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A yeast growth assay to characterize plant poly(ADP-ribose) polymerase (PARP) proteins and inhibitors

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ABSTRACT

Poly(ADP-ribose) polymerases (PARPs) have been implicated in responses of plants to DNA damage and numerous stresses, whereby the mechanistic basis of the interference is often unclear. Therefore, the identification of specific inhibitors and potential interactors of plant PARPs is desirable. For this purpose, we established an assay based on heterologous expression of *PARP* genes from the model plant *Arabidopsis thaliana* in yeast. Expression of *AtPARP*s caused an inhibition of yeast growth to different extent, which was alleviated by inhibitors targeted at human PARPs. This assay provides a fast and simple means to identify target proteins and pharmacological inhibitors of AtPARP1.

Heterologous expression of genes in yeast has proven to be a powerful tool to study their function. In line with this, several authors have described the characterization of human *Poly(ADP-ribose) polymerases* (*HsPARP*s) in yeast [1–5]. Poly(ADP-ribosyl)ation (PARylation) is a posttranslational protein modification, adding poly(ADP-ribose) moieties to nuclear proteins and thereby recruiting DNA repair components to DNA damage sites. Hence, PARPs are key elements of cellular genome integrity, and as such, PARPs have been attributed functions in diseases, such as cancer [6].

Expression of *HsPARP1* and *HsPARP2* results in a growth arrest of yeast cells [1–5], which has been attributed to the production of poly(ADP-ribose) moieties [3,4]. The presence of a DNA-binding domain was shown to be indispensable for HsPARP1-induced yeast growth inhibition [1]. The expression of the PARP antagonist *Poly(ADP-ribose) glycohydrolase* (*PARG*), which removes ADP-ribose moieties from target proteins of PARylation, as well as the application of PARP inhibitors can revert the growth inhibition [1,3,4]. This action of known PARP inhibitors has been exploited to establish a screening procedure to identify novel HsPARP inhibitors [3]. In addition, such a growth assay provides a means to study potential targets of poly(ADP-ribosyl)ation.

Recently, PARP proteins have gained an increased interest in plant science. As in animals, they have been linked to plant DNA damage responses [7–9], but, in addition, they were also considered to be regulators of plant stress responses [10,11]. However, the mechanistic basis of many presumed plant PARP functions has not been fully resolved yet [12]. To study functional similarities of HsPARP1 and the Arabidopsis PARP proteins, and to develop a screening procedure for potential pharmacological inhibitors, we expressed the three canonical *AtPARP*s and *HsPARP1* heterologously in *Saccharomyces cerevisiae*. To this end, *HsPARP1* and *AtPARP1* (At2g31320) cDNAs were PCR-amplified from plasmids IRATp970E1051D (imaGENES, Germany) and U19185 (ABRC), respectively. cDNAs of *AtPARP2* (At4g02390) and *AtPARP3* (At5g24470) were amplified as described previously [9]. Primers used to amplify the cDNA sequences are listed in Table 1 in Supplementary Material. The resulting cDNAs were cloned into the yeast expression vector pYES2 by homologous recombination performed in the yeast

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wild type strain W303-1A as described elsewhere [3]. The pYES2 expression vector contains a galactose (Gal)-inducible promoter allowing a temporally controlled expression, which is required because *PARP* expression was supposed to inhibit yeast cell growth. Recombinant plasmids were purified and employed for yeast transformation. Transformed cells were transferred to liquid synthetic complete (LSC) medium (Formedium, UK) without uracil (Ura) containing 2% glucose (Glc) as carbon source and incubated at 30 °C overnight. 150 μL of liquid yeast culture were mixed with 850 μL of sterile glycerol, aliquoted, and frozen at −80 °C to produce cryo-starter aliquods. Those starter cultures ensured identical starting conditions in all experiments.

For yeast growth assays, an overnight culture in LSC-Ura + Glc was started from a 50 μL cryo-starter aliquod. After *c.* 24 h of growth, the culture was diluted with LSC-Ura + Glc to yield 1×10^6 cells mL^{-1} next morning. The cell suspension was washed with distilled water, diluted to 1×10^6 cells mL^{-1} in LSC medium containing 2% Gal to induce *PARP* gene expression, and 100 μL of cell suspension were transferred into wells of a 96-well plate. All incubations were carried out at 30 °C. OD_{600} was measured at the indicated time points using a microplate reader (Mithras LB940S3, Berthold Technologies, Germany). As determined by RT-PCR analysis, all *PARP* genes were expressed under inducing conditions.

As expected, induction of Hs*PARP1* expression in W303-1A yeast cells resulted in growth inhibition compared to the control cells carrying the empty vector (Fig. 1A). The same was observed for At*PARP1*-expressing cells, where OD_{600} increased only slightly within a 43 h growth period. The expression of At*PARP2* only partially delayed yeast cell growth, and At*PARP3* expression did not affect cell growth consistently (Fig. 1A). In contrast to the zinc-finger DNA-binding domains of HsPARP1 and AtPARP1, AtPARP2 has a SAP domain involved in DNA binding [13]. AtPARP3 apparently lacks any DNA binding domain [12]. Since a truncation of the DNA binding site has been shown before to repress the HsPARP1-induced growth inhibition in yeast cells [1], these differences in the structure of the N-terminal region of AtPARP2 and AtPARP3 are likely to be the reason for the absence of full yeast growth inhibition.

To analyze if growth inhibition of yeast cells expressing At*PARP1* is related to poly(ADP-ribosyl)ation activity, PARylation of yeast protein was determined. To this end, *PARP* expression was induced in LSC-Ura + Gal medium for 6 h. Approx. 100 mg yeast cells were harvested and frozen at −80 °C. 500 μL protein extraction buffer [25 mM Tris-HCl (pH 7.4), 10 mM $MgCl_2$, 10 mM β-mercaptoethanol, 1% protease inhibitor cocktail (Sigma-Aldrich)] and 600 μL acid-washed glass beads (425–600 μm, Sigma-Aldrich) were added to the frozen yeast cells, and cells were lysed in a tissue lyser (Qiagen) at 30 Hz for 5 min. The lysate was centrifuged in a microfuge for 3 min at max. speed and 4 °C. The supernatant was transferred to a new tube, and total protein concentration was determined by Bradford assay. 8 μg protein lysate were mixed with 1 volume of 40% methanol and spotted onto a nitrocellulose membrane using a dot blot apparatus (Biometra, Germany). Protein spots on the nitrocellulose membrane were excised by a hole-punch and transferred to a 96-well plate. PAR residues were determined using a PAR monoclonal antibody (10H) (Enzo Life Sciences, Farmingdale, USA) and a secondary anti-mouse antibody coupled to horseradish peroxidase. ECL reagent (250 mg L^{-1} luminol, 0.1 M Tris-HCl pH 8.6, 1% DMSO, 1 g L^{-1} para-coumaric acid) was added, and luminescence was quantified by using a microplate reader (Mithras LB940S3). Yeast cells expressing *HsPARP1* exhibited PARylation activity (Fig. 1B) as described previously [1,3–5]. Similarly high PAR modification was also observed in At*PARP1*-expressing yeast cells (Fig. 1B). Hence, AtPARP1 shares cellular functions with HsPARP1 in yeast. Previous authors used the activity of HsPARP1 in yeast to identify potential poly(ADP-ribose) acceptor proteins homologous to human nuclear proteins [5]. As yeast and plants also share homologous genes, the identification of AtPARP1 target proteins in yeast cells can provide a shortcut to identify putative AtPARP target proteins *in planta*.

Pharmacological inhibition of Arabidopsis PARP proteins *in planta* has been widely used to study their function, but the results are not unequivocal [12]. We modified the yeast growth assay to determine the activity of known human PARP inhibitors to AtPARP1, i.e., 3-aminobenzamide (3-AB), 6-(5H)-phenantridinone (PHE), and 4-amino-1,8-naphtalimde (4-ANI), and to establish a method to identify new inhibitors of AtPARP1. Since yeast cells lacking efflux pumps exhibit an increased drug sensitivity, the yeast

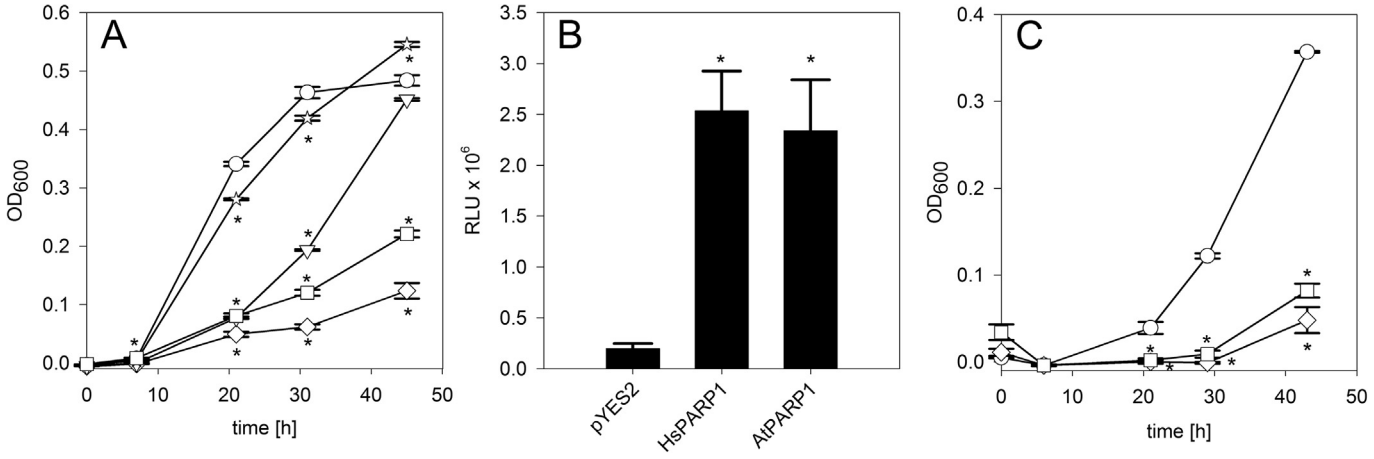

Fig. 1. Expression of Hs*PARP1* and At*PARP1* genes in yeast cells inhibits growth. (A) Growth of the W303-1A strain transformed with pYES2 empty vector (circles), or pYES2 carrying Hs*PARP1* (diamonds), At*PARP1* (squares), At*PARP2* (triangles), or At*PARP3* (stars). *PARP* expression was induced by the addition of galactose to the medium. Growth was scored by determining OD_{600} at the indicated time points. (B) Protein PARylation in W303-1A yeast cells as determined by PAR antibody after 6 h of PARP gene expression. (C) Growth of the AD1234567 strain transformed with pYES2 empty vector (circles), or pYES2 carrying Hs*PARP1* (diamonds) or At*PARP1* (squares). *PARP* expression was induced by the addition of galactose to the medium. Growth was scored by determining OD_{600} at the indicated time points. (A–C) Error bars represent SE of three replicates. An asterisk indicates that the mean of the yeast strain transformed with the *PARP* gene is significantly different from the mean of the pYES2-transformed strain according to two-sample two-sided Welch T-test at significance level 0.05. Experiments were repeated twice with similar results.