and *ThPOD3* cDNA (Fig. 3b).

Enhanced stress tolerance of recombinant *E. coli* harboring *ThPOD1* and *ThPOD2*, *ThPOD3* fragments.

To determine the function of the expressed fusion protein under stress, the effects of salt and drought stress on the growth of transformed *E. coli* and control strains were examined. The growth curve assay showed no apparent differences among recombinant *E. coli* harboring different plasmids or the control strain containing an empty vector

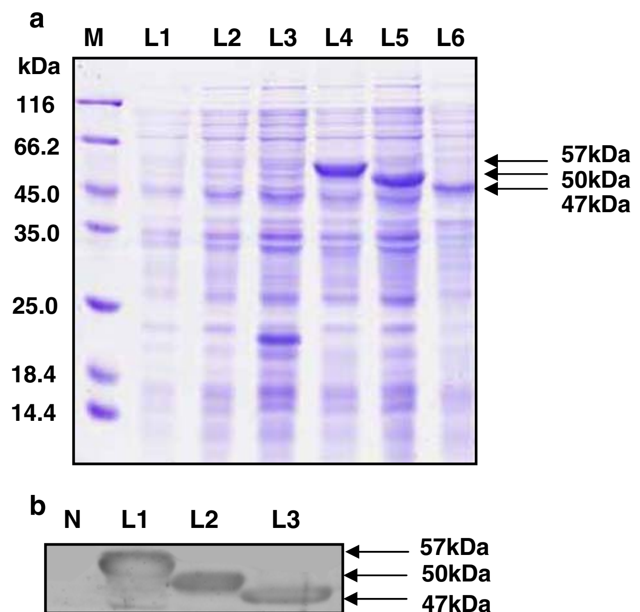

Fig. 3 **a** SDS–PAGE (12%) analysis of fusion protein expression in *E. coli* Rosetta gami (Coomassie blue staining). *Lane 1* (M), Protein marker; *lane 2* (L1), whole cell lysate of Rosetta gami *E. coli* cells containing the empty vector pET32a without IPTG induction; *lane 3* (L2), whole cell lysate of non-induced Rosetta gami *E. coli* cells containing the plasmid pET32a–ThPOD1; *lane 4* (L3), whole cell lysate of Rosetta gami *E. coli* cells containing the empty vector pET32a with IPTG induction; *lanes 5–7* (L4, L5 and L6), whole cell lysate of Rosetta gami *E. coli* cells containing the plasmid pET32a–ThPOD1, pET32a–ThPOD2 and pET32a–ThPOD3 obtained 4 h after induction with 0.1 mM IPTG, respectively. The bands corresponding to the products of *ThPOD1*, *ThPOD2* and *ThPOD3* cDNA are indicated by an *arrow* (~57, ~50 and ~47 kDa, respectively). **b** Western blot analysis using specific antibody against the ThPOD1 protein. *Lane 1* (N) negative control i.e., protein sample from *E. coli* cells containing empty vector (pET32a); *lanes 2–4* (L1, L2 and L3) samples from *E. coli* cells containing pET32a–ThPOD1, pET32a–ThPOD2 and pET32a–ThPOD3, and incubated with IPTG, respectively. The bands corresponding to the products of *ThPOD1*, *ThPOD2* and *ThPOD3* cDNA are indicated by an *arrow* (~57, ~50 and ~47 kDa, respectively)

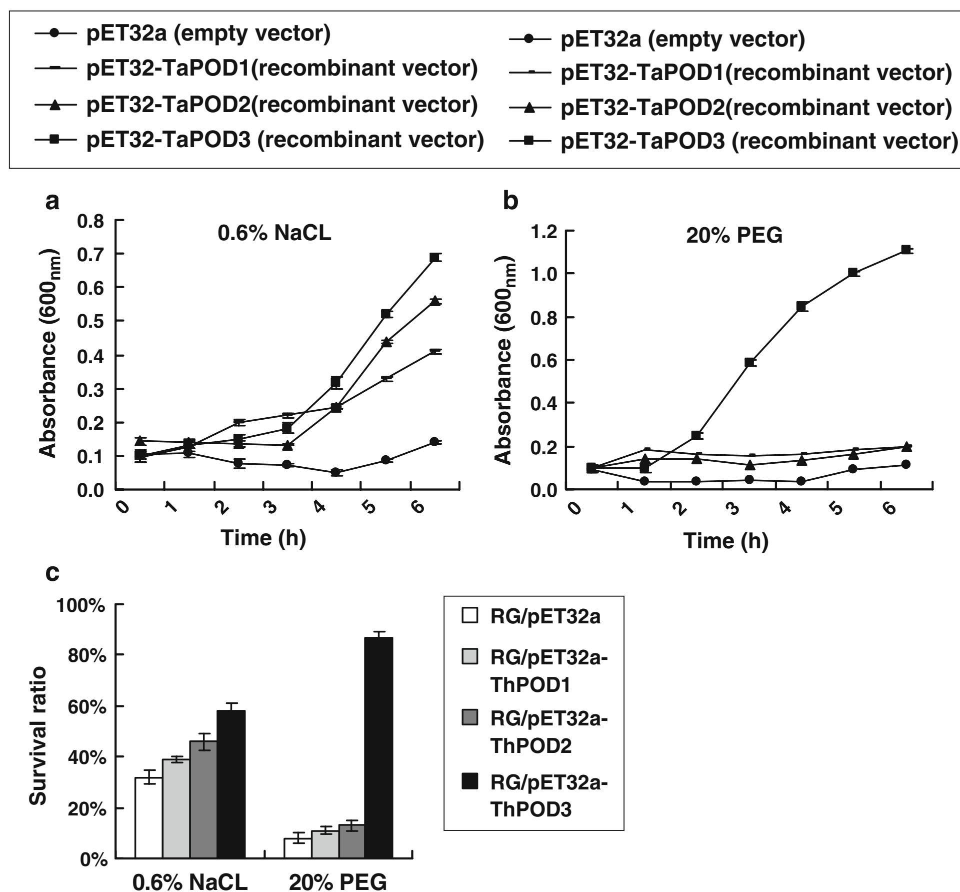

Fig. 4 Effect of **a** salt or **b** drought on the growth of *E. coli* cells transformed with recombinant plasmids (pET32a–ThPOD1, pET32a–ThPOD2 and pET32a–ThPOD3) or empty vector (pET32a). The means of six independent experiments are plotted with error bars indicating standard deviations. **a**, **b** Represent the *growth curves* of *E. coli* cells in liquid medium on exposure to salt and drought, respectively. **c** Survival ratio of transformed *E. coli* under abiotic stress conditions. Cultures of *E. coli* harboring ThPOD1, ThPOD2, ThPOD3 or an empty vector were spread on LB plates with additional NaCl and PEG. The number of colonies appearing on the plates were counted and used for calculating the survival ratios as described in "Materials and Methods". The means of six independent experiments are plotted with *error bars* indicating standard deviations

before abiotic stress (data not shown). However, under salt stress, the *E. coli* strain containing the empty vector showed reduced growth speed when compared to the transformed *E. coli* (Fig. 4a). Under drought stress, only *E. coli* harboring *ThPOD3* presented a close to normal growth rate; the other *E. coli* strains barely survived (Fig. 4b).

The experimental evidences provided by Lan et al. [26] and Yamada et al. [27] proved that the expression of foreign plant genes can directly contribute to increasing stress tolerance of the bacteria host cells. In plants, biotic and abiotic stresses can trigger the generation of reactive oxygen species (ROS), which disrupt cellular homeostasis and induce expression of genes involved in defense mechanisms [28]. Peroxidases play a key role in the detoxification of reactive oxygen species because they have an almost 1000-fold higher affinity for H_2O_2 when compared to catalases, and their activities can be modified by different stress factors [29]. Our results of enhancement of abiotic tolerance of the recombinant bacteria cells can indicate that expression of these polypeptides in host cells could confer protective function against damage of proteins, cellular membrane, and cells. For this reason, we speculated that ThPOD2 and ThPOD3 contain specific conserved regions such as POD active site and distal and proximal heme binding site (Fig. 1 indicated with black boxes). When abiotic stress was applied, these protein or polypeptides in the cells might be interacting with excessive ROS, and could protect the host cells survival from excess H_2O_2 toxicant by stabilizing the structure of protein and cellular membrane in stressed cells. These results are consistent with those observed for other POD proteins in transgenic wheat [30], tobacco [31].

In additional, on a relatively solid medium, the survival ratio of *E. coli* harboring *ThPOD* 3 was over two and tenfold higher than that of control *E. coli* grown on LB

plates supplemented with 0.6% NaCl and 20% PEG, respectively (Fig. 4c). Based on the results, we speculated that ThPOD3 encodes acrobate peroxidase, which in higher plants has been shown to increase in response to various stressors such as drought, ozone, chemicals, salt, heat and microbial infection [32–35], and the enzyme activity of ThPOD3 in protecting *E. coli* cells from abiotic stress might show some difference from that of ThPOD1 or ThPOD2. The more details in protective mechanism as for the aa 73-337 functional region of ThPOD1 on abiotic tolerance of cells are under study.

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