Does proline accumulation play an active role in stress-induced growth reduction?

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Summary

An interesting observation, reported for transgenic plants that have been engineered to overproduce osmolytes, is that they often exhibit impaired growth in the absence of stress. As growth reduction and accumulation of osmolytes both typically result from adaptation, we hypothesized that growth reduction may actually result from osmolyte accumulation. To examine this possibility more closely, intracellular proline level was manipulated by expressing mutated derivatives of tomPRO2 (a Δ^1-pyrroline-5-carboxylate synthetase, P5CS, from tomato) in Saccharomyces cerevisiae. This was done in the presence and absence of a functional proline oxidase, followed by selection and screening for increased accumulation of proline in the absence of any stress. Here we show, in support of our hypothesis, that the level of proline accumulation and the amount of growth are inversely correlated in cells grown under normal osmotic conditions. In addition, the intracellular concentration of proline also resulted in increases in ploidy level, vacuolation and altered accumulation of several different transcripts related to cell division and gene expression control. Because these cellular modifications are common responses to salt stress in both yeast and plants, we propose that proline and other osmolytes may act as a signaling/regulatory molecule able to activate multiple responses that are part of the adaptation process. As in previous studies with transgenic plants that overaccumulate osmolytes, we observed some increase in relative growth of proline-overaccumulating cells in mild hyperosmotic stress.

Keywords: osmolytes, ploidy, vacuolation, microarray, signal transduction.

Introduction

Recent attempts to improve plant growth and/or survival under stress by manipulating the level of compatible solutes through genetic engineering have been only marginally successful. The assumption behind this approach has been that overproduction of these osmolytes may help plants to tolerate stress by improving their ability to adjust osmotically (Hayashi et al., 1997; Holmström et al., 1996; Kavi Kishor et al., 1995; Pilon-Smits et al., 1995; Sheveleva et al., 1997; Tarczynski et al., 1993). The constitutive or inducible concentrations of compatible solutes reported in most of these studies, however, has not exceeded a few millimolar, an amount that can account for only a very small proportion of the total osmotic adjustment typically observed when plant cells are osmotically challenged (Morgan, 1984). Considering the relatively minor impact of these organic osmolytes on cellular water relations and the resulting controversy of osmotic adjustment as a mechanism of tolerance observed in transgenic plants (Blum et al., 1996; Sharp et al., 1996), others have suggested that the principal role of these substances may be related to their ability to stabilize proteins, membranes and subcellular structures (Rhodes...
and Samaras, 1994; Yancey, 1994), or to protect cellular functions against oxidative damage by reactive oxygen species (Bohnert and Shen, 1999).

Importantly, neither osmotic adjustment nor osmoprotection has been conclusively demonstrated to be a result of recent efforts to engineer genetically such traits. In plants, the metabolic constraints limiting overproduction are not well understood for most compatible solutes. The biochemical basis for controlling accumulation of proline has been partially revealed, however (Csonka, 1981; Hu et al., 1992; Maggio et al., 1997; Samaras et al., 1995). The proline biosynthetic pathway has been described in bacteria and plants (Csonka and Baich, 1983; Delauney et al., 1993), and the rate-limiting enzyme also has been identified. In plants this is a 1,4-pyrroline-5-carboxylate synthetase (P5CS), a bifunctional enzyme with both γ-glutamyl kinase (GK) and γ-glutamyl phosphate reductase activities.

Limited accumulation of proline has been obtained in transgenic tobacco plants overexpressing the bifunctional enzyme P5CS (Hong et al., 2000; Kavi Kishor et al., 1995). Two potential problems might explain why proline failed to accumulate constitutively to osmotically significant levels. First, proline inhibits its own synthesis as an allosteric feedback regulator of γ-glutamyl kinase (Hu et al., 1992; Samaras et al., 1995). Second, proline is quickly catabolized to glutamate (Thompson, 1980). As a result, overexpression of GK and GPR activities alone may not allow the maximal potential for proline accumulation in transgenic plants.

In bacteria it has been clearly established that expression of GK-GPR mutations with loss of feedback inhibition by proline can significantly increase the accumulation of proline and improve salt tolerance (Csonka, 1981). Mutants of Salmonella typhimurium with enhanced osmotolerance have been selected as strains which overproduce proline due to mutations affecting the feedback sensitivity of γ-glutamyl kinase (GK), the enzyme involved in the first step of proline biosynthesis (Csonka, 1998). Transfer of the mutated genes conferred enhanced osmotolerance to other S. typhimurium recipient strains. These spontaneous mutations were generated by selection for resistance on the toxic proline analogue L-azetidine-2-carboxylate (AZ) (Grant et al., 1975). Strains with a high intracellular level of proline were able to survive because the high proline concentration can competitively block the toxic analogue. As there are orthologous proline biosynthetic pathways in bacteria and plants, and evidence exists that the plant P5CS enzyme pathway is also under proline feedback regulation (Hu et al., 1992; Samaras et al., 1995), we used a similar approach to isolate mutations in the GK domain of tomPRO2, a 1,4-pyrroline-5-carboxylate synthetase (P5CS) previously cloned from tomato. This enzyme also encodes both γ-glutamyl kinase (GK) and γ-glutamyl phosphate reductase (GPR) (Maggio et al., 1996). Here we report on the isolation of mutated versions of tomPRO2 which, when expressed in yeast, allow enhanced accumulation of proline. Although we found only marginal increases in relative salt tolerance of proline overaccumulating cells, we observed that growth and intracellular proline content are inversely correlated in yeast. Furthermore, we observed that the intracellular proline accumulation in yeast was directly correlated with increased ploidy level and vacuolation. As reduced growth, endopolyploidy and formation of multiple vacuoles are common responses to hyperosmotic stress in yeast (Bone et al., 1998) and plants (Chang et al., 1996), our results suggest that proline acts as a signal/regulatory molecule able to activate multiple stress responses. In fact, microarray analysis indicates that the levels of several transcripts are altered in proline-overproducing cells. Enhancement and/or constitutive activation of these responses does not lead to increased salt tolerance, presumably because the adaptive response is incomplete.

Results

Isolation of proline-overproducing mutants

Proline-overproducing mutants were selected as Escherichia coli strains resistant to DHP, a toxic proline analogue (Csonka, 1981). Despite the appealing simplicity of this approach, similar experiments performed on bacteria revealed that only a very small percentage (1%) of resistant Salmonella mutants overaccumulated proline. Proline accumulation is, in fact, also conferred by a more frequent mutation (put1) inactivating the major proline permease which functions in the uptake of proline (and its analogues) (Csonka, 1981). Our approach to by-passing this potential problem and increasing the percentage of desirable mutations was to randomly mutagenize the plasmid carrying the tomPRO2 gene in a separate host, XL1Red, which is deficient in three of the primary DNA repair pathways (Figure 1). The mutagenized plasmids were then used to transform a normally sensitive E. coli host strain (CSH26) prior to plating on the toxic analogue. The E. coli strain CSH26 is a proline auxotroph that requires a functional tomPRO2 to survive on minimal media (M63), but is inhibited by toxic analogues of proline. Any acquired resistance would probably be the result of mutations in the tomPRO2 gene, and not in genes encoding the permease, which is carried on the bacterial chromosome. The mutated tomPRO2 plasmids were transformed in CSH26 and selected as resistant to 1.5 mM DHP (a threshold concentration lethal for the strain carrying the wild-type plasmid). Two rounds of selection confirmed the DHP-resistant phenotype. Before determining proline content of the mutants, a preliminary screen...
was performed by growing DHP-resistant colonies on media containing 0.6 M NaCl. Fifteen colonies clearly exhibited faster growth on NaCl when compared to the cells carrying the wild-type \textit{tomPRO2} gene. Plasmids were isolated from the 15 DHP/NaCl-resistant colonies and retransformed in CSH26 to confirm plasmid-specific transmissibility of DHP resistance. Determination of the cellular proline content by the TL131 bioassay identified three categories of proline overproducers: mild, strong and very strong (5, 10 and >20 times more proline than \textit{tomPRO2}). Three clones from the last category, designated number 4, 13 and 20, were chosen for further analysis.

\begin{table}
\centering
\begin{tabular}{|c|c|c|}
\hline
Mutation No. & Nucleotide change & Amino acid substitution & Position \\
\hline
4 & Ctt \rightarrow Ttt & Leu \rightarrow Phe & 70 \\
13 & gGc \rightarrow gCc & Gly \rightarrow Ala & 99 \\
20 & gGa \rightarrow gAa & Gly \rightarrow Glu & 171 \\
\hline
\end{tabular}
\caption{Amino acid substitution in \textit{tomPRO2-4}, \textit{tomPRO2-13} and \textit{tomPRO2-20}}
\end{table}

Capital letters indicate a single nucleotide change.

The \textit{GK} amino acid terminal domain. However, the amino acid substitution for mutations 4 (Leu \rightarrow Phe) and 13 (Gly \rightarrow Ala) were only 19 amino acids apart from each other, whereas mutation 20 (Gly \rightarrow Glu) was nearly 100 amino acids away from the other two (Table 1).

\textit{TomPRO2} gene functions in \textit{Saccharomyces cerevisiae pro1}

To determine whether a plant P5CS could be functionally expressed in yeast, we transformed a \textit{Saccharomyces cerevisiae pro1} mutant strain (DT1000) with \textit{tomPRO2} and with the three mutated derivatives of \textit{tomPRO2}. Both \textit{tomPRO2} and its mutated versions complemented the \textit{pro1} mutants, re-establishing proline prototrophy (Figure 2a). The plant gene(s) that encodes for a bifunctional enzyme with both $\gamma$-glutamyl kinase (GK) and $\gamma$-glutamyl phosphate reductase (GPR) was also able to confer proline prototrophy in a yeast \textit{pro2} mutant strain (Orser \textit{et al}., 1988; data not shown). Proline content in DTP2 was 20 times higher than that measured in wild-type MB1433, whereas the average proline content of the three mutants (DTP2-4, DTP2-13 and DTP2-20) was 25-fold higher than the wild-type yeast and significantly different from the proline level in the strain carrying the wild-type plant gene (DTP2) (Figure 2c).

\textit{Proline accumulation is associated with reduced growth}

As hypothesized, the growth of the different yeast transformants bearing the \textit{tomPRO2} genes was inversely correlated to their proline content when grown in the absence of any stress. To further examine the relationship between proline accumulation and growth, we also inactivated \textit{put1}, the gene encoding the yeast proline oxidase, in the strains carrying the tomato \textit{tomPRO2} (DTP2) and two of its mutated derivatives (DTP2-13 and DTP2-20) (Figure 2b). Using this strategy, we were able to obtain an average 63-fold increase in the proline pool of the wild-type yeast strain (Figure 2c). There continued to be a very strong inverse relationship between growth and proline accumulation over a very large range of cellular
proline levels (0.5–50 nmol μg\(^{-1}\) cellular protein) which were brought about only by genetic manipulation of genes controlling proline metabolism/catabolism. Thus the observed reductions in growth appear to be directly related to the intracellular level of proline.

To describe the kinetics of yeast cell growth, OD\(_{600}\) values were measured throughout 55 h growth periods, and a second-order logistic growth curve of the form

\[
\text{OD}_{600} = \frac{a}{1 + be^{-(ct + dt^2)}}
\]

was fitted to each data set (Joly and Hahn, 1989), where parameter \(a\) is the final (asymptotic) value attained by OD\(_{600}\), \(b\) is related to the initial starting size, \(c\) defines the duration of the lag phase prior to the onset of accelerated growth, \(d\) is the system’s inherent rate of increase, and \(t\) is time. Visual inspection of the fitted curves revealed key differences among strains in the general shape of the

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**Figure 2.**
(a) Complementation of strain DT1000 (1) with tomPRO2 and tomPRO2 derivatives tomP2-4, tomP2-13 and tomP2-20 to obtain strains DTP2 (2), DTP2-4 (3), DTP2-13 (4) and DTP2-20 (5), respectively.
(b) Knock-out of the structural proline oxidase in strains MB1433 (DT1000 parental strain); DTP2; DTP2-13; and DTP2-20. The derived strains MB1433p (1); DTPP2 (2); DTPP2-13 (3); and DTPP2-20 (4) were grown on media containing ammonium sulfate (top panel) or proline (bottom panel) as nitrogen source.
(c) Proline accumulation in the strains studied. Strains MB1433 and MB1433p are labelled MB and MBP, respectively. Error bars represent LSD, \(P = 0.05\). Means are different where error bars do not overlap.

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**Figure 3.** Growth curves of strains MB1433, DT1000G (vector only), DTP2, DTPP2, DTP2-20, and DTPP2-20 in Yeast extract Peptone Dextrose (YPD) media without salt. Lines are fitted curves of a second-order logistic function.

function. First, there was a strong negative association between proline accumulation and final biomass attained (Figure 3). This result appears to be due to reduced cell division, as microscopic examination of single cells from different strains revealed an increase in cell diameter in proline overproducer strains. Final growth was similar in strains carrying the mutated synthetase alone (DTP2-20) or in combination with the knock-out of the structural proline oxidase (DTPPP2-20). Second, proline accumulation delayed the onset of exponential growth. Analysis of the fitted logistic curves permitted us to compute both the maximum rate of growth (the rate at which the first derivative of the line was maximized) and the time at which the growth rate stops increasing (the inflection point of the sigmoid curve). Both parameters of cell-growth kinetics were strongly associated with proline content, with overaccumulating strains (DTPP2 and DTPPP2-20) showing lower rates of maximal growth and a delayed inflection point of maximal growth. The maximal rate of growth for wild-type strain MB1433, for example, was 0.53 OD units h\(^{-1}\), a value approximately four times greater than that measured in either DTP2 or DTPPP2-20. In addition, the time at which growth rate stopped increasing was delayed in these strains by approximately 7.5 h, relative to the wild type.

**Proline accumulation, DNA content and cell morphology**

Compared with MB1433 (wild type) and vector-only transformed yeast, an increased ploidy level was observed in proline overproducing strains DTP2-20 and DTPPP2-20 (Figure 4). The DNA content analyzed by flow cytometry was also directly proportional to the cellular proline content, with strains DTP2-20 (\(\sim 25 \times \) proline) and DTPPP2-20 (\(\sim 65 \times \) proline) having 1.5 and 2.0 times, respectively, the normal DNA content (Figure 4a). Cell size also increased with increasing ploidy (Figure 4b). Strains DTP2-20 (long axis = 7.12 ± 0.18 μm, \(n = 50\)) and DTPPP2-20 (long axis = 9.32 ± 0.25 μm, \(n = 50\)) were each significantly larger than either the wild-type strain (MB1433, long axis = 5.6 ± 0.07 μm, \(n = 50\)) or the vector-only transformant (long axis = 5.9 ± 0.14 μm, \(n = 50\)) (Figure 4b). Microscopic analysis of strains MB1433 (wild type), DT1000G (vector control), DTP2-20 (\(\sim 25 \times \) proline) and DTPPP2-20 (\(\sim 65 \times \) proline) (Figure 4b) revealed significant cytological differences after proline accumulation. In addition to the increased size and elongated shape previously noted, strain DTPPP2-20 had increased vacuolation (Figures 4b and 5). We observed a higher number of vacuoles in mother and daughter mature cells (Figure 5). However, we noticed the presence of several small vacuoles and a large central vacuole only in mature cells (WT = 2.3 ± 0.3, \(n = 10\); DTPPP2-20 = 16.4 ± 0.8, \(n = 10\)), suggesting that the numerous small vacuoles of the neo-formed cell will eventually coalesce in the main vacuole (Figure 5). This syndrome of alterations that are associated with increased proline accumulation could have resulted from a perturbation of signal transduction which would probably also result in gene expression changes.

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**Figure 4.** DNA content (a) and yeast cells photographed in the optical microscope using Nomarsky contrast (b) of strains MB1433 (WT), DT1000G (vector only), DTP2-20, and DTPPP2-20.

Gene expression changes associated with increased proline accumulation

The MB1433 and DTPP2-20 yeast strains are isogenic, except for the expression of the mutated version of the plant tomPRO2 gene and the knock-out of proline oxidase carried by DTPP2-20. The only known major metabolic consequence of these genetic changes is the accumulation of proline. If signal transduction perturbations are initiated by altered proline metabolism and result in the observed changes in ploidy and vacuolization, many alterations in transcript abundance should also occur. Microarray analysis of all 6218 expressed ORFs of the yeast genome revealed that numerous genes were either significantly induced or repressed in response to altered proline metabolism. After averaging repeated hybridizations, a total of 140 ORFs showed a ratio higher (and 184 ORFs a ratio lower), than 2 twofold deviation between the two lines (Figure 6; Table 2).

Several interesting features of these changes are pertinent to the phenotypic differences between MB1433 and DTPP2-20. The genes displaying the most dramatically altered levels of transcripts between these two lines were mostly annotated as ORFs with regulatory functions such as transcription factors, chromatin structure/remodeling proteins, DNA/RNA synthesis-metabolism, and G-protein regulators (Table 2). Also, many of the highly differentially accumulated transcripts were encoded by genes of unknown function. It is interesting to note that an unknown transcription factor represented the transcript most dramatically affected by proline over accumulation. Galitski et al. (1999) identified 17 genes that displayed altered transcript accumulation patterns most reliably related to ploidy level in yeast. Curiously, none of those genes was found among our list of most altered transcripts (Table 2). Because Galitski et al. (1999) altered the ploidy of yeast cells via genetic manipulation of mating type, this suggests that specific genetic perturbations may give rise to particular sets of ploidy-associated gene expression in yeast.

Can proline accumulation confer tolerance to NaCl-induced stress?

Preliminary dose-response experiments identified an NaCl concentration threshold of 500 mM that was somewhat inhibitory to the growth of MB1433 (and GPD), but did not affect the growth of proline overaccumulating strains. In the presence of 500 mM NaCl, strain MB1433 had a 10% reduction in growth relative to its non-stressed control. In contrast, the growth of strains DTP2 and DTP2-13 was not affected by the presence of NaCl at these concentrations (Figure 7). We were unable to demonstrate any dramatic changes in NaCl tolerance, even with cells accumulating 100 times normal proline levels. As reported for transgenic plants that overaccumulate other osmolytes (Holmström et al., 1996; Romero et al., 1997; Tarczynski et al., 1993), increased tolerance to NaCl could be demonstrated only relative to growth without stress or during a particular stage of development.

Discussion

Isolation of tomPRO2 mutations and enhanced accumulation of cellular proline

As the relationship between enhanced accumulation of cellular proline and increased tolerance to osmotic stress remains uncertain, despite being studied for more than...
three decades, we sought to use the powerful molecular genetic model \textit{S. cerevisiae} to help examine this relationship more closely. The three \textit{tomPRO2} mutants analyzed in this study were selected according to their ability to accumulate at least 20 times more proline in \textit{E. coli} than the wild-type \textit{tomPRO2}, indicating that the mutations probably had affected the \textit{tomPRO2} feedback inhibition sites. Sequence analyses revealed a new class of single-nucleotide mutations, none of which resembled, in terms of amino acid substitution and localization within the GK domain, the mutations obtained in genes from other organisms (Table 1; Csonka, 1981; Zhang \textit{et al.}, 1995).

The advantage of our selection strategy compared to others (site-directed mutagenesis) is that it was targeted to the isolation of functional mutations determining proline accumulation. In fact, although Zhang \textit{et al.} (1995) removed the feedback inhibition in the P5CS from \textit{Vigna aconitifolia}, replacing an alanine for a phenylalanine at amino acid residue 129, they did not provide any data concerning the level of proline accumulation in \textit{E. coli} (or any other organism) harboring this mutated enzyme. It is likely that the mutations we isolated also resulted in reduced feedback inhibition of the P5CS by proline. Regardless of the biochemical mechanism, these mutations resulted in significant increases in intracellular proline levels, allowing us to examine the specific effect of proline accumulation on growth with and without osmotic stress.

**Proline accumulation, growth and salt tolerance**

Although plant species can differ considerably in the amount of proline that accumulates upon stress, there is also no clear relationship between ability to accumulate proline on stress imposition and stress tolerance among species. Species from the Solanaceae family, for example, can increase their proline pool by more than two orders of magnitude (Fujita \textit{et al.}, 1998; Handa \textit{et al.}, 1986). For many other species, only a moderate increase in proline content has been observed (Aspinall and Paleg, 1981). \textit{Arabidopsis}, for example, is not considered to be a strong proline accumulator as its proline pool typically exhibits...
only a 10-fold increase when plants are exposed to stress (Liu and Zhu, 1997). Ideally, plants with identical genetic backgrounds differing only for their constitutive/inducible proline content should be compared for salt tolerance. Attempts to accomplish this by genetic transformation of plants with genes encoding proline metabolic enzymes have not achieved this goal — only modest increases in proline accumulation were found in the engineered plants (Hong et al., 2000; Kavi Kishor et al., 1995).

We envisaged that *S. cerevisiae* would be an ideal system by which to quantify the relationship between intracellular proline level and salt tolerance because it does not synthesize proline when osmotically challenged and is easily manipulated genetically (Blomberg and Adler, 1992). Therefore, any advantage of accumulation of proline by *tomPRO2* transformants/mutants would not be confounded by a stress-induced proline accumulation in wild-type cells. Our best proline-overaccumulating lines exhibited only modest tolerance to NaCl, and tolerance was evident only when growth under stress was considered relative to growth without stress (Figure 8). Relative tolerance to NaCl may actually result from an effect of proline accumulation on growth, not on tolerance per se. Such an effect would be generally consistent with other reports in the literature, and highlights a complex and intriguing relationship between overproduction of osmolytes, growth and osmoprotection. For instance, Tarczynski et al. (1993) demonstrated that transgenic tobacco plants overproducing the sugar alcohol mannitol were also salt-tolerant. However, later experiments by Karakas et al. (1997) showed that the relatively greater salt tolerance of plants overproducing mannitol was associated with their generally slower growth. They suggested that the slower growth of the transgenic plants, and not the presence of mannitol per se, may have been the cause of greater relative salt tolerance. These transgenic plants did not achieve an amount of fresh or dry mass greater than the non-transgenic controls under any conditions.
tested. Similar results were obtained by Romero et al. (1997) with transgenic tobacco plants overexpressing the TPS1 gene from S. cerevisiae that encodes a trehalase-6-phosphate synthase. Although overaccumulation of solutes could conceivably reduce growth through altered nutrient allocation, growth reduction has been associated in many of these cases, with accumulation of solutes at much less than nutrient-limiting levels. Under some conditions of stress, proline accumulation is not always associated with reduced growth. For example, in water-stressed maize roots, cell elongation is maintained in cells that accumulate proline (Voetberg and Sharp, 1991).

It is clear that cellular and, ultimately, organismal growth rates are intimately linked to the adaptive response to osmotic stress. This close connection is the result of a survival advantage conveyed by reduced growth during stress (Bressan et al., 1990). What is less clear is the way by which organisms, including plants, perceive a stressful environment and maintain an altered growth rate, even after osmotically adjusting to the stress. It is generally thought that initial perception of osmotic stress occurs through an osmotically induced change in the cell membrane, leading to the activation of a receptor, such as the SLN two-component kinase in yeast (Gustin et al., 1998), or its apparent homologue AtHKt which has recently been described in Arabidopsis (Urao et al., 1999). After the cell has re-established osmotic balance with the environment (osmotically adjusted), however, the plasma membrane should no longer be in an osmotically induced altered state. How, then, does the cell maintain an altered growth rate after osmotic adjustment? Perception of the continued osmotically challenging environment may be transduced to elements controlling cell growth by the elevated concentration of a cell solute (proline) which would subside only after the pre-stress osmotic gradient is re-established by decreasing the osmotic strength of the cell’s environment. Although it is as yet unclear exactly how this transduction occurs in yeast or plants, it may involve signaling through the yeast MAPKinase SKN7.

Figure 7. Working model based on what is known about Saccharomyces cerevisiae and Saccharomyces pombe to explain a possible role for proline in mediating growth and adaptation in a hyperosmotic environment.

Figure 8. Osmotic stress in strains DT1000G (vector), MB1433, DTP2, and DTP2-13. Cells were grown in YPD media with of 0, 0.5 or 1.0 M NaCl at 28°C for 36 h, then collected for OD₆₀₀ measurements (see Experimental procedures). Error bars, LSD at P = 0.05. Means are different where error bars do not overlap.
On the other hand, in microorganisms the significance of a proline-induced polyploidization and subsequently altered gene expression may be to generate many changes in expressed genes from which cells with the most beneficial expression pattern are selected by the stress environment. The relationship between salt-induced transcript changes and changes associated with altered proline metabolism deserves further analysis. In particular, the way salt stress- and proline-mediated changes are controlled by key signal components such as HOG and calcineurin could reveal much about the role of proline in stress signal transduction.

Proline-associated vacuolation

Salt-induced vacuolation (formation of multiple vacuoles) of the cell has been reported in plants (Chang et al., 1998) and yeast (Bone et al., 1998). The biological advantage of multiple vacuoles in NaCl environments has been described in detail, especially with respect to Na⁺ compartmentation and cytoplasm detoxification (Frommer et al., 1999; Hasegawa et al., 2000). In yeast, the Na⁺/H⁺ antipporter responsible for intracellular compartmentation is localized in the membrane of prevacuoles that eventually coalesce into the large vacuole (Frommer et al., 1999). A similar mechanism has been proposed for plant cells exposed to NaCl. Indeed, salt-adapted tobacco cells contain numerous small vacuoles and transvacuolar strands (Hasegawa et al., 2000), indicating that cytological and physiological bases for such a mechanism exist. As suggested by Hasegawa et al. (2000), this process would contribute to osmotic adjustment and simultaneous detoxification of the cytosol. Our results suggest that, in addition to their role in osmotic homeostasis, compatible solutes may function as activators of the vacuolation process. We have shown that constitutive overproduction of proline in yeast, as a result of specific genetic manipulation of proline metabolism, resulted in the proliferation of multiple vacuoles. This indicates that proline and possibly other solutes, perhaps through their effect on internal osmotic potential, may serve as initiators of signal transduction that lead to vacuole fusion. Although it is not possible to predict the precise genes that would be affected by such signal perturbations, microarray results (Figure 6; Table 2) indicate that changes in expression of many genes of unknown function result from *tomPRO2* expression and proline overaccumulation. In addition, signal events triggered by metabolic changes may be manifested at many levels of control besides effects on transcript abundance.

Vacuolar fission upon salt stress has been thoroughly characterized in the fission yeast *Schizosaccharomyces pombe*, but it is also common in *S. cerevisiae* (Bone et al., 1998). In response to increased external osmolar-
ity, the Hog1p kinase pathway and the Sty1p kinase pathway are activated in *S. cerevisiae* and *S. pombe*, respectively. Phosphorylation of Sty1p (Hog1p) in turn initiates both glycerol synthesis via the transcription factor Atf1, and vacuole fission through the activation of unknown components (Bone et al., 1998). In addition, Pmk1, a MAP kinase acting in the PKC pathway, is also required for vacuolar fission, suggesting that this process is highly regulated. Because osmolyte synthesis and vacuolar fission are both involved in osmotic adaptation, it is likely that in hypertonic media these two responses are co-ordinately activated through a mechanism of feedback regulation, as outlined in Figure 7. Co-ordination of osmotic stress responses in yeast also is exemplified in this model. Control over growth and osmotic adjustment is achieved via a dual-sensing mechanism, mediated by the two-component histidine kinase SLN, which thereby facilitates a balanced control of both the HOG and SKN7-mediated pathways (Figure 7). It appears that accumulation of intracellular proline is able to alter parts of the signal transduction system involved with perception of and adaptation to osmotic stress, but the accumulation itself does not result in a fully co-ordinated response that leads to subsequent adaptation and tolerance.

**Proline as a stress-related signal and the identification of novel components in the stress-adaptation response**

Hare and Cress (1997) have also proposed that proline might function as an osmoticum and regulatory signal at the same time. Recently published results with *Arabidopsis* reduced salt-sensitive mutants (rss; Werner and Finkelstein, 1995) and salt overly sensitive mutants (sos; Liu and Zhu, 1997) substantiate this possibility. A defective K⁺ high-affinity uptake system in the *sos1* mutant causes K⁺ deficiency and inhibits growth in NaCl-treated plants (Wu et al., 1996). At 50 mM NaCl stress, *sos1* plants have a dramatically reduced growth and also accumulate eight to ten times more proline than wild-type plants. Conversely, *rss* mutants, which showed reduced sensitivity to Na⁺, accumulated significantly less proline than the wild type on NaCl and sorbitol stress (Werner and Finkelstein, 1995). We note that it is now well established that a molecular signal system capable of sensing proline levels acts in plant cells to control gene expression (Garcia et al., 1997; Hellmann et al., 2000; Shinozaki and Yamaguchi-Shinozaki, 1996).

Much more work is required to fully understand the relationship between solute accumulation, stress adaptation and control of cellular growth. These slow-growing yeast strains which overaccumulate proline may provide a valuable tool to help elucidate these relationships. If, indeed, the proline concentration of the cell is 'sensed' and this signal is transduced to activate responses to NaCl stress, it should be possible to identify components of the sensing/signal pathway through mutation screening in yeast. Selection for restored growth rates in proline-overaccumulating lines should produce mutations that include genes encoding these signal components.

**Experimental procedures**

*Bacteria and yeast culture media*

Bacteria were grown in minimal medium (M63) (Cohen and Rickenberg, 1966) containing 10 mM glucose, 0.1 mM thiamine-HCl, unless otherwise stated. To facilitate cell growth, minimal medium was supplemented with a mixture of 19 amino acids (minus proline) or 18 amino acids (minus proline and cysteine). The complex medium used was Luria-Bertani (LB) supplemented with ampicillin at 100 μg ml⁻¹ concentration, unless otherwise stated.

Yeast were grown aerobically at 28°C in yeast nitrogen base medium (Difco, Becton Dickinson & Co., Sparks, MD, USA) without amino acids, supplemented with glucose at 20 g l⁻¹ and the required amino acids. The nitrogen source was 20 g l⁻¹ (NH₄)₂SO₄ or proline (10 mM). The strains used in this study are listed in Table 3.

**Isolation of dehydroproline-resistant mutants**

The *tomPRO2* cDNA utilized in the present study was cloned by screening a tomato cDNA library with a 1.6 kb genomic fragment from *Arabidopsis*, previously obtained by PCR amplification using degenerate primers derived from a highly conserved region of P5CS (Maggio et al., 1996). The *tomPRO2* coding sequence was subcloned in pET-32a(+) (Novagen, Madison, WI) as an NcoI/BamHI fragment to create plasmid pPRO2. Plasmid pPRO2, pET-32a(+) harboring *tomPRO2*, was transformed in the *E. coli* mutator strain XL-1 Red according to the manufacturer's instructions (Stratagene, La Jolla, CA). Transformants were plated on LB media and incubated at 37°C for 26 or 32 h, the time necessary to introduce random mutations. Cells were collected and the mutated plasmids were isolated with a Qiagen (Valencia,

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**Table 3 Yeast strains used in this study**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Reference/source</th>
</tr>
</thead>
<tbody>
<tr>
<td>MB1433</td>
<td>α trp1 ure3-52</td>
<td>Brandriss (1979)</td>
</tr>
<tr>
<td>DT1000</td>
<td>α pro1-4 trp-1 ure3-52</td>
<td>M. Brandriss</td>
</tr>
<tr>
<td>DT1000G</td>
<td>DT1000 + pGPD</td>
<td>This study</td>
</tr>
<tr>
<td>DTP2</td>
<td>DT1000 + pGPDP2</td>
<td>This study</td>
</tr>
<tr>
<td>DTP2-4</td>
<td>DT1000 + pGPDP2-4</td>
<td>This study</td>
</tr>
<tr>
<td>DTP2-13</td>
<td>DT1000 + pGPDP2-13</td>
<td>This study</td>
</tr>
<tr>
<td>DTP2-20</td>
<td>DT1000 + pGPDP2-20</td>
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</tr>
<tr>
<td>MB1433p</td>
<td>MB1433 + pBW12</td>
<td>This study</td>
</tr>
<tr>
<td>DTPP2</td>
<td>DTP2 + pBW12</td>
<td>This study</td>
</tr>
<tr>
<td>DTPP2-13</td>
<td>DTP2-13 + pBW12</td>
<td>This study</td>
</tr>
<tr>
<td>DTPP2-20</td>
<td>DTP2-20 + pBW12</td>
<td>This study</td>
</tr>
</tbody>
</table>

Proline measurements

The colonies that grew better in the final screening were ranked according to their proline content. Proline was measured using a bioassay. Briefly, an extract from liquid cultures (5 ml) of each colony was used as the sole proline source for *Salmonella typhimurium* strain TL131, which is auxotrophic for proline. Proline content was then quantified via a standard curve, which expressed growth of TL131 as a function of known amounts of proline in the culture medium. Proline content was then confirmed using the acid ninhydrin procedure of Troll and Lindsley (1955). The acid ninhydrin procedure also was used to measure yeast proline content after autoclaving the cells for 20 min.

DNA and amino acid sequence analyses

DNA sequence was determined using an automated fluorescence sequencer (Pharmacia, Piscataway, NJ). Nucleotide and predicted amino acid sequences were analyzed using the Genetic Computer Group package of the University of Wisconsin (Madison, WI).

Recombinant DNA Procedures and Yeast Transformation

tomPRO2 and tomPRO2-mutated derivatives were each sub-cloned as *KpnI/BamH1* fragments in the pT7Blue vector (Novagen) and then inserted into the p414GPD vector as *EcoR1/Sal1* fragments. The resulting plasmids GPDP2 (tomPRO2), GPDP2-4, GPDP2-13 and GPDP2-20 (tomPRO2-mutated derivatives) were used to complement the yeast proline auxotroph strain DT1000. The transformant strains were then designated DTP2, DTP2-4, DTP2-13 and DTP2-20, respectively. To further enhance the accumulation of cellular proline, we inactivated *put1*, the structural gene for proline oxidase (Wang and Brandriss, 1986). The *put1::URA3* mutations were obtained by transformation of strains DTP2, DTP2-4, DTP2-13 and DTP2-20 with plasmid pWB12 (Wang and Brandriss, 1986), using the one-step gene-disruption method (Guthrie and Fink, 1991). The resulting strains carrying the plant gene for proline biosynthesis in combination with knock-outs of the structural oxidative enzyme were designated DTPP2, DTTP2-13 and DTPP2-20. Transformation was carried out by the lithium acetate method.

Osmotic stress

Yeast strains were grown in liquid media until stationary phase and subcultured in fresh media until an OD600 of 0.4 was reached. Cultures were then diluted again to an OD600 of 0.1 before inoculating fresh media containing NaCl (500 mM). After 36 h at 28°C, cells were collected for growth measurements, total protein and proline determination.

Flow cytometric analysis

Yeast strains were grown as described. When the OD600 was equal to 0.8, 500 µl samples were centrifuged. Cells were fixed in 70% ethanol for 30 min at −20°C, washed by centrifugation with 50 mM sodium citrate, then treated with 20 µg ml⁻¹ Rnase A (Sigma, St Louis, MO) in the same buffer for 2 h at 37°C. The cell populations were analyzed for distribution of DNA content according to Yun et al. (1998).

Microscopy

Yeast strains were grown as described above until an OD600 of 0.8 was reached. Samples were collected and immediately used for light microscopy analysis. For electron microscopy analysis, samples were fixed in 3% formaldehyde and 0.5% glutaraldehyde in 0.05 M potassium phosphate buffer supplemented with 0.5 M sorbitol (pH 6.7), dehydrated in ethanol series, and embedded in LR white resin (London Resin Co., UK). Ultrathin sections were viewed and photographed with a TEM200 (Phillips Electronics Instruments, Mahaws, NJ).

Preparation of labeled probes and microarray hybridizations

Yeast strains were grown in selective minimal media with (NH4)2SO₄ as nitrogen source to an OD600 of 0.8, and total RNA was isolated from cells using the RNEasy MiniKit (Qiagen). Messenger RNA was prepared using the polyATract mRNA isolation systems (Promega, Madison, WI, USA). Labeling and hybridization followed established protocols (Kawasaki et al., 2001). Yeast microarray slides (yeast Y6.4k1_v2) were obtained from the Microarray Centre, Clinical Genomics Centre, University of Toronto, Canada (http://www.oci.utoronto.ca/services/microarray/index.html).

Fluorescence signatures were captured using GenePix 4000B (Axon Instruments Inc., Union City, CA, USA), and analyzed using GENEPIX PRO 3.0 software. Local background was subtracted from the value of each spot on the array. Spots covered by dust particles, missing spots, spots with low signal intensity, and spots in high background areas were flagged as candidates for exclusion after further analysis. Transcript regulation is expressed as the ratio of intensities between stress and control (log ratio, LR). Normalization between the Cy3 and Cy5 fluorescent dye channels was achieved by calculating the ratio between the total Cy3 signal from all spots in the microarray in relation to the total Cy5 signal from all spots (Hardwick et al., 1999).

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