

MOLECULAR CLONING AND FUNCTIONAL ANALYSIS OF THE OSGLYOXALASE II GENE UNDER SALT STRESS TOLERANCE USING *Saccharomyces cerevisiae* AS A EUKARYOTIC MODEL SYSTEM

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The glyoxalase pathway constitutes a primary cellular defense mechanism against the accumulation of methylglyoxal (MG), a reactive cytotoxic byproduct generated under normal and stress-induced metabolic flux; through the coordinated action of glyoxalase I (GLY I) and glyoxalase II (GLY II), MG is detoxified to D-lactate using glutathione (GSH), thereby safeguarding proteins, nucleic acids, and other biomolecules from glycation and oxidative damage under adverse environmental conditions (Singla-Pareek *et al.*, 2005 and Mzid *et al.*, 2007). In plants, GLY II has emerged as a critical enzyme conferring enhanced tolerance to abiotic stresses as GLY II overexpression was shown to reduce MG accumulation and improve salt and osmotic stress resilience, indicating its essential role in stress-defense machinery and redox homeostasis (Mishra *et al.*, 2023). Salt stress is considered as one of the most prevalent abiotic stresses refers to excessive salinity of soil or water, which leads to ionic stress, osmotic

imbalance, and oxidative stress; such stress classification ranges from mild to severe depending on salt concentration, duration, and ion composition (Munns and Tester, 2008). In rice, high-salinity conditions disturb ion homeostasis and prompt overproduction of reactive oxygen species (ROS) and MG, triggering deleterious effects on growth, metabolism, and yield; upregulation of the glyoxalase pathway, including OsGLYII, has been correlated with improved salt-stress tolerance through more efficient MG detoxification and maintenance of cellular redox balance (Gill and Tuteja, 2010). The budding yeast *Saccharomyces cerevisiae* serves as an excellent eukaryotic model system for functional expression studies due to its well-characterized genetics, conserved stress response pathways, and ease of cultivation, which allow effective testing of heterologous genes' roles under stress conditions (Kumar *et al.*, 2022 and Wu *et al.*, 2022). Particularly, yeast expression vectors such as pYES2 provide a strong,

inducible GAL1 promoter, high copy number replication, and selectable markers for efficient cloning and expression of plant genes in yeast, facilitating functional assays of stress-tolerance genes under controlled salinity or osmotic challenges (Piraine *et al.*, 2021 and Xiao *et al.*, 2023). Gene cloning remains a cornerstone tool in molecular biology because it enables precise manipulation, overexpression, or mutagenesis of target genes, thereby allowing direct functional characterization and potential biotechnological applications, such as engineering improved stress tolerance by overexpressing detoxification enzymes such as GLY II. Cloning of the rice OsGlyII gene is thus particularly significant: it offers a promising strategy to confer salt-stress tolerance by enhancing cellular capacity for methylglyoxal detoxification in heterologous systems and, potentially, in transgenic crops (Hammadi and Al-Mousawi, 2021 and Liu *et al.*, 2022). Accordingly, the aim of this study is to clone OsGlyII gene into pYES2, express it in *S. cerevisiae*, and functionally analyze its contribution to salt-stress tolerance via growth and survival assays under saline conditions.

MATERIALS AND METHODS

1. Plant material and salt stress induction

1.1. Plant material used

Seeds of the rice cultivar *Oryza sativa* cv. Gz 178 were provided by the

Rice Department, Field Crops Research Institute, ARC, Giza, Egypt) were used in all experiments.

1.2. Surface sterilization and germination

Seeds were surface-sterilized and rinsed under aseptic conditions, then germinated on half-strength Murashige & Skoog medium ($\frac{1}{2}$ MS) supplemented with $30 \text{ g}\cdot\text{L}^{-1}$ sucrose and solidified as required; seedlings were grown in the dark initially and then transferred to light for subsequent growth (Lindsey *et al.*, 2017).

1.3. Upregulation of Osglyoxalase II expression via salt stress induction

Four-day-old seedlings were transferred to $\frac{1}{2}$ MS medium supplemented with 200 mM NaCl and incubated under light for 6 days to induce salt stress prior to RNA extraction. Such NaCl treatment is commonly used to induce salinity stress responses and upregulate glyoxalase genes in rice and other plants (Yadav *et al.*, 2007).

2. RT-PCR amplification of the Osglyoxalase II gene

2.1. Isolation of total RNA and cDNA synthesis

Total RNA was isolated from salt-treated rice seedlings using Qiagen RNeasy Plant Mini Kit (cat. no.74104). The First-strand cDNA was synthesized

from 1–2 µg of total RNA using reverse transcriptase under conditions that minimize genomic DNA carry-over; DNase treatment was applied when necessary (Del Aguila *et al.*, 2005).

2.2. RT-PCR Optimization and Amplification

Gene-specific primers were designed from the registered rice glyoxalase II mRNA sequence database with GenBank accession ID of AY054407 to amplify the obtained cDNA of Osgly II gene. RT-PCR was performed using a high-fidelity polymerase or an RT-PCR system under the conditions clarified in Table (1). products were resolved on 1% agarose gels to verify the single band of expected size. Conditions were selected to minimize errors and ensure full-length amplification as described in glyoxalase cloning reports (Biswas *et al.*, 2018).

2.3. Subcloning of the amplified Osgly II gene into pGEM-T (TA cloning)

2.3.1. Subcloning strategy

In order to select and isolate the insert of the target sequence of the amplified RT-PCR products that contains the sequence of Osgly II gene, the products have undergone digestion through exposure to EcoR I and Hind III restriction enzymes. After that, the purified PCR products carrying 3' A-overhangs were ligated into a pGEM-T vector using T4 DNA ligase and standard TA-ligation conditions, followed by

bacterial transformation for plasmid rescue (Zhao *et al.*, 2009).

2.3.2. Blue/white screening and colony selection

Transformants were plated on LB agar containing ampicillin, IPTG and X-gal, then colonies that were transformed by pGEM-T vector have been screened through visualization of a set of parameters which include survival against antibacterial features of ampicillin, white colored colonies that result from suppression of lacZ gene expression that produces β-galactosidase enzyme responsible for X-Gal cleavage (Litterer, 2009).

2.3.3. Plasmid confirmation and sequencing

Plasmid DNA from positive clones was analyzed by restriction mapping and Sanger sequencing (universal primers) to confirm insert identity and integrity; sequences were compared to NCBI entries (Zhao *et al.*, 2009).

2.4. Construction of pYES2 Construct and *S. cerevisiae* transformation

2.4.1. Restriction enzymes digestion and ligation

The confirmed Osgly II insert in the aforementioned colonies that carry pGEM-T vector and the pYES2 yeast expression vector were digested with compatible restriction endonucleases (SacII and NotI) to enable directional cloning into the multiple cloning site

downstream of the GAL1 inducible promoter (Fig 1), after that, the digested insert and vector were ligated (Drew and Kim, 2012).

2.4.2. preparation of competent yeast using Li-acetate approach

Competent *Saccharomyces cerevisiae* cells were prepared and transformed using the LiAc/SS-carrier DNA/PEG method. The transformation mixtures were heat-shocked and cells were plated on SC medium lacking uracil for selection of pYES2 transformants (URA3 marker). Colonies were then screened by colony PCR for the presence of the Osgly II insert and maintained on selective medium prior to functional assays (Gietz and Schiestl, 2007).

2.5. Functional assays in yeast under salt stress

2.5.1. Growth assay under NaCl gradients

Initially, the experimental design has consisted of the utilization of 3 types of *Saccharomyces cerevisiae* samples, Transgenic yeast strains that harbor the Osgly II gene in their pYES2 vector, a negative control sample consisting of non-transformant yeast without pYES2, and a positive control yeast sample that harbors the pYES2 vector without the Osgly II gene in it. Sample were incubated for two days in a 5 mL volume of selective synthetic complete (SC) medium lacking uracil, which enables selection of transformants since the *URA3*

marker on pYES2 complements uracil auxotrophy. Following this pre-culture, 300 µL of each yeast sample suspension was inoculated into 25 mL of SC medium supplemented with a range of sodium chloride concentrations (0, 1.7, 1.8, 1.9, 2.0, 2.1, 2.2, and 2.3 M) and incubated for 7 days to assess salt-stress tolerance. After incubation, yeast growth was monitored spectrophotometrically at OD600 using a Smart spec 3000 spectrophotometer (Chen *et al.*, 2002).

2.5.2. Transgene expression analysis by RT-PCR

In a similarity with the aforementioned RT-PCR reaction conditions, total RNA from stressed and control yeast cultures was isolated using the SV total RNA isolation kit (Promega TM, Cat. No. Z3100), first cDNA strand was formed after an initial reverse-transcription reaction at 42°C for 60min., employing a mixture of 20 µL reaction volume contained 11 µL RNA, 2µL dNTPs, 1µL oligo(dT), 1 µL reverse transcriptase, 4 µL 5 x reverse transcription buffer, and 1 µL RNase inhibitor in order to accomplish cDNA formation. After that, cDNA strand was amplified using Osgly II gene specific primer and gel electrophoresis verified expected amplicon size of the produced PCR products (Green and Sambrook, 2021).

2.5.3. Intracellular proline measurement

After subjecting yeast cultures to stress conditions, intracellular proline a well-known osmoprotectant often used in

stress physiology studies was extracted from harvested cells and quantified by a ninhydrin-based colorimetric assay. Specifically, proline was measured as the red chromophore developed by the proline–ninhydrin reaction, with absorbance read at 520 nm (OD₅₂₀). All procedures adhered to established protocols for free-proline quantification under stress conditions (Zulkifli *et al.*, 2020).

RESULTS AND DISCUSSION

The integrity and specificity of the RT-PCR product were confirmed by agarose gel electrophoresis, demonstrating successful isolation and amplification of the target gene from the cDNA template derived from salt-stressed rice seeds. The amplification reaction yielded a single, distinct DNA fragment of approximately 1,011 bp, matching the expected size of the Osglyoxalase II coding region (Fig. 2). The presence of a discrete amplicon at the predicted size confirms the successful recovery of the full-length Osgly II open reading frame, enabling its use for subsequent cloning and expression analysis. Within the framework of this research, we aimed to obtain Osglyoxalase II gene from salt-treated *Oryza sativa* seedlings and clone it into pYES2, express it in *Saccharomyces cerevisiae*, and assess its role in salt tolerance through growth and survival assays. Our findings proved that the successful amplification of the full-length Osglyoxalase II cDNA (1,011 bp) from salt-treated rice seedlings, followed by

cloning into pGEM-T vector aligns with the stress-responsive behavior previously described by Hasanuzzaman *et al.* (2017). They confirming its role as a salt-induced gene

3.2. Directional cloning and verification of the pYES2–Osgly II recombinant construct

Restriction digestion of the recombinant pYES2–Osgly II by SacII and NotI restriction endonucleases constructs produced two distinct bands: a 1011 bp fragment corresponding to the Osgly II insert and a 5.9 kbp fragment representing the linearized pYES2 backbone (Fig 3). The presence of both fragments at their expected sizes confirms successful directional insertion of the gene into the vector. Sequencing of selected clones further verified that the Osgly II was intact and free of mutations, confirming the integrity of the final expression construct. Furthermore, The Osgly II insert of pGEM-T/Osgly II construct was directionally subcloned into the SacII/NotI sites of the pYES2 vector under the GAL1 promoter that were then used to transform *Saccharomyces cerevisiae* (LiAc method), with transformants selected on uracil-deficient medium, the approach is in line with established protocols for functional expression of plant stress genes in yeast as Assem *et al.* (2017) in his study similarly cloned plant glyoxalase genes into pYES2 and selected transformants on SC–Ura to assess stress tolerance (Assem *et al.*, 2017).

3.3. Evaluation of transgenic *S. cerevisiae* harboring Osgly II under salt stress

3.3.1. Growth performance of transgenic and control yeast strains under salt stress

Growth of yeast strains was quantified as optical density at 600 nm over a range of NaCl concentrations. Under non-saline conditions (0 M NaCl), both the OsGlyII-expressing strain and the empty-vector control reached high cell densities, whereas the strain lacking the expression vector showed very low OD values. This pattern establishes that the plasmid-bearing strains are physiologically competent under control conditions and that the low readings for the non-transformed cells represent a genuine difference in growth rather than an assay artifact. Upon exposure to elevated NaCl concentrations, all strains exhibited reduced growth, but the magnitude of this reduction differed substantially. At moderate salinity (1.7–1.8 M NaCl), the Osgly II-expressing strains have maintained OD₆₀₀ values close to 1.9, while the empty-vector control dropped to around 1.17–0.86 and the non-transformed strain remained near or below 0.07. At the highest concentration tested (2.3 M NaCl), the Osgly II strain still retained measurable growth (OD₆₀₀ ≈ 0.61), whereas the empty-vector control declined to

approximately 0.18 and the non-transformed control to about 0.04. Across the upper part of the gradient, the OsGlyII strain therefore sustained several-fold higher optical density than both control groups, indicating that the introduced gene is associated with a marked increase in tolerance to severe osmotic stress (Table 2). Over and above, yeast transformed with Osgly II showed markedly better growth under high-salt conditions than control strains as it maintained high OD₆₀₀ readings even at 1.9–2.2 M NaCl, whereas both empty-vector and untransformed controls exhibited greatly reduced growth, such enhanced salt tolerance is consistent with previous reports on glyoxalase enzymes, Mudalkar *et al.* (2017). demonstrated that yeast cultures expressing glyoxalases from *Jatropha curcas* sustained growth under 200 mM NaCl (Mudalkar *et al.*, 2017). Similarly, Ghosh *et al.* (2014) showed that ectopic expression of a rice Osgly II gene in other microorganisms and tobacco conferred improved tolerance to salinity (Ghosh *et al.*, 2014).

3.3.2. Verification of Osgly II gene expression in transgenic *S. cerevisiae* under salt stress

Expression of the heterologous gene in yeast was evaluated by RT-PCR using RNA isolated from transformed and control cultures (Fig 4). In the OsGlyII-expressing strain, amplification produced

a single band at approximately 1,011 bp, matching the size of the coding sequence cloned from rice. No corresponding band was detected in RNA samples from yeast carrying only the empty vector, and the size marker confirmed that the fragment in the transgenic sample aligned with the expected length. These results show that the inserted *OsGlyII* sequence is actively transcribed in yeast under the conditions tested and that the transcript corresponds to the full-length coding region. Moreover, RT-PCR of total RNA from salt-treated cultures confirmed *Osgly II* transcription in transformed yeast, yielding a 1011 bp band detectable only in *Osgly II* transformants and absent in empty-vector controls. This verifies active gene expression under the induction conditions. Comparable expression validation using gene-specific RT-PCR is routinely reported in heterologous plant gene expression studies, such as the study conducted by Jana and Yaish., (2020).

3.4. Intracellular proline accumulation in response to salt stress

Proline levels were quantified in all yeast strains across the same NaCl gradient used for growth measurements. Under control conditions, intracellular proline concentrations were similar among the three groups, indicating a comparable basal osmolyte status in the absence of stress. As salinity increased, however, the patterns diverged clearly

between *OsGlyII*-expressing cells and the controls. In the *OsGlyII* strain, proline content rose progressively with increasing NaCl, reaching its highest value at 2.3 M. At this concentration, the measured proline level in the transgenic yeast was more than twice the baseline value at 0 M NaCl and several-fold higher than in either control group. In contrast, the non-transformed strain showed a steady decline in proline with rising salt, and the empty-vector control exhibited only a modest increase at lower salinities followed by a decrease at higher concentrations. Overall, the data reveal a consistent association between expression of *OsGlyII* and enhanced accumulation or retention of proline under salt stress, in parallel with the improved growth performance observed at high NaCl levels (Table 3). Yeast expressing *Osgly II* accumulated markedly more intracellular proline under salt stress 2-4 folds higher than controls across all NaCl levels tested. This concurring with previous findings at the same context as wild-type of *S. cerevisiae* generally do not boost proline under osmotic stress, but engineered or mutant strains that accumulate proline show broad stress tolerance. These studies support the conclusion that the elevated proline in *Osgly II* yeast aligns with the enhanced salt tolerance observed in other high-proline strains (Matsuura and Takagi, 2005 and Sasano *et al.*, 2012).

It concluded that From the standpoint of this study, the key findings showed that *Osglyoxalase II* gene enhances yeast tolerance to high salinity

by supporting better growth, maintaining active transcript levels under stress, and promoting greater intracellular proline accumulation. These combined responses indicate that Osgly II strengthens osmotic stress resilience in the transgenic strain. To fully establish the gene's broader potential, future work should evaluate its function in plant systems, clarify its interactions within the glyoxalase pathway, and determine whether similar protective effects occur under other abiotic stress conditions.

SUMMARY

Salt stress is known for its relation with increasing cellular methylglyoxal, which is detoxified through the glyoxalase pathway. The Osglyoxalase II gene enhances the GLYII step of this pathway, thereby improving MG clearance and contributing to greater stress tolerance. In this study, The Osglyoxalase II gene was isolated from salt-stressed *Oryza sativa* cv. Gz 178 seedlings by total RNA extraction, cDNA synthesis and RT-PCR amplification. The resulting 1,011 bp amplicon was cloned into pGEM-T, verified by restriction mapping and sequencing, then excised with SacII/NotI and directionally subcloned into the yeast expression vector pYES2. Furthermore, the directional subcloning was verified by restriction digestion showing the expected 5.9 kbp vector and 1011 bp insert, with sequencing confirming an intact structure. Recombinant plasmids were used to transform *Saccharomyces cerevisiae* via

the Li-acetate method, transformants were selected on SC-Ura medium. Functional evaluation comprised: growth assays (OD₆₀₀) across a NaCl gradient, RT-PCR confirmation of transgene expression in yeast, spectrophotometric quantification of intracellular proline, and statistical analysis of replicate experiments. Transgenic yeast expressing Osgly II displayed consistently higher OD₆₀₀ values across all NaCl concentrations, maintaining substantial growth even at 2.3 M salt. RT-PCR detection of a 1,011 bp transcript exclusively in transformed yeast confirmed active gene expression. Intracellular proline levels increased progressively with salinity in the Osgly II strain, while both controls showed minimal or declining accumulation.

Data Availability Statement

The article's Material section highlights the study's original contributions; you may get in touch with the study's corresponding author for further details or clarifications.

Conflict of Interest Statement

During the study, the authors confirmed that there were no corporate or financial relations that may be believed to pose a potential conflict of interest.

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Table (1). Target Genes, Primer Sets, and RT-PCR Cycling Conditions

Salt concentration (M NaCl)	negative control (-)	positive control pYES2 (+)	Osgly II
0 M	0.1026 l	2.5670a	2.8650 a
1.7 M	0.0676 l	1.1678 e	1.8613 b
1.8 M	0.0633 l	0.8564 fg	1.8910 c
1.9 M	0.0556 l	0.7003 h	1.4569 cd
2.0 M	0.0543 l	0.6432 hi	1.5421 e

Table (2). OD₆₀₀ of Osgly II-expressing and control yeast under increasing NaCl concentrations

Target gene	Amplification primers	Reverse transcription step	Primary denaturation step	30-cycle amplification phase			Final extension
				Secondary denaturation	Annealing	Extension	
Osgly II gene (cDNA)	5' ATG CGG ATG CTG TCC AAG GCG'3	45°C/45 min	94°C/2 min.	94°C/30 sec.	60°C/45 sec.	72°C/1 min.	72°C/10 min.
	5' TTA AAA GTT ATC CTT CGC TCG'3						

Table (3). Intracellular proline content in OsglyII-expressing and control yeast strains under salt stress

Salt concentration (M NaCl)	Negative control (-)	Positive control pYES2 (+)	Osgly II transgenic yeast
0 M	1.450 l	1.5025 j	1.490 ij
1.7 M	0.9745 n	1.2433 m	1.840 h
1.8 M	0.8501 o	0.9773 n	1.925 f
1.9 M	0.8099 o	0.9476 n	1.987 de
2.0 M	0.7236 p	0.8206 o	2.102 cd
2.1 M	0.5761 r	0.6583 q	2.179 b
2.2 M	0.5092 s	0.6280 qr	2.145 b
2.3 M	0.3651 t	0.5310 s	2.345 a

Numbers with the same letters do not differ appreciably at 1%.

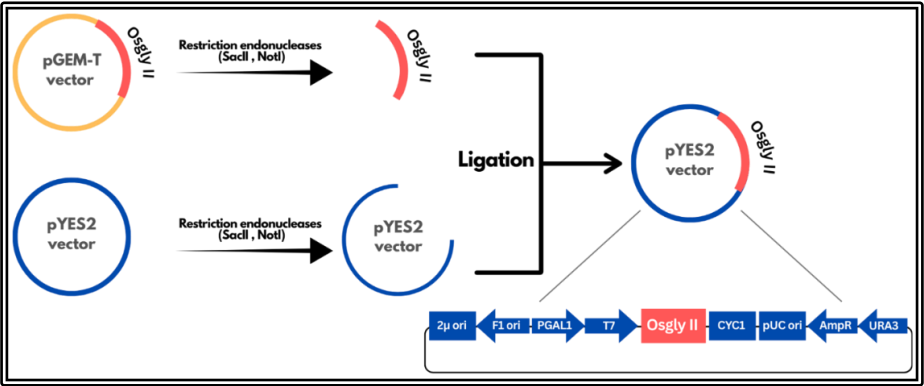


Fig. (1). Schematic workflow of directional cloning of the Osgly II Gene from pGEM-T into the pYES2 vector

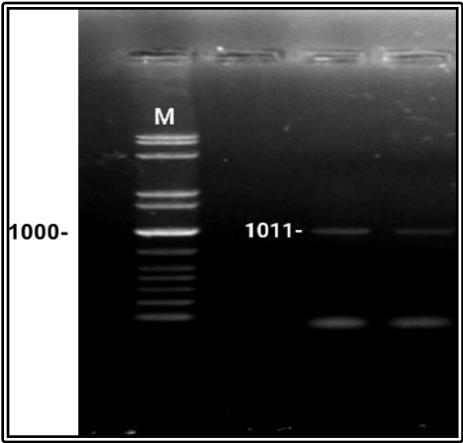


Fig. (2). RT-PCR amplification of the Osgly II gene from rice cDNA showing a single 1011 bp band

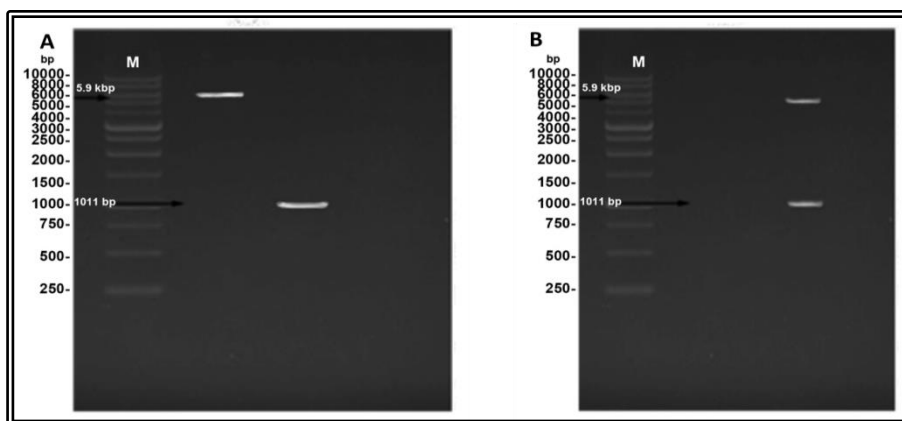


Fig. (3). Restriction digestion analysis of recombinant pYES2–Osgly II plasmids.

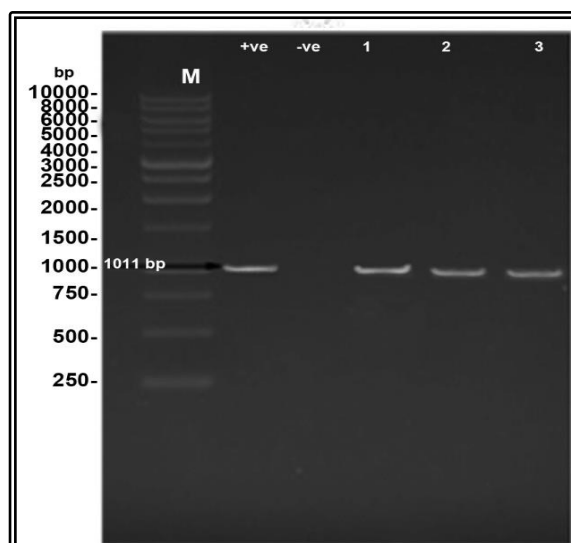


Fig. (4). RT-PCR verification of *Osgly II* expression in yeast showing a single 1,011-bp amplicon in the positive control and in lanes 1–3 (independent transgenic yeast transformants), with no amplification in the negative control.