

Phytoene Synthase Gene (*PSY*) from Sweet Potato (*Ipomoea batatas* Lam.) Enhances Tolerance to Abiotic Stress

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ABSTRACT

Phytoene synthase (*PSY*) is the rate-limiting enzyme for carotenoid biosynthesis. To date, several studies focused on *PSY* genes in the context of abiotic stress responses. In this study, two phytoene synthase encoding genes, *IbPSY1* and *IbPSY2*, were identified from a published transcriptome and bioinformatic analysis showed that they shared conserved domains with phytoene synthases from other plants. The *IbPSY1* gene was cloned and carefully characterized. Digital gene expression profiling (*DGE*) showed that the highest transcription level of *IbPSY1* was in young leaves, and the lowest level was in stems. *In vivo* expression levels of *IbPSY1* under abiotic stress were observed to be highest in stems at day 11. Over-expression of *IbPSY1* in *Escherichia coli* and yeast cells endowed the cells with better growth under salt and drought stress than the control cells. This study demonstrated that *IbPSY1* not only played an important role *in vivo*, but also in *E. coli* and yeast to improve tolerance to salinity and drought stress. Thus, *IbPSY1* may be aid in the development of transgenic plants with enhanced stress tolerance.

Keywords: *Ipomoea batatas*, phytoene synthase, abiotic stress.



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INTRODUCTION

Sweet potato (*Ipomoea batatas* Lam.) is a major root crop that is grown for human consumption, particularly in Sub-Saharan Africa, parts of Asia and the Pacific Islands. Many parts of the plant are edible including its leaves, roots and vines, and varieties exist with a wide range of skin and flesh colors, ranging from white to yellow-orange and deep purple¹⁻³. Its rich nutritional content and high adaptability to marginal land allow it to support human nutrition and food security in the developing world⁴. However, environmental stresses, such as salinity and drought, have adverse effects on the growth and productivity of sweet potato crops⁵⁻⁷.

Carotenoids are the most common group of pigments found in nature, featuring rich conjugated double bond systems⁸. In plants, carotenoids are essential components involved in photoprotection⁹, photosynthesis¹⁰ and the production of carotenoid-derived phytohormones¹¹. Phytoene synthase (PSY) catalyzes the conversion of two molecules of geranylgeranyl diphosphate (GGPP) to phytoene, playing a pivotal role in the carotenoid pathway as the first committed biosynthetic step and controlling flux through the pathway¹². Multiple *PSY* genes have been well characterized in many plants and exhibit tissue-specific expression that might affect the ability to control carotenogenesis independently of photosynthesis or occur in response to certain stresses¹³. For instance, *PSY1* is expressed in fruits and flowers of tomatoes, while *PSY2* is primarily expressed in leaves¹⁴.

In previous studies, over-expression of the endogenous *Arabidopsis* phytoene synthase gene results in increased levels of carotenoids¹⁵. *PSY* from *Salicornia europaea* was found to alter responses to reactive oxygen species under salt stress in transgenic *Arabidopsis*¹⁶. Hence, studies have increasingly focused on the anti-stress functions of *PSY*. To date, the function of *PSY* from sweet potato has not been studied in the context of abiotic stress. Here, we cloned the *PSY* gene (*IbPSY1*) from sweet potato and characterized its molecular function. We determined that the gene responded significantly to both salinity and drought stress. The functionality of the *IbPSY1* gene was also determined in prokaryotic (*E. coli*) and eukaryotic (*Saccharomyces cerevisiae*) cells.

MATERIAL AND METHODS

Plant materials and growth condition

Sweet potato (*Ipomoea batatas* Lam., cv. Xushu 18) was used in this study, planted at 26 ± 1 °C air temperature and grown under cool white fluorescent lamps providing a 16-h photoperiod. The salt and drought stress experiments were performed by supplementing the media with 300 mM NaCl and 10% (w/v) PEG 6000 for 20 days, respectively¹⁷. Young leaves, stems and expanding tuberous roots were collected from various samples, immediately washed and frozen in liquid nitrogen. At least three independent replicates of each experiment were performed.

Amplification and sequence analysis of *IbPSY1*

Total RNA was extracted using Trizol (Invitrogen, USA). The quality and the concentration of the RNA was determined by 1.0% agarose gel electrophoresis and a NanoDrop 2000 spectrophotometer (Thermo, USA). One microgram of total RNA was used for first-strand cDNA synthesis by a PrimeScript First Strand cDNA Synthesis Kit (Invitrogen, USA). The full-length coding sequence of *IbPSY1* was amplified from cDNA using the gene-specific primers *IbPSY1*(SLIC)-F and *IbPSY1*(SLIC)-R (Table 1). The vector DNA fragment was cloned from the pET32a(+) using primers PET(SLIC)-F and PET(SLIC)-R. Next, the PCR products were subcloned into pET32a(+) vector using sequence and ligation-independent cloning (SLIC)¹⁸.

Genomic analysis was based on our high-throughput sequencing data (GenBank accession no. JP104589 to JP160056) ¹⁹. Protein modification sites were predicted with Proscan (http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_proscan.html). An analysis of protein physical properties was carried out by the On-line Analysis System (http://web.expasy.org/compute_pi/). Sequence similarities were examined using the BLAST program (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) and searching against the GenBank database. The amino acid sequences of the cloned cDNA fragments were deduced, and protein sequences were aligned using the program DNAMAN 6.0 (Lynnon Biosoft, Quebec, Canada). Phylogenetic relationships were determined using Clustal X2 with the Neighbor-Joining method and 1000 bootstrap replicates ²⁰.

Table 1. Primer sequences used for gene cloning and expression analysis in this work

Primers	Sequences (5' to 3')
IbPSY1(SLIC)-F	GACAAGGCCATGGCTGATATCATGTCTAGTGCCTTGCTGTGGG
IbPSY1(SLIC)-R	GTCGACGGAGCTCGAATTCTCAAGCTTTTGCCAGAGGGG
PET(SLIC)-F	CCCCTCTGGCAAAGCTTGAGAATTCGAGCTCCGTCGAC
PET(SLIC)-R	CCCACAGCAAGGCACTAGACATGATATCAGCCATGGCCTTGTC
IbPSY1-qF	CTGTGGGTCGCTTGTTTT
IbPSY1-qR	CAAGACGATGTCGCTGAGTAT
IbActin-qF	GGTGTATGGTTGGGATGGGAC
IbActin-qR	GGTAAGAAGGACAGGGTGCTC

F: Forward primer sequence R: Reverse primer sequence

Digital gene expression profiling

Using the Illumina pipeline, DGE tags were generated from three different tissues: young leaves, stems and expanding tuberous roots. Next, the tags were mapped to the complete CDS of *IbPSY1* by Bowtie with only one base mismatch using Galaxy's web platform ^{21, 22}. All clean tags corresponding to the *IbPSY1* gene provided a raw expression level in the different tissues, and the expression levels were normalized using the TPM algorithm. This approach generated an accurate and reasonable measurement of gene expression.

Expression analysis of *IbPSY1* by real-time RT-PCR

Total RNA was extracted from young leaves, stems and expanding tuberous roots of sweet potato under three growth conditions: normal, high-salt and drought. After first-strand cDNA synthesis, qRT-PCR was carried out with primers *IbPSY1*-qF and *IbPSY1*-qR (Table 1) with the SsoFast™ EvaGreen® Supermix (BIO-RAD, Singapore) on a Bio-Rad CFX96 Real-time PCR machine. The amplification procedure was 95 °C for 10 s initially, followed by 40 cycles, each comprising 95 °C for 10 s, 60 °C for 15 s and 72 °C for 20 s and 3 min at 72 °C thereafter. In addition, the *actin* gene (GenBank accession no. EU250003.1) from *I. batatas* was used as the internal reference.

Expression of *IbPSY1* in *E. coli* BL21 and yeast

The recombinant plasmid (pET32-*IbPSY1*) constructed by the SLIC method was transformed into *E. coli* BL21. A single colony of *E. coli* strain BL21 harboring pET32-*IbPSY1* or the empty vector pET32a(+) was inoculated at 37 °C in LB liquid medium containing ampicillin (100 mg/L) with shaking (180 rpm) until the OD600 value reached 0.6. Protein expression was induced by addition of isopropyl-β-D-thiogalactoside (IPTG) to a final concentration of 1 mM and the cultures were grown at 18 °C for 16 hours. Expressed proteins were visualized by SDS-PAGE.

To construct the yeast expression vector, pYES2 and pET32-*IbPSY1* were digested with *EcoRV* and *EcoRI* and later re-ligated to construct the expression vector pYES2-*IbPSY1*. The recombinant plasmid was extracted from *E. coli* and validated by

sequencing. pYES2-IbPSY1 and pYES2 were transformed into *S. cerevisiae* INVSc1 by electroporation with a capacitance of 25 μ F, a resistance of 186 Ω and a voltage of 1500 V. Samples were plated onto SD-URA solid medium at 30 °C and incubated for 48 h before being confirmed by PCR.

Functional analysis of IbPSY1 in *E.coli* and yeast

For *E. coli*, the OD600 value of the induced culture was diluted to 0.8. Next, 500 μ L of culture from each treatment was inoculated in 50 mL LB medium containing 0.8 M NaCl and 30% PEG. The effects of salt and drought on the growth of transformed *E. coli* BL21(DE3) cells with pET32a(+) (empty vector), and the pET32-IbPSY1 were examined every 4 h. The growth curve of the recombinant strains was compared to the control cells.

To evaluate the functional significance of IbPSY1 with respect to salinity and drought stress in yeast, transformants containing pYES2-IbPSY1 and pYES2 (empty vector) were grown on SC-URA solid medium with 2% glucose and incubated for 24 h at 30 °C²³. Subsequently, a fixed number of cells from each treatment (approximately 1×10^7 cells) were used for the abiotic stress experiments. For high-salinity stress, 5 M NaCl solution was used. After centrifugation, yeast cells were incubated in the presence of 5 M NaCl and placed at 4 °C for 24 h. Next, cells were diluted 1000-fold and plated on SC-URA solid medium. Cells were cultured at 30 °C until single colonies were grown, and the number of colonies was determined. Similarly, to analyze the tolerance to drought stress in transgenic yeast, 4 M sorbitol solution was used following the above method.

RESULTS

Isolation and bioinformatic analysis of IbPSY1

We identified two full-length *PSY* encoding sequences based on the results of transcriptomic database established previously in our laboratory. The two encoded proteins shared 80.2 % amino acid sequence identity (Figure 1A). The *IbPSY1* gene was isolated and characterized from the cDNA of sweet potato. The PCR product was approximately 1.3 Kb, as expected. The predicted protein molecular weight was 49 kD, and its theoretical pI was 9.18. An evaluation of the hydrophilicity of IbPSY1 was -0.253 such that it could be a hydrophilic protein.

Putative post-translational modification sites were also found in IbPSY1, including four N-glycosylation sites, five protein kinase C phosphorylation sites, four casein kinase II phosphorylation sites, one tyrosine kinase phosphorylation site, two N-myristoylation sites and one amidation site. Homology analysis for the *PSY* protein sequence obtained in this study was carried out using BLAST software at the NCBI server. The results demonstrated that IbPSY1 was homologous to known *PSY* sequences from other species, including *Ipomoea sp. Kenyan* (96% sequence identity), *Nicotiana tabacum* (84%), *Nicotiana glauca* (84%), *Gardenia jasminoides* (83%), *Nicotiana glauca* (83%), *Coffea canephora* (83%), *Lycium barbarum* (82%), *Solanum tuberosum* (82%) (Figure 1B). Based on analysis using Clustal X2, the homology tree for the deduced amino acid sequences of IbPSY1 with eight other plants showed that IbPSY1 had the highest sequence identity with the *PSY* of *Ipomoea sp. Kenyan* (Figure 1C).

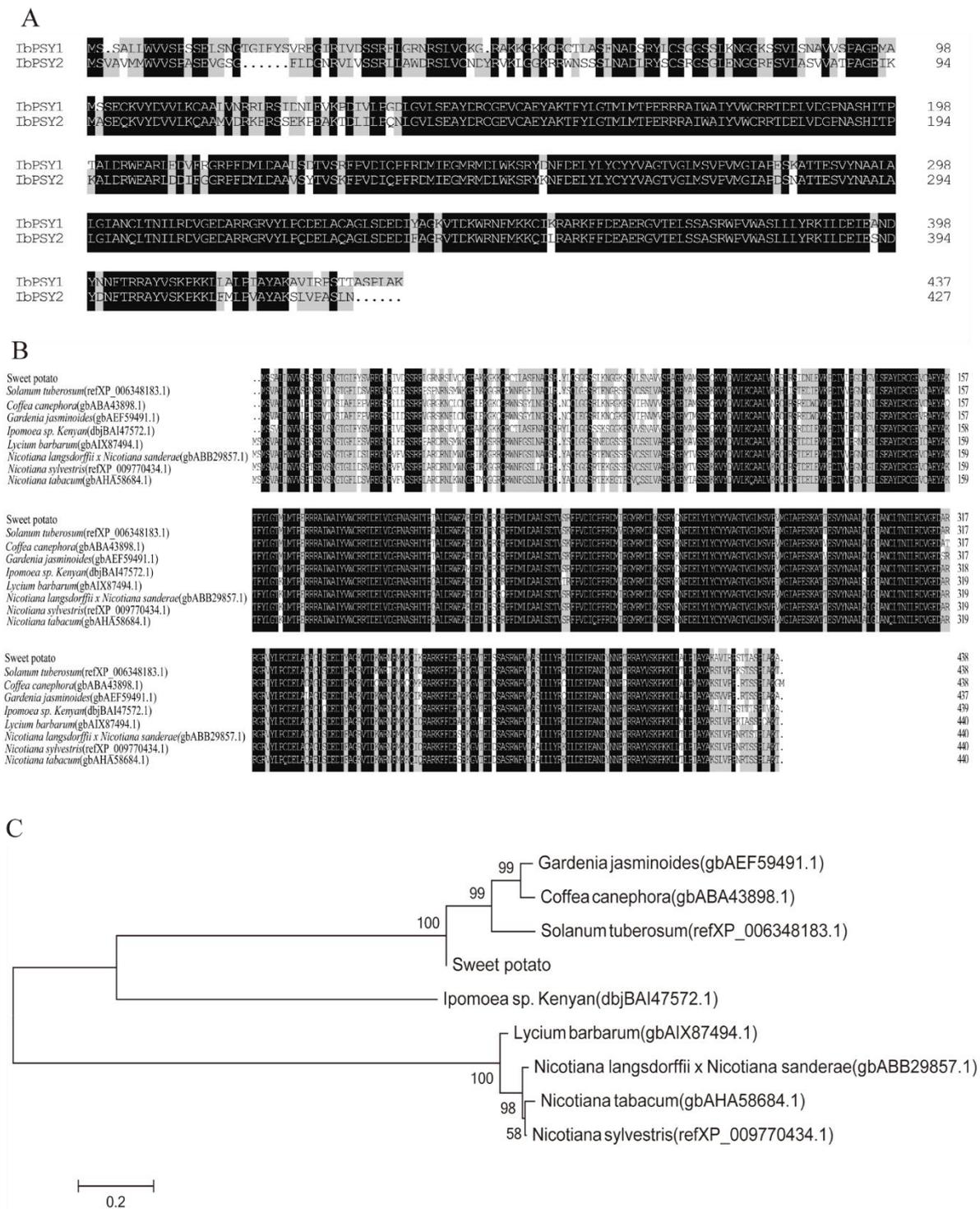


Figure 1. IbPSY1 protein sequence alignment and phylogenetic analysis. (A) Alignment of IbPSY1 and IbPSY2 based on full-length amino acid sequences; (B) Multiple sequence alignment of IbPSY1 with PSY sequences from other plants. The highly conserved regions are shaded; (C) Phylogenetic analysis of IbPSY1 with homologues from other plants.

Digital gene expression profiling

To further characterize the *IbPSY1* gene, DGE profiling was used to determine expression levels in various tissues. The transcriptional level of *IbPSY1* in different tissues is shown in Figure 2. Young leaves (53 transcripts per million clean tags) had

higher levels of *IbPSYI* expression than did stems (5 TPM) or expanding tuberous roots (8 TPM).

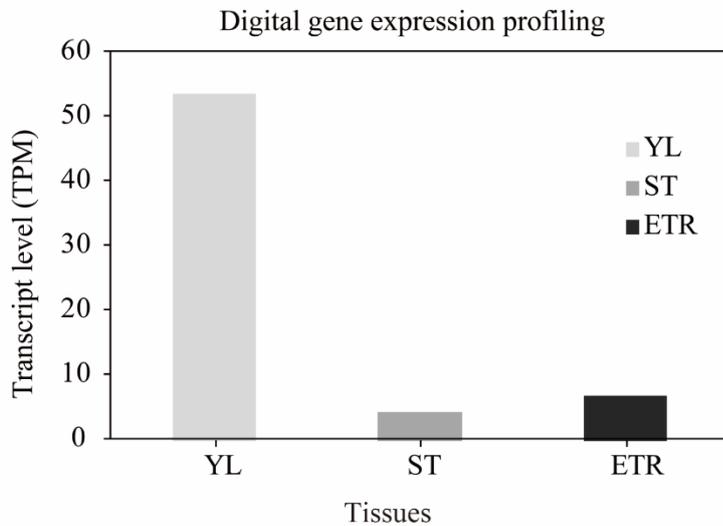


Figure 2. The expression level of *IbPSYI* in different sweet potato tissues by digital gene expression profiling (TPM means transcripts per million). YL, young leaf; ST, stem; ETR, expanding tuberous root.

Expression patterns of *IbPSYI* under abiotic stress *in vivo*

To determine the tissue specific expression patterns of *IbPSYI*, mRNA isolated from seedlings under different abiotic stresses was analyzed by qRT-PCR at 4 time points (0 D, 2 D, 11 D, 20 D). As shown in Figure 3, *IbPSYI* mRNA was induced and reached a maximum at 11 days post treatment with NaCl (300 mM) and PEG6000 (10% (w/v)). Expression of *IbPSYI* in both young leaves and stems was strongly induced by 300 mM NaCl during the first two days and continuously increased until day 11. As shown in Figure 3, *IbPSYI* mRNA reached a maximum at day 11 in stems, whereas young leaves showed lower levels of induction under the same stress. *IbPSYI* expression was reduced from day 11 onwards under salt stress. During PEG6000 treatment, *IbPSYI* mRNA also peaked at day 11, but its transcriptional level was low, and only a faint signal could be detected at other time points. All of the results mentioned above showed that *IbPSYI* could be induced by NaCl or PEG6000 treatment.

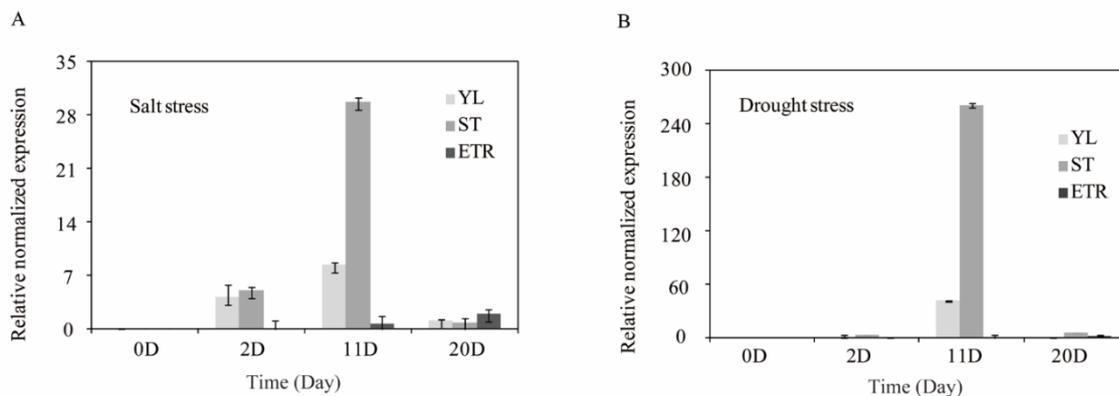


Figure 3. Expression patterns of *IbPSYI* under abiotic stress *in vivo*. (A) Normal growth conditions and after NaCl (300 mM) treatment; (B) Normal growth conditions and after PEG6000 (10% (w/v)) treatment.

Expression of the recombinant *IbPSYI*

The *IbPSYI* gene was expressed in *E. coli* BL21 (DE3) as a fusion protein with TrxA (approximately 17.6 kDa) and visualized by SDS-PAGE (Figure 4). The over-

expressed recombinant protein was observed at 66.6 kDa, which was in agreement with its predicted size. However, there was no difference in protein expression level when it was induced by different concentrations of IPTG (0.1, 0.5, 1 and 2 mM).

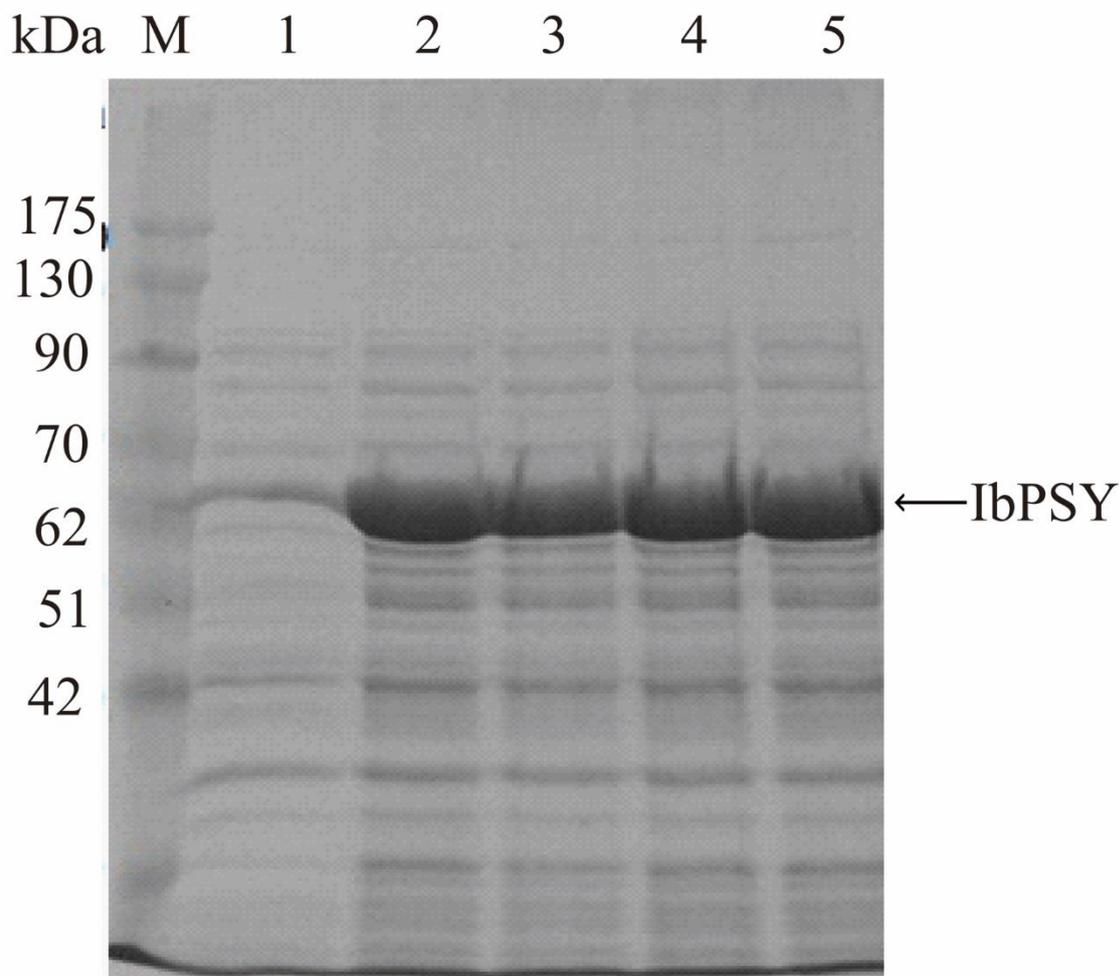


Figure 4. Analysis of recombinant IbPSY1 protein expression. SDS-PAGE analysis of IbPSY1 over-expression in *E. coli* BL21; Lane M, protein marker; lane 1, crude extracts of *E. coli* BL21 containing the pET32- IbPSY1 without IPTG induction; lane 2-5, crude extracts of *E. coli* BL21 containing the pET32-IbPSY1 with different concentrations of IPTG (0.1, 0.5, 1 and 2 mM) respectively.

Expression of IbPSY1 in *E. coli* enhances growth during stress

Standard growth curves showed that there was no significant difference in growth rate between the control strain and recombinant strain (Figure 5A), with the control strain showing only slightly better growth than the cells harboring pET32-IbPSY1. This phenomenon is probably due to the burden imposed by the over-expression of a foreign protein on the growth of *E. coli*.

The growth of the recombinant strain was first arrested, but exponential growth began after a lag phase of 20 h when grown in LB liquid medium containing 800 mM NaCl. For the control strain, the lag phase lasted only 3 h. In general, the recombinant strain displayed improved growth compared with the control strain when exposed to 800 mM NaCl.

To determine if the expression of IbPSY1 altered the drought tolerance of *E. coli*, the two strains were grown in LB liquid medium containing 30% PEG6000. Similarly, the drought tolerance of *E. coli* cells harboring pET32-IbPSY1 was improved compared to

that of the control strain. The recombinant strain exhibited a shorter lag period (24 h) than the control strain (44 h) (Figure 5B).

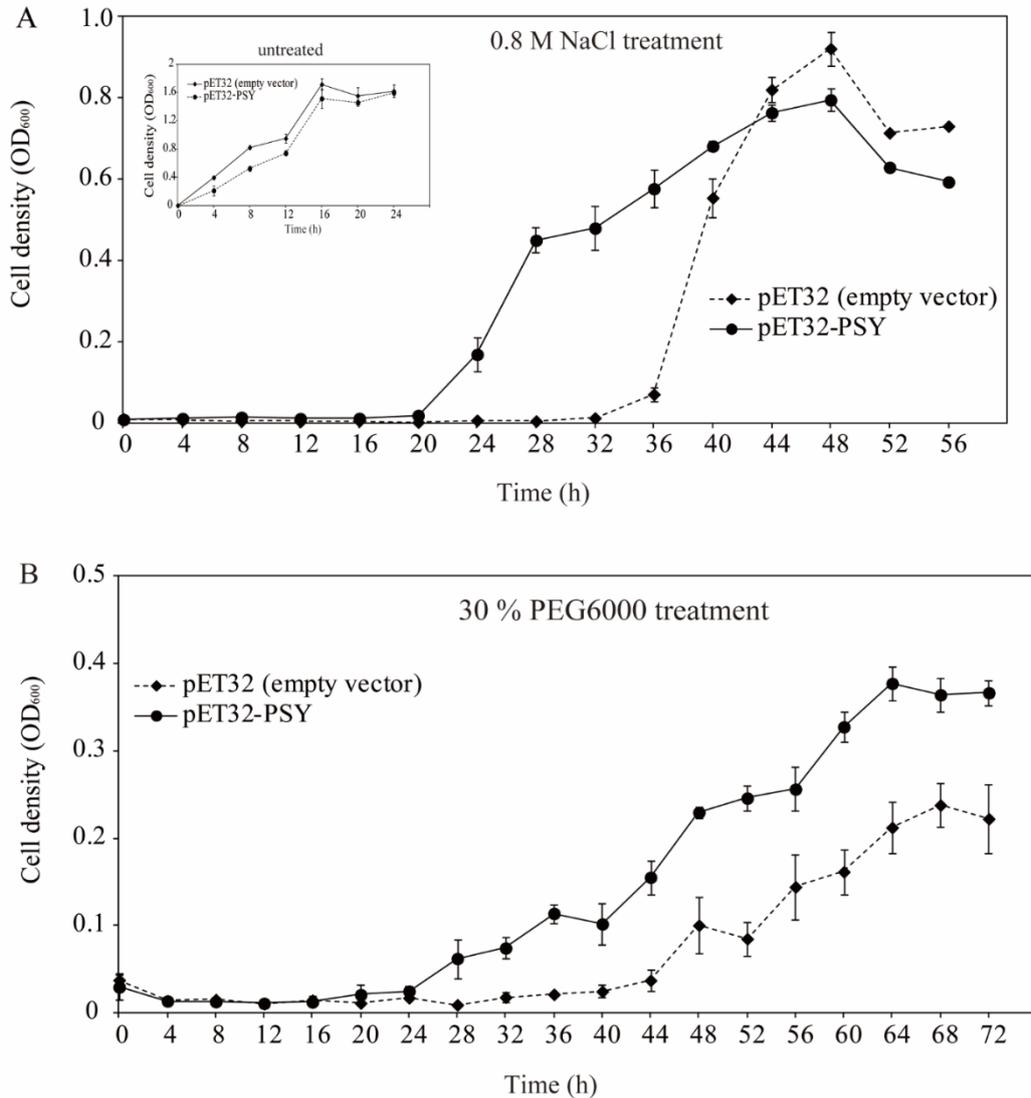


Figure 5. The growth curves of IbPSY1 in *E. coli* BL21 under salt or drought stress. (A) Growth curves of IbPSY1 in *E. coli* BL21 harboring pET32-IbPSY1 (circles), pET32a(+) (diamond) under salt stress; (B) Growth curves of IbPSY1 in *E. coli* BL21 harboring pET32-IbPSY1 (circles), pET32a(+) (diamond) under drought stress.

Expression of IbPSY1 in yeast enhances growth during stress

To analyze the possible function of IbPSY1, IbPSY1 was over-expressed in *S. cerevisiae* from the expression vector pYES2. Yeast cells were pre-treated by either salt (5 M NaCl) or drought (4 M sorbitol) stress and were then diluted and spread onto SC-URA solid medium plates, with the number of colonies on the plates being counted thereafter. The result showed that there was an obvious difference in yeast cells harboring IbPSY1 compared to the control. Figure 6 shows that cell viability decreased rapidly in the presence NaCl or PEG6000, but yeast cells expressing IbPSY1 exhibited higher survival rates than control cells. Following a 24-h salt shock, the survival rate of cells without IbPSY1 was approximately 52.4% of the cells expressing IbPSY1. Similarly, after 24 h of drought treatment, the survival rate of cells without IbPSY1 was approximately 44.8 % of the cells expressing IbPSY1. These results indicated that IbPSY1 improved tolerance to salt and drought stresses in both *E. coli* and *S. cerevisiae*.

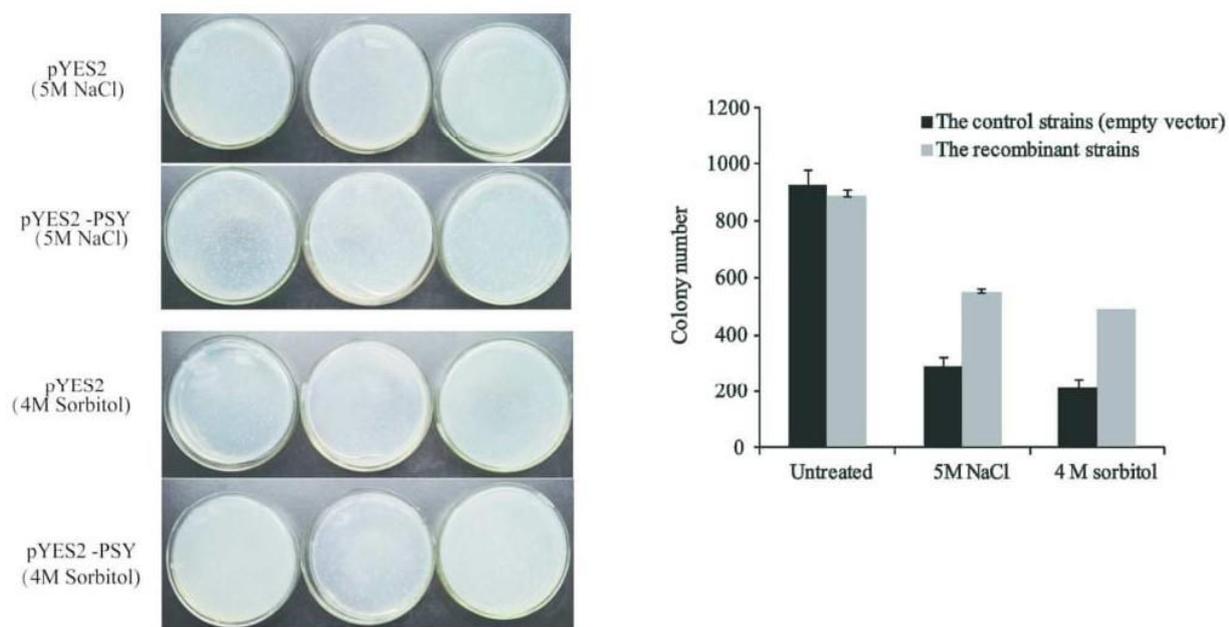


Figure 6. Growth of IbPSY1 in yeast under salt or drought stress. (A) Salt tolerance of yeast cells harboring pYES2-IbPSY1 or pYES2; (B) Drought tolerance of yeast cells harboring pYES2-IbPSY1 or pYES2. (C) Number of yeast cells under different abiotic stress conditions.

DISCUSSION

At present, the continuous deterioration of the environment means that plants are much more likely to suffer a variety of stresses. High salinity and drought stress are major environmental factors that limit crop productivity in arid and semi-arid regions²³⁻²⁷. Carotenoids are essential for photosynthesis and for photoprotection. In addition, carotenoids also serve as precursors to signaling molecules that influence plant development and the biotic/abiotic stress response²⁸. PSY is an important rate limiting regulatory enzyme in the carotenoid pathway, constituting the first committed step and a bottleneck in carotenogenesis⁸. Overexpression of the *PSY* gene in *Narcissus pseudonarcissus* L²⁹, *Solanum tuberosum*³⁰, and *Zea mays*³¹ resulted in significantly increased carotenoid levels. PSY is also transcriptionally responsive to drought, temperature, photoperiod, ABA, salt and post-transcriptional feedback regulation³².

Previous work on the genetic manipulation of *PSY* isolated from *Salicornia europaea* conferred tolerance to salt and oxidative stress in transgenic *Arabidopsis*¹⁶. To date, there have been no reports regarding whether PSY from sweet potato (*Ipomoea batatas*) could improve salt and drought stress tolerance in plants. In this study, we cloned and characterized a full-length cDNA clone encoding IbPSY1 from sweet potato. Bioinformatic analysis showed that IbPSY1 contained an ORF of 1314 bp and encoded a protein of 438 amino acids. Generally, most protein functions are regulated by phosphorylation/ dephosphorylation³³. Six types of putative phosphorylation sites were found in IbPSY1, including N-glycosylation, protein kinase C phosphorylation, casein kinase II phosphorylation, tyrosine kinase phosphorylation, N-myristoylation and amidation sites. These modification sites are important for the function of phytoene synthase³⁴⁻³⁶, especially the N-myristoylation site, which is necessary for salt tolerance in plants³⁶. Therefore, we speculated that this protein may be involved in the regulation of stress responses. We examined the transcript levels of IbPSY1 in seedlings that were treated with salt and drought stress and found that the expression of IbPSY1 in both young leaves and stems was strongly induced by salt or drought

stress at day 11 and continuously increased for the next 20 days. Interestingly, *IbPSY1* mRNA peaked at day 11 in stems in both stress conditions. However, transcription of *IbPSY1* was highest in young leaves of sweet potato grown under normal conditions. According to the expression profiles of carotenoid biosynthetic genes in *Brassica rapa*, the *BrPSY* gene exhibited a higher expression level in leaves than in stems³⁸. Similarly, when maize inbred B73 seedlings were subjected to salt stress, *PSY* transcript levels were barely altered in leaves, but increased in roots within 30 min³⁹. These results are consistent with the expression level of *IbPSY1*, implying that *IbPSY1* may play an important role in stems in the response to salt or drought stress. Growth measurements in salt or drought stress media revealed that *E. coli* cells with pET32-*IbPSY1* exhibited a shorter lag period and better growth than the control cells. This finding indicates that *IbPSY1* may have different protective functions in *E. coli*. Our study also shows that the expression of *IbPSY1* confers salt and drought tolerance by enhancing the survival of yeast cells. Salt and drought stress affect plant growth and development, resulting in a knock-on effect on crop yields. In summary, analysis of the stress tolerance effect of *IbPSY1* suggests that this gene functions not only in sweet potato but also in microbes. This study improves our understanding of how *IbPSY1* functions during stress and might help us to enhance the abiotic stress tolerance of crops by genetic engineering methods in the future.

CONCLUSION

In this study, *IbPSY1* was shown to increase tolerance to abiotic stresses in *E. coli* and yeast cells. In addition, the expression level of *IbPSY1* was affected by drought or salt stress. Therefore, we conclude that *IbPSY1* plays an important role in the defense response of plants to abiotic stresses *in vivo* and may be beneficial in plant anti-stress genetic engineering.

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