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ASR1, a stress-induced tomato protein, protects yeast from osmotic stress

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Asr1, a tomato gene induced by abiotic stress, belongs to a family, composed by at least three members, involved in adaptation to dry climates. To understand the mechanism by which proteins of this family seem to protect cells from water loss in plants, we expressed *Asr1* in the heterologous expression system *Saccharomyces cerevisiae* under the control of a galactose-inducible promoter. In a mutant yeast strain deficient in one component of the stress-responsive high-osmolarity glycerol (HOG) pathway, namely the MAP kinase *Hog*1, the synthesis of ASR1 protein restores growth under osmotic stress conditions such as 0.5 *M* NaCl and 1.2 *M* sorbitol. In contrast, the rescuing of this phenotype was less evident using a wild-type strain or the upstream MAP kinase kinase (Pbs2)-deficient strain. In both knock-out strains impaired in glycerol synthesis because of a dysfunctional HOG pathway, but not in wild-type, ASR1 led to the accumulation of endogenous glycerol in an osmotic stress-independent and unrestrained manner. These data suggest that ASR1 complements yeast HOG-deficient phenotypes by inducing downstream components of the HOG pathway. The results are discussed in terms of the function of ASR proteins in planta at the molecular and cellular level.

Introduction

Plants constantly have to cope with abiotic stress. Water deficit, salty soils and cold are the common stress conditions affecting plant development. Among them, high salinity is one of the most serious limiting factors in plant growth and productivity (Boyer 1982). In response to environmental stress, most plants produce an array of proteins that lead to adaptation of cell metabolism to these conditions (Ingram and Bartels 1996; Thomashow 1998, 1999).

Our interest is focused in one of the many stress-induced proteins occurring in plants, named ASR1, encoded by a gene first reported in tomato (Iusem et al. 1993; Rossi and Iusem 1994). Rossi et al. (1996) demonstrated that *Asr1* actually belongs to a gene family composed by at least three genes (*Asr1*, *Asr2* and *Asr3*). Since then, several homologs were isolated from many different species (reviewed by Maskin et al. 2001).

It is known that tomato *Asr1* is expressed under salt and osmotic stress (Amitai-Zeigerson et al. 1995) and differentially induced by water deficit (Maskin et al. 2001). ASR1 protein is highly hydrophilic as a consequence of its content of charged residues, particularly

Abbreviations – HOG, high-osmolarity glycerol; LEA, late embryogenesis-abudant; SC, synthetic complete.
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His, Lys and Glu (Amitai-Zeigerson et al. 1995; Iusem et al. 1993) and binds to a specific DNA sequence (Kalifa et al. 2004), in agreement with evidence in favour of its localization in the nucleus (Iusem et al. 1993; Wang et al. 2005). It is worth mentioning that the high hydrophilicity, along with a robust water stress induction in vegetative tissues, is feature shared by ASR (Maskin et al. 2001) and most proteins of the late embryogenesis-abundant (LEA) superfamily (Skriver and Mundy 1990), expressed at high levels during late embryo maturation (Chung et al. 2005). Although the biochemical function of ASR1 remains unclear, its nuclear localization and DNA-binding activity are in good agreement with the proposed role of ASR as a regulatory transcription factor involved in the activation of sugar transport (Cakir et al. 2003).

The baker's yeast S*accharomyces cerevisiae* is a well-developed and widely used model organism (Ton and Rao 2004; Wheeler and Grant 2004) as it offers a compact and fully sequenced genome, simple culturing conditions and a conservation of basic signal transduction pathways with higher eukaryotes. We decided to take advantage of this model organism to investigate if there is any osmoregulatory response mediated by ASR1, in an attempt to outline the in vivo function of tomato ASR1. Therefore, the intrinsic physiological response of *S. cerevisiae* to an osmotic challenge was to be taken into account.

Adaptation to osmotic stress in yeast is controlled by the high-osmolarity glycerol (HOG) MAP kinase pathway (Brewster et al. 1993). This pathway consists of two transmembrane osmosensors, Sho1p and Sln1p, a branched MAPK cascade and common downstream elements Pbs2p MAPKK and Hog1p MAPK. A high osmolarity estimulus causes rapid nuclear accumulation of Hog1p, the sole terminal MAPK (Gustin et al. 1998; Mager and Siderius 2002). Activated MAPKK Pbs2 phosphorylates the MAPK Hog1p by dual phosphorylation. Once activated and after translocation to the nucleus (Sharma and Mondal 2005), Hog1p modulates the expression of several osmo-inducible genes such as *GPD1*, encoding glycerol 3-phosphate dehydrogenase (Albertyn et al. 1994; Ferrigno et al. 1998; Lee et al. 2002; Reiser et al. 1999). This enzyme is involved in the synthesis of glycerol, which accounts for the major compatible solute of *Saccharomyces*. By increasing endogenous glycerol, cells regain turgor. Importantly to this work, the HOG pathway seems to be structurally and functionally conserved in plants (Covic et al. 1999).

To test the possible osmoprotective function of ASR1, we forced expression of this protein in the heterologous system *S. cerevisiae* under osmotic stress conditions. We demonstrated that ASR1 is able to rapidly improve yeast cell growth under saline stress conditions. Moreover, we found that ASR1 is able to complement osmotolerance deficiencies in *hog1*Δ and *pbs2*Δ mutants concomitantly with an increased accumulation of the osmolyte glycerol.

Materials and methods

Yeast strains and growth conditions

S. cerevisiae strains used in this study are the wild-type YPH102 (*MATa ura3-52 lys2-801^{amber} ade2-101^{ochre} his3-Δ200 leu2-Δ1*) and the isogenic mutants JBY13 [YPH499 (*MATa ura3-52 lys2-801^{amber} ade2-101^{ochre} trp1-Δ63 his3-Δ200 leu2-Δ1*) + *hog1Δ::TRP1*] and MAY1 (YPH102 + *pbs2Δ::LEU2*). Yeast was grown at 30°C in YPD medium (1% yeast extract, 2% peptone and 2% glucose) or in synthetic complete (SC) medium (0.17% yeast nitrogen base without amino acids and ammonium sulphate supplemented with Ura-Dropout and 10 m*M* ammonium sulphate). Carbon sources for synthetic medium were 2% raffinose or 1% galactose.

Stress assays

Yeast was cultivated in SC medium containing 2% raffinose for 24 h. SC media containing 2% raffinose or 1% galactose were supplied with 1/5 volume of the prepared preculture, and cells were incubated for different periods of time as indicated in the figures. Cells were then subjected to osmolarity stress at various NaCl concentrations, and growth was determined by measuring absorbance at 600 nm.

To test osmosensitivity in solid medium, we grew cells in 2% raffinose SC for 24 h, then transferred to 1% galactose SC and incubated for 3 h. Serially diluted (1:10) cell suspensions (3 µl) were spotted on YPD plates supplemented with various concentrations of NaCl or sorbitol. After three days at 30°C, plates were photographed.

Construction of the recombinant vector

All DNA manipulations were performed by standard procedures (Sambrook et al. 1989). The tomato *Asr1* full-length cDNA was PCR amplified using primers containing Xba1 and Kpn1 restriction sites (upper primer: 5′-GCTCTAGACTAAAATAACAATTTAGAAGAGAT-3′; lower primer: 5′GGGGTACCATGGAGGAGGAGAAACACCACCAC-3′). The cDNA was then cloned into the Xba1/Kpn1 site of the high-copy-number shuttle vector pYES2 (Invitrogen, CH Groningen, the Netherlands) that contains the GAL1 promoter. Wild-type and mutant

strains were transformed with the resulting recombinant vector using the method described by Chen et al. (1992). As a control, the same strains were transformed with the empty vector.

Protein extraction and Western blot assay

Yeast total protein was extracted with a buffer containing 0.06 *M* Tris HCl pH 6.8, 5% (v/v) β-mercaptoethanol, 10% glycerol, 2% sodium dodecyl sulphate (SDS). Western blots were performed according to Sambrook et al. (1989), loading 20 μg of total protein. After SDS-PAGE, proteins were electroblotted onto Hybond-C Extra nitrocellulose membrane (Amersham Pharmacia Biotech, Piscataway, NJ, USA). Membranes were stained with Ponceau red to ensure equal loading and then incubated with ASR1-specific antiserum (1/4000). Bound antibodies were detected using an anti-rabbit immunoglobulin G-peroxidase conjugate (Biorad, Hercules, CA, USA) diluted 1/4000. For visualization of enzyme activity, the ECL chemiluminescence detection system was used (Amersham Pharmacia Biotech).

Glycerol measurements

Cells from an overnight culture in 2% raffinose SC medium were transferred to fresh SC medium containing 2% raffinose or 1% galactose and incubated for 1 h. Cells were then transferred again to similar fresh media in the presence or absence of 0.25 *M* NaCl. After 3 h of incubation, two 10-ml samples were taken out for glycerol and total protein measurements. Cells were pelleted, washed with cold water and centrifuged again at 4°C. For glycerol determinations, pellets were resuspended in 1 ml 50 m*M* Tris-HCl pH 7.5, boiled for 15 min and centrifuged to remove cellular debris. The supernatant was used for glycerol quantification according to the manufacturers' instructions (glycerol measurement kit, Boehringer Mannheim, Mannheim, Germany).

Protein content was determined by the Bradford method (Bradford 1976).

Results

Effect of ASR1 expression on yeast growth

To test the possible role of ASR1 in protecting cells from water loss, we chose *S. cerevisiae* as a convenient heterologous expression host system. The wild-type strain was transformed with the plasmid pYES2 containing the *Asr1* clone driven by the galactose-inducible promoter GAL1. Cells transformed with the empty vector were used as a control. Cells derived from inducible conditions were transferred to fresh 1% galactose media containing or not 0.5 *M* NaCl. ASR1 seemed to confer an advantage in cell survival at early times of exposure to the high saline environment (Fig. 1). Under normal conditions, a detrimental effect of ASR1 in cell growth was observed (data not shown), as also reported for other heterologous proteins overexpressed in yeast (Choi et al. 1994; Kiel et al. 1999). The slight protective effect of ASR1 on wild-type cell growth under saline stress conditions was detected only at early times probably due to the establishment of yeast intrinsic adaptation mechanisms as time passes by. It should be noted that the observed behaviour may be the outcome of both a positive osmoprotective effect and the simultaneous and detrimental effect brought about by ASR1.

The presence of ASR1 in the culture was confirmed by Western blot (Fig. 1, inset).

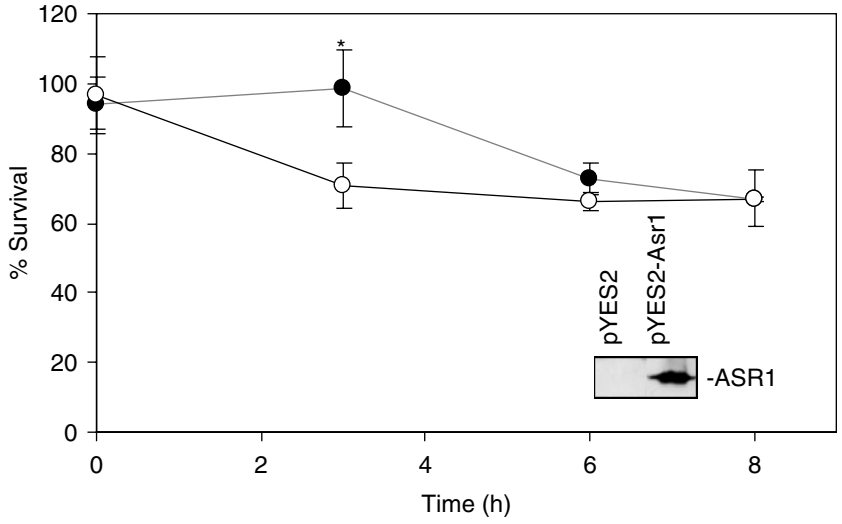

Fig. 1. Effect of ASR1 on yeast survival under salt stress. YPH102 cells transformed with pYES2 (○) or pYES2-Asr1 (●) were grown on 1% galactose medium for 17 h and then transferred ($t = 0$) to fresh 1% galactose synthetic complete medium supplemented or not with 0.5 *M* NaCl, and absorbance at 600 nm (A600nm) was determined at the indicated times. Survival percentage was expressed as 100 × A_{600nm} of the culture with NaCl/A_{600nm} of the culture without NaCl. Values presented are the mean of three independent experiments. Error bars denote ±mean standard error. *, P value < 0.05 (according to student's t-test). Inset: total protein extracts from YPH102 cells at 0 min were analysed by Western blot using a specific ASR1 antiserum.

As high expression levels of ASR1 in wild-type yeast cells produced some deleterious effect on cellular growth, we decided to diminish intracellular ASR1 amounts by shortening the time of induction by galactose. Total protein was extracted from cultures induced with galactose for different periods of time and analysed by immunoblot (Fig. 2). Although the maximum expression levels were reached after 8 h of induction, 3 h were enough to obtain a significant ASR1 expression.

Consequently, cells preinduced for 3 h were plated on complete media with different NaCl concentrations and assayed for growth. An overall detrimental effect caused by high osmolarity could be observed (Fig. 3, first row of each panel). The expression of ASR1 resulted in a slight, but consistently observed, improved salt resistance of wild-type cells (Fig. 3). It must be noted that this result was obtained after 3 days of growth in non-inductive conditions. The turnover of the heterologous protein was slow enough to allow its immunodetection even 48 h after the induction period (Fig. 4).

NaCl concentrations higher than those indicated allowed no growth at all even in Asr-bearing cells (not shown).

Functional analysis of ASR1 in yeast

To get further insight into the osmoprotective function of ASR1 in yeast cells, the effect of this protein was analysed in cells deficient in the HOG pathway, which bear a phenotype markedly sensitive to osmotic stress due to their inability to synthesize normal amounts of glycerol (Brewster et al. 1993). ASR1 turned out to provide protection to *hog1*Δ cells, as it allowed them to grow at 0.5 *M* NaCl, a concentration otherwise lethal for this strain (Fig. 5). However, we failed to detect ASR1 overcoming the growth impairment of salt-stressed *pbs2*Δ cells (Fig. 5), even when different stress conditions (not shown) were used (0.6 and 0.75 *M* NaCl; 0.5, 0.6 and 0.75 *M* KCl; 0.9 *M* sorbitol). A possible explanation is

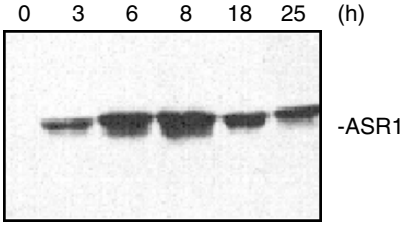

Fig. 2. Analysis of ASR1 expression levels in yeast cells after different times of galactose induction. YPH102 cells transformed with pYES2-Asr1 were grown on 2% raffinose synthetic complete (SC) medium and transferred ($t = 0$) to 1% galactose SC medium. At the indicated times, total protein extracts were prepared and analysed by Western blot.

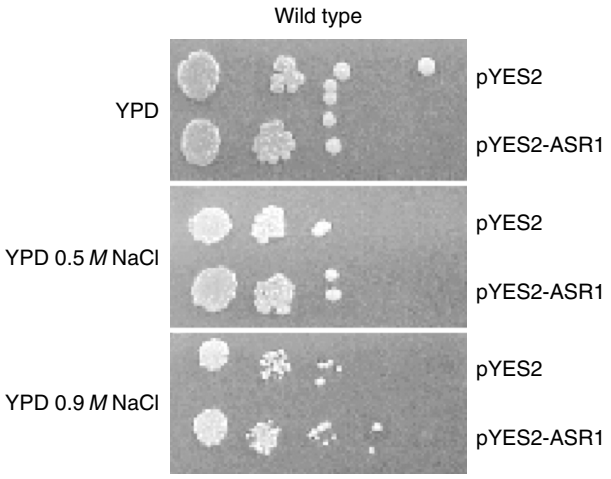

Fig. 3. Effect of ASR1 on yeast growth under different saline concentrations. YPH102 cells transformed with pYES2 or pYES2-Asr1 were grown on 2% raffinose synthetic complete (SC) medium and then incubated for 3 h with 1% galactose. Serially diluted (1:10, starting $A_{600nm} = 0.1$) cell suspensions (3 µl) were spotted on YPD plates without or with 0.5 *M* NaCl and 0.9 *M* NaCl. After 3 days at 30°C, plates were photographed.

that the higher amounts of endogenous ASR1 found in the latter mutant (Fig. 4) may be the cause of its poor performance. It is noteworthy that although the number of colonies for this mutant did not change, they became smaller in size in the presence of an osmolyte, indicating a lower number of cell divisions and thus the occurrence of stress.

We also incubated yeast mutant cells in a medium containing sorbitol, a non-charged osmolyte. The resulting effect was in the same direction but slighter than that caused by NaCl (Fig. 5, bottom panels), suggesting that the change in cellular ion balance is an important, but not exclusive, component of the overall stress against which Asr protects.

These results prompted us to determine intracellular glycerol content in cells challenged to stress. The stress conditions used were mild (0.25 *M* NaCl) to allow normal growth of the deficient yeast strains. In wild-type cells (Figs. 6A and B), accumulation of glycerol depended on the presence of NaCl, irrespective of the expression of ASR1. Interestingly, the expression of ASR1 in *hog1*Δ cells produced a significant accumulation of glycerol, and this effect was independent on the imposition of any salt stress (Fig. 6C). Similar results were observed in *pbs2*Δ cells, although the glycerol content was lower than that measured in *hog1*Δ cells. The results indicate that ASR1 produces an important increase in glycerol accumulation only when the HOG pathway is dysfunctional. In contrast, when this pathway is normally operating, ASR1 per se does not modify

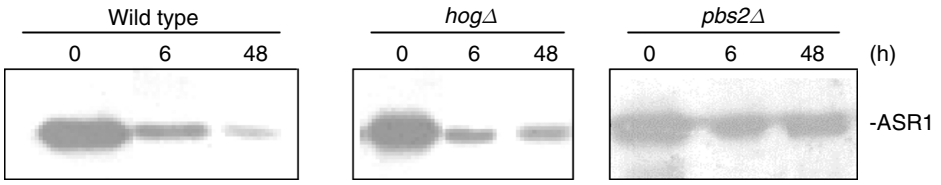

Fig. 4. Life time of ASR1 protein in yeast cells. Wild-type (YPH102), *hog1*Δ (BJY13) and *pbs2*Δ (MAY1) cells carrying pYES-Asr1 were grown on 2% raffinose synthetic complete (SC) medium and then transferred to 1% galactose SC medium. After 3 h of incubation, cells were transferred to YPD medium ($t = 0$) and incubated at 30°C. Samples were withdrawn at the indicated times, and total protein extracts were prepared and analysed by Western blot.

the glycerol phenotype, suggesting a tight regulation of glycerol content by the HOG pathway.

Discussion

Asr1 is one of the numerous plant gene products that become overexpressed under water and salt stress conditions (Ramanjulu and Bartels 2002). On the other hand, yeast cells are considered an attractive eukaryotic model expression system used to address the function of plant genes (Cadinanos et al. 2003; Hashimoto et al. 2004; Yamaguchi et al. 2003; Zhuang et al. 2005). Inasmuch as plant responses to environmental salt and water deficit stress have much in common, because salinity reduces the ability of plants to take up water (Munns 2002), we expressed the water deficit stress-induced tomato *Asr1* gene in the *S. cerevisiae* host under osmotic stress to gain insights into the in planta function of ASR1 protein.

The inability of *pbs2*Δ and *hog1*Δ to grow in the presence of high salt concentrations conforms the previously observed dramatic reduction of osmoresistance of such yeast mutant cells lacking a functional HOG pathway (Brewster et al. 1993). Interestingly, the osmoprotective effect of ASR1 turned out to be more evident in such yeast strains. In fact, our results demonstrate that ASR1, in spite of being a source of mild stress itself, is able to complement the growth impairment phenotype inherent to the *hog1*Δ mutant. Although both strains are defective in the same pathway, the absence of the terminal kinase *Hog*1p leads to a stronger osmosensitivity than that observed in the absence of the kinase kinase *Pbs*2p. Although many stress conditions were tested, we found no overt restoring of *pbs2*Δ growth by ASR1. However, such a scenario could be foreseen, if more severe but not lethal stress conditions were imposed.

Yeast cells respond to a shift to higher osmolarity by increasing the cellular content of the osmolyte glycerol (Shen et al. 1999). In this context, the mere production of heterologous proteins might as well trigger broad stress responses, including synthesis of glycerol and would thus lead to misinterpretation of the results associated with osmoprotection. Nevertheless, this was not the case as ASR1 alone produced no increase in the accumulation of glycerol in wild-type cells. The puzzling fact that *hog1*Δ cells are able to produce substantial amounts of glycerol in basal conditions is in good

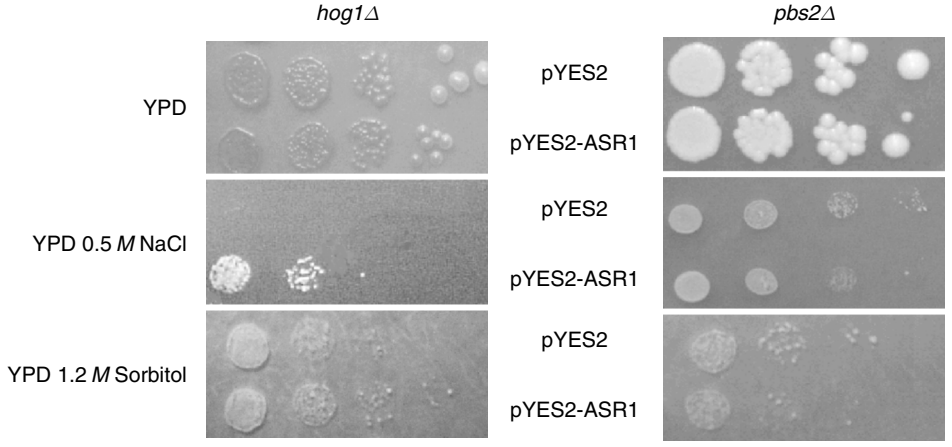

Fig. 5. Effect of ASR1 on growth of HOG pathway-defective mutants under osmotic stress. Wild-type (YPH102), *hog1*Δ (JBY13) and *pbs2*Δ (MAY1) cells transformed with pYES2 or pYES2-Asr1 were grown on 2% raffinose synthetic complete (SC) medium, then transferred to 1% galactose SC medium. After 3 h of incubation, serially diluted (1:10, starting $A_{600nm} = 1.0$) cell suspensions (3 μl) were spotted on YPD plates and on YPD plates with 0.5 *M* NaCl or 1.2 *M* sorbitol. After 3 days at 30°C, plates were photographed.

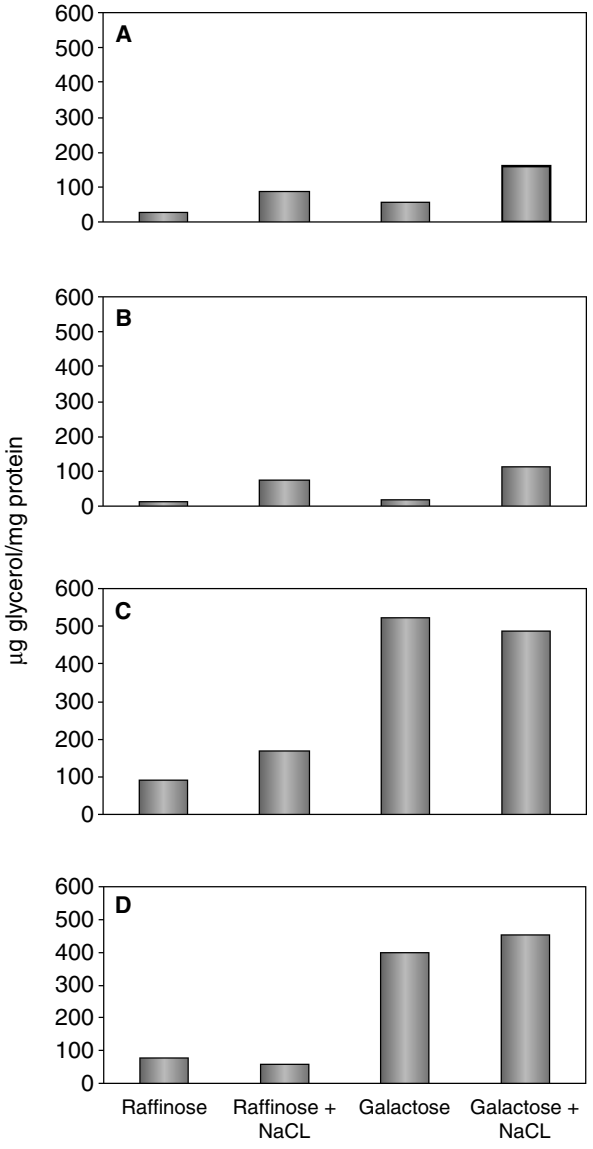

Fig. 6. Intracellular glycerol levels in yeast cells. Cells from an overnight culture in 2% raffinose synthetic complete (SC) medium were transferred to fresh SC medium containing 2% raffinose or 1% galactose and incubated for 1 h. Then, cells were transferred again to similar fresh media containing or not 0.25 *M* NaCl. After 3 days of incubation, two 10-ml samples were taken for glycerol and protein quantification. Standard deviations were lower than 15%. (A) YPH102 wild-type cells transformed with empty plasmid (pYES2). (B) YPH102 wild-type cells transformed with the recombinant plasmid (pYES2-Asr1). (C) JBY13 cells (*hog1*Δ) transformed with the recombinant plasmid (pYES2-Asr1). Glycerol value for control cells with empty plasmid induced with galactose was 110 µg glycerol/mg protein. (D) MAY1 cells (*pbs2*Δ) transformed with the recombinant plasmid (pYES2-Asr1). Glycerol value for control cells with empty plasmid induced with galactose was 88 µg glycerol/mg protein.

agreement with the findings by Albertyn et al. (1994), who postulated a *hog1*-independent mechanism mediating osmostress-induced glycerol accumulation. If this putative alternative pathway indeed exists, it seems to be more active in the mutant cells than in the wild-type genotype, probably because *hog1p* normally inhibits shared upstream components of the HOG pathway, as part of a feedback control on the signal cascade, as envisaged by O'Rourke and Herskowitz (1998).

Only in mutant strains bearing a dysfunctional HOG pathway, ASR1 led to the accumulation of glycerol in an osmotic stress-independent manner. The mechanisms by which ASR1 restores adequate intracellular glycerol levels in such an unrestrained fashion are still obscure to understand. The complementation experiments in yeast suggest a genetic mode of action for ASR1 by inducing downstream components of the HOG pathway, which is known to exist in plants as well (Covic et al. 1999). However, based on its abundant expression in several plant tissues and its high degree of hydrophylicity (Iusem et al. 1993; Maskin et al. 2001), an alternative, but not mutually exclusive, structural function such as the promotion of direct protection from water loss cannot be ruled out, as is the case for a mitochondrial LEA protein able to protect enzymes from drying (Grelet et al. 2005). Independently of what the mechanism might be, the involvement of ASR proteins in physiological adaptation of wild tomato to dry climates is strongly supported by our studies through evolutionary approaches concluding that *Asr2* has been the target of positive natural selection (Frankel et al. 2003).

Very recently, ASR from lily pollen has been reported to confer drought and salt resistance in Arabidopsis (Yang et al. 2005), although no precise physiological mechanism could be concluded. Current research using transgenic plants is in progress in our laboratory with the intention to fully understand the molecular and cellular mechanism of action of these widespread proteins underlying adaptation of plants to environmental stress.

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