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Use of the *Saccharomyces cerevisiae* endopolygalacturonase promoter to direct expression in *Escherichia coli*

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Abstract In *Saccharomyces cerevisiae*, an endopolygalacturonase encoded by the *PGL1* gene catalyzes the random hydrolysis of the α-1,4 glycosidic linkages in polygalacturonic acid. To study the regulation of the *PGL1* gene, we constructed a reporter vector containing the lacZ gene under the control of *PGL1* promoter. Surprisingly, when *Escherichia coli* DH5α was transformed by this vector, cells harboring the constructed plasmid produced blue colonies. Sequence analysis of this promoter revealed that *E. coli* consensus sequences required to express an in-frame lacZ alpha product were present. We next decided to investigate how the *PGL1* promoter is regulated in *E. coli* compared to yeast. In this study, we examined the modulation of the *PGL1* promoter in *E. coli*, and the results indicated that its activity is greatly induced by saturated digalacturonic acid and is indirectly regulated by the transcriptional regulators the 2-keto-3-deoxygluconate repressor. Moreover, *PGL1* expression is enhanced under aerobic conditions. We found that *β*-galactosidase activity in *E. coli* could reach 180 units, which is 40-fold greater than the activity produced in *S. cerevisiae*, and greater than recombinant protein expression previously reported by other researchers. We thus demonstrate that this vector can be considered as a dual expression plasmid for both *E. coli* and *S. cerevisiae* hosts. So far, no modulation of endoPG promoters expressed in *E. coli* has been reported.

Keywords Endopolygalacturonase · *Escherichia coli* · Gene expression · *PGL1* gene · *Saccharomyces cerevisiae* · Dual expression plasmid

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Introduction

Pectin is a major constituent of plant cell walls, mainly composed of D-galacturonic acid residues, either esterified or not and joined by α-1,4-linkages, forming homogalacturonan chains. In both bacterial and fungal pectin-degrading systems, the enzymatic hydrolysis of pectin involves the action of pectinases. Among these enzymes, endopolygalacturonase (endoPG; EC.3.2.1.15) hydrolyzes the α-1,4-glycosidic bonds between two non-methylated galacturonic acid (GA) residues. Polygalacturonic acid (PGA) hydrolysis products consist mainly of digalacturonic acid (DGA) and highly soluble oligosaccharides [4]. Pectinases have a share of 25% in the global sales of food enzymes because they are relevant in numerous biotechnological applications such as in fruit juice extraction and its clarification, scouring of cotton, degumming of plant fibers, waste water treatment, vegetable oil extraction, tea and coffee fermentations, bleaching of paper, in poultry feed additives, and in alcoholic beverages and food [27, 40]. Otherwise, analysis of expression has shown that the PGL1p activity by degradation of pectin could play another function. Thus, it was reported that the *PGL1* gene was a target of the MAPK regulatory pathway and was also required in pseudohyphae development [30] and therefore involved in plant pathogenesis [14, 15, 18].

Until now, many *Saccharomyces cerevisiae* strains screened for their ability to produce endoPG have been deficient or have produced a low activity of this enzyme [11]. On the other hand, when the *PGL1* ORF was isolated under its own promoter, endoPG activity was hardly detectable [16] by conventional methods [33, 37]. To bypass this problem and to improve expression efficiency, the *PGL1* ORF was placed under the control of a strong promoter [3, 17, 20]. These latter approaches are used to get a sufficient amount of enzyme to study its properties [2, 12] or simply to produce it on an industrial scale. This production can be justified by the fact that when pectolytic yeasts are used directly under winemaking conditions, the actual composition of the obtained wines was only sparingly altered [10, 46]. These slight changes in aroma profile according to us were due to the high glucose concentration of the musts that repress the biosynthesis of the enzyme during the process.

We previously reported that the *S. cerevisiae* SCPP strain is the only yeast strain to express a high level of endoPG activity [11], and that the *S. cerevisiae* X2180 strain produces a low basal activity despite possessing an intact *PGL1* gene. The main difference in the 5′ non-coding sequences of the two strains is the insertion of delta Ty2 transposon in the intergenic *DAL5-PGL1* fragment, which regulates expression of the *PGL1* ORF in *S. cerevisiae* [16]. It was suggested [22] that endoPG activity in different *S. cerevisiae* strains is modulated at the transcriptional level and not by the structural gene. Later, this hypothesis was confirmed by works showing that the *PGL1* gene was found not to be regulated by sequence difference in the ORF but by the transcriptional level of the gene [28, 29].

Some regulatory mechanisms of the bacterium *Erwinia chrysanthemi* for pectinases biosynthesis are also conserved in *E. coli* [21, 42]. Thus, the five *pel* (Pectate lyases encoded by the five genes *pelA-E*) transferred in a heterologous host *E. coli* are affected like in *E. chrysanthemi* by growth phase, catabolite repression, and anaerobic growth conditions. They also are induced in the presence of galacturonate, a sugar whose catabolism leads to the formation of 2-keto-3-deoxygluconate (KDG), the inducer of *pel* transcription in *E. chrysanthemi* [21]. All genes involved in pectin degradation are specifically controlled by the 2-keto-3-deoxygluconate repressor (KdgR) and are induced by the pectin catabolic product, KDG. The regulation of *pel* expression by temperature or nitrogen starvation, observed in *E. chrysanthemi,* was not conserved in *E. coli,* suggesting that the mechanisms responsible for these regulations are specific to *E. chrysanthemi* [21].

The present report is the first to identify the possible origin of the gene encoding endoPG from bacteria to yeasts on the basis of the promoter structure and a comparison of gene expression in *E. coli* and *S. cerevisiae* using a *lacZ* reporter; and to verify whether physiological regulation taking place in *S. cerevisiae* is conserved in *E. coli.*

Materials and methods

Plasmid construction

Intergenic *DAL5/PGL1* DNA fragments (Fig. 1a) were generated by PCR amplification using the Pwo DNA polymerase (Eurogentec Seraing, Belgium) with genomic DNA using the *S. cerevisiae* X2180-1B strain as a template and pgprombam (5′-GGGGATCCTGAAGAAACAGAGA ATTTAGAG-3′) and pgpromxba (5′-AAT CTAGAAGAA ATCATTGCGTTTGTCAATCAA-3′) primers. Purified and *Bam*HI ± *Xba*I digested PCR products were ligated to the YEp357 vector [35] linearized with *Xba*I and *Bam*HI using T4 DNA ligase (Biolabs New England, Evry, France). The ligation mixture contained a DNA vector ratio of 10:1 and was carried out overnight at 16°C. Ligation products were used to transform *E. coli* DH5α. Plasmids were extracted from positive *E. coli* clones and used to transform the *S. cerevisiae* MATa, ura3-D, trp1-4 strain for measuring β-galactosidase activity. Recombinant YEppgX plasmids were verified by sequencing (Eurogentec, Seraing, Belgium).

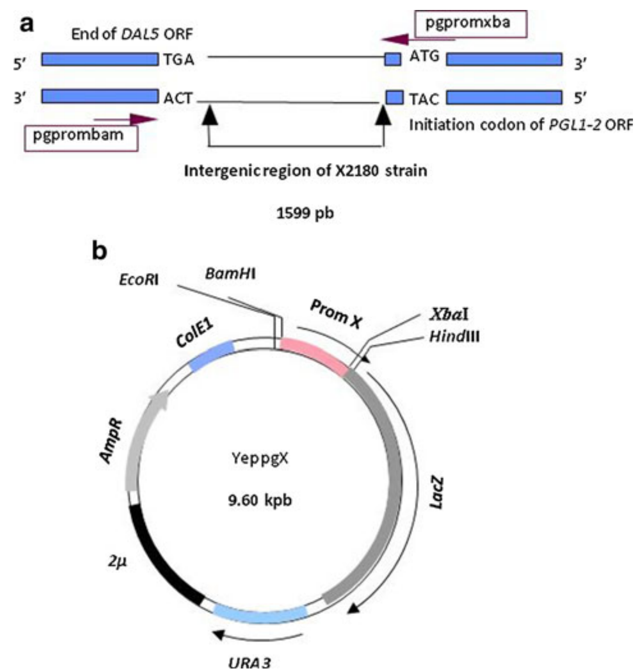

Fig. 1 Construction of the YEppgX plasmid. **a** The putative *PGL1* regulatory region (*DAL5/PGL1*) corresponds to the region bracketed by the two *black arrows* obtained after PCR using the primers pgprombam and pgpromxba (*dark red arrows*) was ligated to plasmid YEp357 to construct plasmid YEppgX (**b**) (colour figure online)

Strains and plasmid

The YEp357: 2μ-URA3 plasmid and yeast/*E. coli* shuttle vector were as previously described [35]. The *S. cerevisiae* X2180-1B (*MATa SUC2 mal mel gal2 CUP1*) strain was obtained from the Yeast Genetic Stock Center and used to amplify the *DAL5*/*PGL1* intergenic region containing the *PGL1* promoter. *E. coli* DH5α [*supE*44 *hsdR*17 *recA*1 *endA*1 *gyrA thi*-1 λ^- *relA*1 Δ(*lacIZYA-argF) U169 deoR* (Φ80*dlacZ*Δ(*lacZ*)M15)] (Bethesda Research Laboratories, Gaithersburg, MD, USA) was used as host for cloning and for β-galactosidase assays. *S. cerevisiae* MATa, ura3-D, trp1-4, a derivative of FL100 (ATCC 28383), was used for the β-galactosidase assays after transformation by the YEppgX plasmid.

Transformation

Escherichia coli DH5α and *S. cerevisiae* MATa transformations were performed, respectively, according the cited methods [7, 8].

Media and growth conditions

Transformed *E. coli* strains were grown in M9 minimum medium (15 g/l Na_2HPO_4, 12 H_2O; 3 g/l KH_2PO_4; 0.5 g/l NaCl; 1 g/l NH4Cl after sterilization at 120°C during 20 min, we added glucose 2 g/l; B1 vitamin 0.002 g/l, $MgSO_4$ 0.120 g/l). *S. cerevisiae* [YEppgX] was grown in SD medium (6.7 g/l yeast nitrogen base without amino acids; DIFCO, Detroit, MI, USA; 20 g/l glucose, supplemented with tryptophan). For experiments, media were supplemented with varying different concentrations (see text for details) of GA, DGA, or PGA (Sigma-Aldrich, Saint Quentin, France). Yeast strains were grown at 30°C and *E. coli* at 37°C. Aerobic conditions were obtained by shaking at 150 rpm.

β-Galactosidase assays

Yeast β-galactosidase activity was measured using the appropriate method [24] and bacterial β-galactosidase activity was measured using the Miller method [32]. Transformed strains were grown in appropriated media (M9 or SD) supplemented with different concentrations of GA, DGA, or PGA (with <5% esterification) at different concentrations (see text for details). Activities are given in Miller units (U) as defined previously [24] and are averaged data from at least three experiments. Statistical significance was calculated according to Student's table. Data are expressed as the mean values ± standard deviation. In the equation, the specific activity is a proportional relationship between the total activity and the biomass (represented by the A600), normalized to a standard sample.

Results

Analysis of the *PGL1* promoter

We identified the A nucleotide at position—40 relative to the *PGL1* start codon ATG (Fig. 2) using the http://bioinfo.md.huji.ac.il/marg/promec website [19, 43]. This transcriptional initiation site is at an appropriate distance for the −10 box (represented by the sequence 5′-TATAAA-3′) and the −35 box (represented by 5′-TTCACA-3′) that are positioned at −59/−64 and −79/−85 nucleotides relative to the ATG codon, respectively. The latter box is separated by 15 nucleotides from the Pribnow box that is found in the majority of *E. coli* promoters.

However, no consensus sequence for the ribosomal binding site (RBS), usually known as the Shine–Dalgarno site, was found in the *PGL1* promoter (Fig. 2). Further, no KdgR consensus binding site (5′-AAATGAAACAn TGTTTCATTT-3′) is present within the *DAL5*/*PGL1* intergenic region.

Modulation of the *PGL1* promoter by GA, DGA, and PGA

Modulation of the *PGL1* promoter in *E. coli* DH5α [YEppgX] and *S. cerevisiae* [YEppgX] by GA, DGA, and PGA was investigated (Fig. 3a, b). For clarity, we report here only results concerning the optimal concentrations of the

5′...ATTTTTTTTTTTGACGCTATTATTTAAAACCTAGGATATCCGTCCCATACAAAACGGCCACGAGT
TTCAATCCCAGAATGTACGAGT***TATAAT***TCTCCTAGATGCATGATACTCGTGCATTCGTTTAACAAT
CATACCACTTTCCCATTTTCGGGA**<u>TATTAA</u>**ACATGAACATACTTTTTTACTGTGAGAATGTGG**[TTCA**
CA]ATTATTCCATACAGG**[TATAAA]**AACGCACAG**a**AACGGGAAGACTATCTACCCACATTGATTGACA
AACGCAA**ATG**ATTTATTCTAGAGTCGACGACCTGCAGGCATGCAAGCTTGCTCCC-3′

Fig. 2 Nucleotides sequence of the first 320 bp of the *PGL1* promoter from *S. cerevisiae* X2180-1B strain (SGD Database). The ATG codon is in *bold*. Predicted prokaryotic elements in the *PGL1* promoter (http://www.fruitfly.org/seq_tools/promoter) indicate the transcription start site in *bold lowercase*. The *boxed* elements represent the −10 (Pribnow-Schaller *box*) and −35 region within the prokaryotic promoter. The sequence in *bold* and *underlined* represent the TATA *box* usable in *S. cerevisiae*

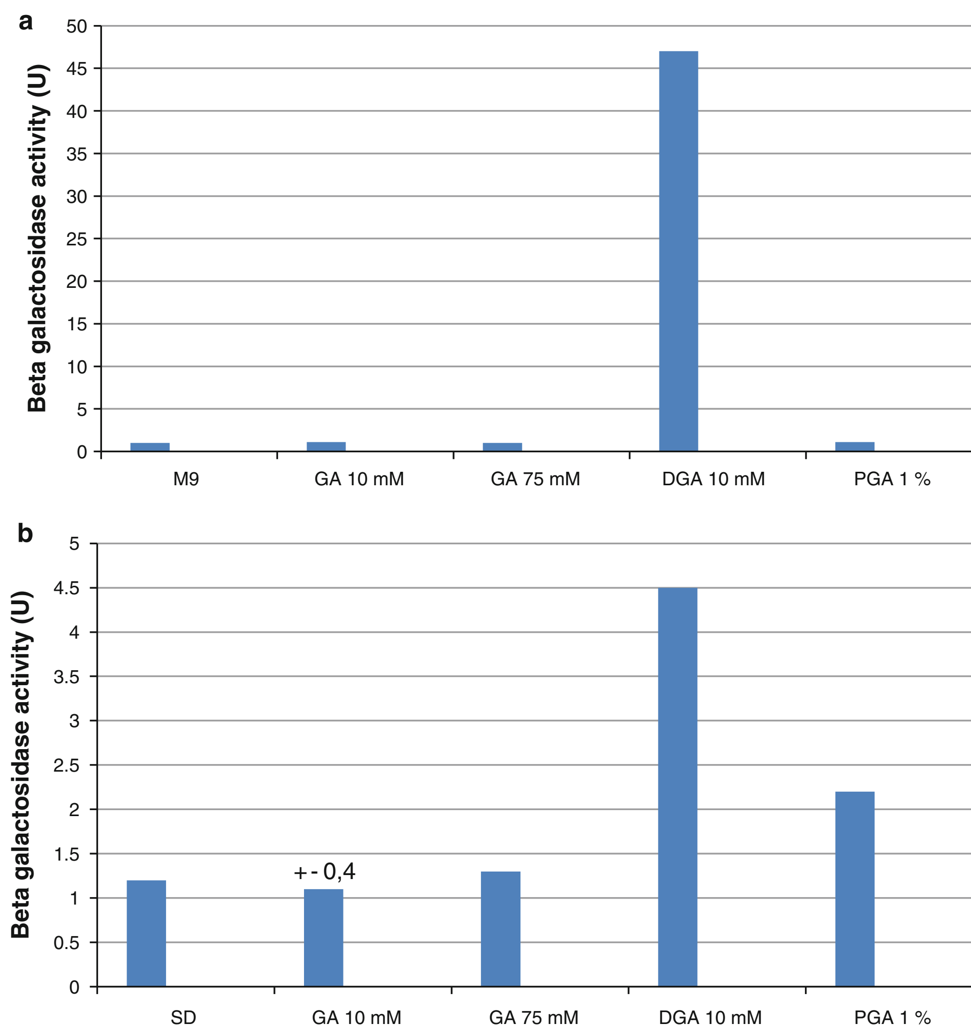

Fig. 3 Levels of *β*-galactosidase activity in the *E. coli* DH5α host strain (**a**) and the *S. cerevisiae* host strain (**b**). *β*-Galactosidase activity was measured in whole cells using the method of Kippert [19] Transformed strains were grown in media supplemented with GA, DGA, or PGA. *β*-galactosidase activity is given in arbitrary units (U) as defined by Kippert [19]. Values are averages of at least three experiments (colour figure online)

different carbon sources for each host. In preliminary experiments, transformed *E. coli* and *S. cerevisiae* were assayed for *PGL1* promoter activity in basal media. For *E. coli*, basal *PGL1* promoter activity was 1.0 *β*-galactosidase units and for *S. cerevisiae* basal *PGL1* promoter activity was 1.2 *β*-galactosidase units.

The addition of 50 or 75 mM of GA to *E. coli* DH5α [YEppgX] or *S. cerevisiae* [YEppgX] cultures had no significant effect on endoPG expression. Similarly, the addition of PGA had no effect on *β*-galactosidase levels in *E. coli* DH5α [YEppgX]. However, in sharp contrast, the addition of PGA resulted in an increase of 2.2 *β*-galactosidase units for *S. cerevisiae* [YEppgX], representing a 66% increase over the basal level. Following the addition of 10 mM DGA, *E. coli* harboring the recombinant plasmid produced 47 *β*-galactosidase units of activity. For *S. cerevisiae*, 10 mM DGA induced a 3.91-fold increase in *PGL1* promoter activity (Fig. 3), indicating that this compound can induce endoPG promoter activity in the both organisms and that induction in *E. coli* was greater than in *S. cerevisiae*. Since the *PGL1* gene is derepressed in *E. coli* by DGA transported into cells via endogenous KdgT [9].

PGL1 activity is regulated by oxygen availability

Under anaerobic conditions, *β*-galactosidase production in transformed yeast was 2.05 *β*-galactosidase units compared to 3.03 units under aerobic conditions. In contrast, *E. coli PGL1* promoter activity was 47 *β*-galactosidase units under aerobic condition and 181 units under anaerobic conditions. The discovery that aeration has opposing effects on *PGL1* promoter activity in *E. coli* and *S. cerevisiae* indicates that different mechanisms of *PGL1* promoter regulation exist in these two systems.

Discussion

The 5′-flanking sequences of some yeast genes have already been shown to activate *E. coli* promoters. This may be explained by the fact that these eukaryotic promoters contain consensus sequences pertaining to prokaryotic-type promoter elements [25, 26]. In bacteria, the most highly conserved promoter elements are the −10 and −35 boxes, upstream of the transcriptional start site. For RNA polymerase containing σ70, canonic consensus sequences for these elements are 5′-TATAAT-3′ and 5′-TTGACA-3′, respectively. It has also been established that for 44% of prokaryotic promoters, the optimal length of spacer region between the two boxes is 17 nucleotides on average [5]. Some reports indicate that 95% of *E. coli* promoters contain at least 3/6 conserved nucleotides within the −10 consensus sequence [5, 34]. The −35 hexamer is not as well conserved as the Pribnow box, since only 17% of prokaryotic promoters contain a −35 hexamer sequence with <50% identity. To date, no prokaryotic promoter has been identified that has intact consensus sequences at both the −10 and −35 sites. This study aimed to investigate whether *S. cerevisiae PGL1* regulation is conserved in the heterologous host, *E. coli*, thus demonstrating the interchangeability of the regulatory systems between both microorganisms, as had previously been reported about for *E. coli* and *E. chrysanthemi* [21]. However, we found that PGA did not stimulate the exogenous *PGL1* promoter in *E. coli*, although it did stimulate the recombinant promoter in *S. cerevisiae*. This may be explained by the presence of extracellular depolymerizing endoPG in *S. cerevisiae* that liberates DGA as a reaction product [4, 12]; in contrast, *E. coli* does not produce endoPG.

Some authors have reported in other microorganisms that pectolytic enzymes are induced by the presence of pectins [38, 41]. They claim that PGA is not expected to penetrate cells and suggest that, instead of pectin, a catabolic product (DGA or GA) is responsible for pectolytic enzyme induction. We observed that β-galactosidase expression in *S. cerevisiae* [YEppgX] is induced 2.5-fold in the presence of 1% PGA (Fig. 3b). However, no such PGA induction was observed for transformed *E. coli* (Fig. 3a). This can be explained by the fact that yeast produces a basal level of secreted endoPG activity, and that DGA removed from the pectin substrate is responsible of the induction of endoPG synthesis. In contrast, *E. coli* is devoid of basal endoPG activity (data not shown) and no PGA induction occurs. Thus, our findings reinforce the previous hypothesis [38, 41]. We found that DGA is a much more potent inducer of the *PGL1* promoter than is GA. This is logical because the *PGL1* ORF encodes an endoPG the major product of which is DGA, not GA [4], and it is generally found that catabolic enzymes are specifically induced by compounds that are structurally similar to their substrates.

As KdgR is present in the genome of all enterobacteria, including *E. coli*, and as we have not detected a KdgR-binding site in the *DAL5/PGL1* intergenic region containing the *PGL1* promoter, it is highly unlikely that the effect of KdgR on the *PGL1* gene is due to direct binding of KdgR to the *PGL1* promoter. A more plausible hypothesis is that KdgR affects *PGL1* expression indirectly via another regulator.

Thus, we have demonstrated that the vector can function as a dual expression plasmid in both prokaryotic and eukaryotic hosts. The sequences of endoPG genes [31] and the published crystal structures of bacterial and fungal endoPG enzymes [39, 45] indicate that fungal endoPGs possess four cysteines that are well conserved and implicated in disulfide (S–S) bridges [45]. While the first two cysteines should be present in all fungal endoPGs, they may be absent from yeast endoPG [31]. Bacterial endoPG has only the first two cysteines, but there is no conservation of positions of these in all bacterial endoPGs. This suggests that genes encoding endoPG in yeast have evolved similarly to those in bacteria. Another interesting fact is that most of the endoPG genes isolated from fungi such as *Aspergillus niger*, *A. awamori, A. flavus*, and *Colletotrichum lindemuthianum*, possess introns [6, 36, 47]. Whereas endoPG genes from yeast contain uninterrupted sequences in their ORFs.

In addition, the endoPG promoter appears to have a prokaryotic structure, as its expression is always stronger in *E. coli* than in *S. cerevisiae* strains have low or no endoPG activity despite the presence of the gene. On this basis, we suggest that the endoPG gene may have been transferred from bacteria to yeast during evolution and that yeast endoPG may share their origins with those of bacteria. Gene transfer from bacteria to yeast is common [1, 13, 44]. Our suggestion is supported by the study [23], who reported that two distantly related species, *S. cerevisiae* and *Helicobacter pylori*, harbor a large complement of evolutionarily conserved pathways, and that many of these pathways appear to have duplicated and specialized within yeast.

So far, only a few of endoPGs expressed in yeast or *E. coli* have been reported, but they did not show higher activity. Our data showed that activity could reach 180 units, which is 40-fold greater than the activity produced in culture of *S. cerevisiae*, and greater than recombinant protein expression reported by other researchers [48].

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