**Biosynthesis of Vitamin C by Yeast Leads to Increased Stress Resistance**

Paola Branduardi1, Tiziana Fossati1, Michael Sauer2,3, Roberto Pagani1, Diethard Mattanovich2,3, Danilo Porro1*

1 Dipartimento di Biotecnologie e Bioscienze, Università degli Studi di Milano-Bicocca, Milano, Italy, 2 School of Bioengineering, University of Applied Sciences, Vienna, Austria, 3 Institute of Applied Microbiology, University of Natural Resources and Applied Life Sciences, Vienna, Austria

Background. In industrial large scale bio-reactions micro-organisms are generally exposed to a variety of environmental stresses, which might be detrimental for growth and productivity. Reactive oxygen species (ROS) play a key role among the common stress factors—directly-through incomplete reduction of O2 during respiration, or indirectly—caused by other stressing factors. Vitamin C or L-ascorbic acid acts as a scavenger of ROS, thereby potentially protecting cells from harmful oxidative products. While most eukaryotes synthesize ascorbic acid, yeast cells produce erythro-ascorbic acid instead. The actual importance of this antioxidant substance for the yeast is still a subject of scientific debate. Methodology/Principal Findings. We set out to enable *Saccharomyces cerevisiae* cells to produce ascorbic acid intracellularly to protect the cells from detrimental effects of environmental stresses. We report for the first time the biosynthesis of L-ascorbic acid from D-glucose by metabolically engineered yeast cells. The amount of L-ascorbic acid produced leads to an improved robustness of the recombinant cells when they are subjected to stress conditions as often met during industrial fermentations. Not only resistance against oxidative agents as H2O2 is increased, but also the tolerance to low pH and weak organic acids at low pH is increased. Conclusions/Significance. This platform provides a new tool whose commercial applications may have a substantial impact on bio-industrial production of Vitamin C. Furthermore, we propose *S. cerevisiae* cells endogenously producing vitamin C as a cellular model to study the genesis/protection of ROS as well as genotoxicity.

**INTRODUCTION**

Micro-organisms maintain optimal growth (and productivity) within a reasonably broad range of physiological conditions, due to a variety of responses that have evolved to cope with many types of environmental insult. Cellular defense mechanisms are able to avoid molecular damages in a wide range of environmental conditions. However, this balance can be disturbed severely in industrial scale bio-reactions where micro-organisms are generally exposed to a variety of environmental stresses [1,2]. In addition to unfavorable exterior conditions the heavy metabolic burden imposed by an efficient production process is another cause for cellular stress. Regardless of their origin, stresses on micro-organisms can have various effects, including lower metabolic activity, growth rate, or productivity or decreased viability. In an industrial process, wherein the micro-organism is used as a means for production, the mentioned effects lead finally to a lower concentration of the product, lower productivity, or a decreased yield. Stress is therefore an undesirable phenomenon, and techniques for minimizing it—particularly in industrial processes—are highly desirable.

Reactive oxygen species (ROS) play a key role among the common stress factors [3–5].

This might be directly—by the generation of ROS due to the incomplete reduction of O2 during respiration—or indirectly—when ROS generation is caused by other stressing factors—metabolical or environmental. Most eukaryotic organisms produce L-ascorbic acid (L-AA or vitamin C), a powerful, water-soluble antioxidant as scavenger of ROS [6–8] to prevent or at least alleviate deleterious effects caused by ROS. However, yeast cells naturally lack the ability to produce L-AA. Instead, erythro-ascorbic acid, a structurally related compound with chemical properties very similar to those of L-AA, is the molecule occurring to a low extent in yeast cells [9]. Its role for stress resistance has been shown, but to which extend it is important is still a question for scientific debate [9–11].

Here, we report for the first time the biosynthesis of L-AA by metabolically engineered *S. cerevisiae* cells starting from D-glucose. We show that the endogenous biosynthesis of ascorbic acid in yeast and its scavenger role against ROS leads to an improved cell viability of the recombinant cells during growth under various stress conditions.

**RESULTS**

Construction of recombinant *S. cerevisiae* strains able to convert D-glucose into L-ascorbic acid

Animals and plants employ two different metabolic pathways to synthesize L-ascorbic acid [12,13]. The plant pathway (shown in fig 1) was chosen for L-AA production in yeast cells for two reasons: First of all, GDP-mannose, a key intermediate in this pathway, is naturally produced in yeasts for cell wall construction [14]. Secondly, the last two steps for biosynthesis of L-AA in plants show similarity to the pathway of production of erythro-ascorbic acid in yeasts. Useful enzymes are therefore already present within the cells. It has been demonstrated that yeast strains incubated in the presence of L-galactose are able to produce L-ascorbic acid [15]. This capacity can be enhanced by overexpression of the
endogenous ALO1 gene and the heterologous Arabidopsis thaliana LGDH gene. In this case, L-AA is even accumulated extracellularly [16] upon incubation of the cells with L-galactose.

Only the three enzymatic activities converting GDP-D-mannose into L-galactose are therefore missing in yeast cells. To close this gap, the following genes were cloned and expressed under the control of the TPI promoter (Table 1): AtME (A. thaliana mannos-6-phosphate epimerase), converting GDP-D-mannose into GDP-L-galactose and AtALO/VTY7/9 (A. thaliana myo-inositol phosphate/L-galactose-1-P-phosphatase), converting L-galactose-1-P into L-galactose. Since the enzyme responsible for the conversion of GDP-L-galactose into L-galactose was only recently identified [17], alternative pyrophosphorylases converting substrates similar to GDP-L-galactose were sought for in the database. L-Glucose guanylyl-transferase (FGT) from Rattus norvegicus [18] appeared suitable and was finally expressed. Additionally, AtLGDH (A. thaliana L-galactose dehydrogenase) which allows the conversion of L-galactono-1,4-lactone into L-galactose-1-P. The level of L-ascorbic acid is already detectable (third column, for both graphs, strain BY4742[ScALO AtLGDH AtME AtMIP] plus strain BY4742[ScALO AtLGDH AtME AtMIP] plus strain BY4742[ScALO AtLGDH AtME AtMIP] plus strain BY4742[ScALO AtLGDH AtME AtMIP]). This result indicates the presence of endogenous pyrophosphorylase activities converting GDP-L-galactose into L-galactose-1-P. The level of L-ascorbic acid becomes even higher when the RnFGT gene is added (fourth column), proving the suitability of this enzyme for our purpose.

While the trend of ascorbic acid production for both strains is similar, the absolute values are significantly different, being almost one order of magnitude lower in the BY background (Fig. 2, left panel). Analogous, the contribution of the RnFGT gene is more effective in the GRF background (Fig. 2, right panel). On the other hand, while such difference could be related to the different genetic background of the two hosts, the basal level of antioxidants appears to be within the same order of magnitude in both yeast

![Figure 1. Ascorbic acid biosynthetic pathway.](https://example.com/figure1.png)

**Figure 1. Ascorbic acid biosynthetic pathway.** Schematic representation of the pathway of L-AA production from D-glucose in plants. The following enzymes are involved: A, hexokinase (2.7.1.1), B, glucose-6-phosphate isomerase (5.3.1.9), C, mannose-6-phosphate isomerase (5.3.1.8), D, phosphomannomutase (5.4.2.2), E, mannose-1-phosphate guanylyltransferase (2.7.7.22), F, GDP-mannose-3,5-epimerase (5.3.1.18), G, GDP-L-galactose phosphomutase (E.C.C not assigned), H, L-Galactose 1-phosphate phosphatase (3.1.3.25), I, L-galactose dehydrogenase, J, L-galactono-1,4-lactone dehydrogenase (1.3.2.3). doi:10.1371/journal.pone.0001092.g001

**Table 1.** List of expression plasmids constructed and used in this study

| Expression vector | Promoter | Expressed protein | Plasmid status | Selection | Transformed strain |
|-------------------|----------|-------------------|----------------|-----------|--------------------|
| p012 MIP          | pTPI     | At Myo-Inositol Phosphatase/L-Galactose-1P Phosphatase | INT | Sc URA3 | BY4742 |
| p012t ME-MIP      | pTPI/pCTPI| At GDP-Mannose-3,5-Epimerase/ Myo-Inositol Phosphatase/L-Galactose-1P Phosphatase | INT | Sc URA3 | GRF18U |
| p022 LGDH         | pTPI     | At L-Galactose Dehydrogenase | INT | Sc HIS3 | BY4742/GRF18U |
| p042 ALO          | pTPI     | Sc D-Arabinono-1,4-lactone oxidase | INT | Sc LEU2 | BY4742/GRF18U |
| p062 ME           | pTPI     | At GDP-Mannose-3,5-Epimerase | INT | Sc LYS2 | BY4742/GRF18U |
| pZv ME            | pTPI     | At GDP-Mannose-3,5-Epimerase | CEN | KanR | GRF18U |
| pZv FGT           | pTPI     | Rn GDP-L-Fucose Pyrophosphatase | CEN | NatR | BY4742/GRF18U |

**Table 1.** List of expression plasmids constructed and used in this study

**Abbreviations: Sc: Saccharomyces cerevisiae; At: Arabidopsis thaliana; Rn: Rattus norvegicus; Zb: Zygosaccharomyces bailii;**

**TPI: Triose Phosphate Isomerase**

**URA3, HIS3, LEU2, LYS2:** gene markers conferring growth to auxotrophic yeast strains in the absence of uracil, histidine, leucine and lysine, respectively.

**NatR:** cassette conferring resistance to nourseothricin.

**KanR:** cassette conferring resistance to Geneticin.

**CEN and INT:** centromeric and integrative plasmids, respectively.

**a complete description of plasmids construction is given in Materials and Methods**

doi:10.1371/journal.pone.0001092.t001
backgrounds. In no case vitamin C was detectable in the culture broth (data not shown).

Effects of endogenous L-AA production on strain robustness: recombinant yeasts behavior under oxidative stress

To test if the endogenous production of vitamin C could have beneficial biological effects, wild type BY4742, GRF18U and the corresponding recombinant L-AA producing strains were challenged by growing the cells under different stress conditions. Since vitamin C acts principally as an antioxidant, the first condition used to test the robustness of the engineered strains was oxidative stress. Yeast cells were inoculated at an initial optical density (660 nm) of 0.1 in the presence of different concentrations of H2O2 (ranging from 2 to 3.5 mM), and their growth was measured as optical density at specific intervals of time over about 80 hours, Figure 3. Cells were also inoculated in the same medium without H2O2, as a control. As expected, in the absence of H2O2 both wild type and recombinant strains grew well (Fig. 3a and 3b). When H2O2 was added to the medium, the growth of both GRF18U and BY4742 wild type strains was negatively affected proportionally to the hydrogen peroxide concentration. Low concentrations (2 mM) lead to a slight delay of growth (data not shown), while the highest H2O2 concentration

Table 2. List of yeast strains constructed and used in this study

| Strain  | Genotype                          | Source |
|---------|-----------------------------------|--------|
| GRF18U  | MATα his3-11 his3-15 leu2-3 leu2-112 ura3 [NRRL Y30320] | [25]    |
| GRF18Uc | MATα his3-11 his3-15 leu2-3 leu2-112 ura3 [pYX012; pYX022; pYX042] | This study |
| GRF18U ALO LGDH | MATα his3-11 his3-15 leu2-3 leu2-112 ura3 [p022AtLGDH; p042ScALO] | [9] |
| GRF18U ALO LGDH ME MIP | MATα his3-11 his3-15 leu2-3 leu2-112 ura3 [p022AtLGDH; p042ScALO; pZ γAIME; p012ATMIP] | This study |
| GRF18U ALO LGDH bTME MIP | MATα his3-11 his3-15 leu2-3 leu2-112 ura3 [p022ATLGDH; p042 ScALO; p012bTAIME ATMIP] | This study |
| GRF18U ALO LGDH bTME MIP FGT | MATα his3-11 his3-15 leu2-3 leu2-112 ura3 [p022ATLGDH; p042ScALO; p012bTAIME ATMIP; pZ5RnFGT] | This study |
| BY4742  | MATα; his3-11; leu2-3; lys2-10; ura3-10 [EuroScarf AN. Y10000] | [26]    |
| BY4742c | MATα; his3-11; leu2-3; lys2-10; ura3-10 [pYX012; pYX022; pYX042; pYX062] | This study |
| BY4742 ALO LGDH | MATα; his3-11; leu2-3; lys2-10; ura3-10 [pYX012; p022ATLGDH; p042 ScALO; pYX062] | This study |
| BY4742 ALO LGDH ME | MATα; his3-11; leu2-3; lys2-10; ura3-10 [pYX012; p022ATLGDH; p042ScALO; p062AIME] | This study |
| BY4742 ALO LGDH ME MIP | MATα; his3-11; leu2-3; lys2-10; ura3-10 [p012ATMIP; p022ATLGDH; p042ScALO; p062AIME] | This study |
| BY4742 ALO LGDH ME MIP FGT | MATα; his3-11; leu2-3; lys2-10; ura3-10 [p012ATMIP; p022ATLGDH; p042ScALO; p062AIME; pZ5RnFGT] | This study |

Figure 2. Conversion of D-Glucose into L-ascorbic acid (milligrams/liter/OD) by transformed S. cerevisiae GRF18U and BY4742 cells. All strains were grown on mineral medium (2% w/v glucose, 0.67% w/v YNB), starting with an initial OD660 of 0.05 for 18 h, when samples were taken and the concentration of L-ascorbic acid inside the cells was determined (GRF18U and BY4742 correspond to the parental strains transformed with the empty plasmids harboring in the productive strains the genes of the L-AA pathway). The control cells, as well as the cells expressing ScALO1 and AtLGDH can not accumulate L-ascorbic acid starting from D-glucose, therefore measured values correspond to the endogenous erythro-ascorbic acid. The standard deviation bars correspond to the data obtained from independent clones, and from independent growth and antioxidant determinations. Please note the different scale of the ordinate axes in the two graphs.

doi:10.1371/journal.pone.0001092.g002
(3.5 mM) lead to an almost complete growth inhibition (Fig. 3c and 3d). A similar behavior has been observed for yeast cells transformed with the ScALO1 and AtLGDH genes (data not shown). Significantly, under the same limiting conditions, all recombinant strains producing L-AA were able to resume growth about 35 hours after inoculation, showing a strong robustness and an increased tolerance to oxidative stress (Fig. 3c and 3d). Moreover, it becomes obvious that this aspect of strain robustness is directly correlated with the amount of L-AA produced (see Fig. 2); in fact, the strains engineered with all five genes necessary to complete the biosynthetic L-AA pathway exhibited even stronger tolerance (Fig. 3c and 3d, triangles) than the corresponding strains, lacking the FGT activity (Fig. 3c and 3d, squares). This phenomenon can be observed for both yeast genetic backgrounds, even if with a slight difference in timing, probably due to a different basal resistance of the two genetic backgrounds (phenomenon not further investigated). The BY background strain, in fact, seems to be more resistant, despite the generally lower ascorbic acid levels measured (see Fig. 2).

Similar results were obtained for cells growing in the presence of 2.5 and 3 mM H2O2. In these cases growth was resumed 20 and 25 hours after inoculation, respectively (data not shown), hence earlier and concentration dependent.

**Effects of endogenous L-AA production on strain robustness: recombinant strain behavior under acidic stress**

To test if the improved strain robustness could be extended to other environmental constraints, wild type and engineered strains were subjected to acidic stress caused by inorganic or organic acids. These are typical environmental conditions occurring during either lab-batch and industrial fermentations.

To this purpose, we analyzed wild type and ascorbic acid producing strains grown in minimal medium brought to pH 2.2 with HCl addition (acidic stress induced by inorganic acid), or with lactic acid (45 g/l) at pH 3 (this value was chosen in order to have
the organic acid almost completely in the undissociated form, since its pKa is 3.78). From the data presented in Figure 4, it is evident that also under these stress conditions the producing strains exhibit a strong robustness and increased tolerance when compared to control strain. At pH 2.2 (Fig. 4a) the growth rate of the wild type strains are significantly delayed in respect to the growth rate of the engineered strains. Furthermore, the addition of 45 g/l of lactic acid to the medium has dramatic effects on the wild type strains, which are completely inhibited in growth, and also on the [ScALO AtLGDH AtME AtMIP] expressing cells which are severely compromised, while the recombinant strains expressing all genes for L-AA production are still able to grow (Fig. 4b). Interestingly, the effect of L-AA produced intracellularly is much more profound than the effect of vitamin C added to the culture broth. Fig. 4c shows the growth curve of wild type control cells in presence of lactic acid (40 g/l) at low pH. The addition of ascorbic acid relieves the growth inhibition evidently, but even with 60 mg/l the effect is not as profound as with the low amounts produced intracellularly. The growth is resumed much later even while less lactic acid was added to the culture.

**Correlation of endogenous L-AA concentration, strain robustness and viability**

Having proven the positive effects on growth under stress conditions as described above, we wanted to deeper investigate the correlation between ascorbic acid endogenously produced by engineered yeast cells, enhanced tolerance to oxidative damage and cell robustness. The wild type and the recombinant GRF18U and BY strains were grown in minimal glucose medium with addition of 3.0 mM H2O2, following the same experimental protocol described in Figure 3. At the time in which all strains start to recover from the imposed stress (each with a different kinetic: approximately after 2–3 duplications from the preinoculum—see example see Figure 3), samples were taken. Each sample was stained with Dihydrorhodamine123 for the detection of ROS [20], and PI (propidium iodide) to detect severely damaged and/or dead cells [21]. Samples were analyzed with a flow cytometer. The resulting dot plots are compared in Figure 5. On the dot plots (DHR123 vs. PI) each individual cell is represented by a single dot and it is quite easy to recognize at least four distinct yeast subpopulations. A first healthy subpopulation (named A in Fig. 5a, used as a schematic representation for the benefit of the following ones), having only the background signal (autofluorescence) for both fluorochromes (low DHR and low PI signal); a second subpopulation (named B in Fig. 5a) of still viable but ROS accumulating cells (low PI, high rhodamine signal); a third subpopulation (named C in Fig. 5a) made of damaged cells displaying high ROS and high PI signals and finally a fourth subpopulation (named D in Fig 5a) of dead cells, presumably originated from subpopulation C by loosing all DHR signal. The data reported in the upper (GRF18U background) panels of
Figure 5 (b, c, d) clearly show that in the wild type strain the oxidative stress induced by \( \text{H}_2\text{O}_2 \) strongly influences the intracellular ROS content and affects cell vitality. In fact, as clearly shown by Fig. 5b, a considerable portion of the analyzed cells (28%) shows high intracellular levels of ROS, and the fraction of dead cells (33%) exceeds that of viable cells (28%). By contrast, in the recombinant strains producing L-AA (Fig. 5c), we registered a clear reduction of ROS formation, (15% of the total population), together with a decrease of cell mortality (30%) and a consequent improved cell viability (44%). This trend is maximized in the strain expressing the \( \text{RnFGT} \) gene (Fig. 5d), where the majority of the cells (89.5%) is still viable and healthy, while ROS formation (1.1%) and cell mortality (8.7%) are almost negligible. As already observed for the growth curves in limiting conditions, also in this experiment there is a direct and positive correlation between the levels of vitamin C produced (see Fig. 2) and cell viability.

A corresponding result has been observed for the BY yeast background (lower panels). While the general trend for both strains is similar, in this case, accordingly to the higher robustness observed for this genetic background (compare the behaviors of the GRF18U and BY cells in Figure 3 and 4) a lower accumulation of ROS has been detected.

Taken together these data allow a more deeper analysis of the data shown in Figure 3 where increasing \( \text{H}_2\text{O}_2 \) concentration lead to longer delays of growth. These longer delays are clearly due to decreasing fractions of viable cells at the time of inoculum.

The same cells analysed by flow cytometry can also be analysed with a fluorescence microscope for ROS accumulation. In the wild type strain, cells with a strong and granular rhodamine staining are quite frequent and well represented, while in the producing strain almost no signal for ROS is detectable (data not shown).

All the described data demonstrate that the endogenous production of the antioxidant L-AA protects cells from oxidative damage, scavenging the intracellular production of ROS and/or increasing cell viability.

**DISCUSSION**

We report for the first time the biosynthesis of vitamin C by recombinant *S. cerevisiae* cells starting from D-glucose. Accumulation of ascorbic acid was proven to be successful in two different strains (Figure 2). Furthermore, we proved that the intracellular accumulation of L-AA leads to an improved robustness of the recombinant yeasts during growth under different stress conditions. The recombinant yeasts that are functionally transformed to produce L-ascorbic acid generate lower levels of ROS (Fig. 5) and exhibit improved growth and a higher viability under conditions of organic (Fig. 4b) and inorganic (Fig. 4a) acid stresses as well as oxidative stress (Fig. 3c and d). These are physiological conditions often encountered during industrial large scale fermentations. For each yeast background, the degree of robustness seems to be directly related to the amount of intracellularly produced vitamin C (Fig. 2, 3 and 4).

In accordance with our data it has been shown before that the addition of ascorbic acid to the growth medium has beneficial effects for heterologous protein production in yeasts [22]. Furthermore, the respective yeast strains are a major step for the development of a cell factory for the production of vitamin C itself, one of the most important specialty chemicals manufactured in the world. This aim has been long sought for, but never reached up to now [23–25]. In this respect, it is noteworthy that we have previously shown that (i) yeast cells do have one or more transporters which allow the accumulation of intracellularly produced ascorbic acid in the culture medium (i.e., thus greatly facilitating the purification procedures) and (ii) that *S. cerevisiae* cells are quite tolerant to high concentrations of ascorbic acid (at least 40–50 g/L) [16]. Consequently, the industrial production of ascorbic acid by a one-step fermentation from D-glucose appears closer then ever.
Cloning the Vitamin C Pathway

One final general consideration can be made considering the role of ROS. It is well known that the production of ROS is common for many types of cancer cells and that vitamin C has a positive effect in reducing the incidence of stomach, lung, and colorectal cancer [26,27]. On the other hand vitamin C can under certain circumstances even increase the generation of ROS and have detrimental effects at least under certain circumstances [28–30]. Hopefully, S. cerevisiae cells endogenously producing vitamin C could represent a well suited cellular model to study the genesis/protection of ROS and genotoxicity in higher eukaryotic organisms.

MATERIALS AND METHODS

Yeast strains, transformation, media and cultivation

The S. cerevisiae parental strains used in this study were GRF18U [31] (MATα; ura3; leu2-3,112; his3-11,15; cir)5, and BY4742 (MATa; ura3Δ; his3Δ1; leu2Δ0; fy2Δ0; cir)5, EuroScarf Accession No. Y10000-http://www.rz.uni-frankfurt.de/FB/fb16/mikro/euroscarf). Yeast transformations were performed according to the Li/PEG/ss-DNA protocol [32] and both strains were transformed with one or more of the constructs described below, in parallel with the corresponding empty plasmids(s). The presence of the heterologous genes was confirmed by PCR analysis. For each set of transformation at least three independent clones were initially tested, showing no meaningful differences among them. The resulting S. cerevisiae strains constructed in this study are listed in Table 2, with their respective genotypes. In GRF18U the plasmid integration is locus specific (the auxotrophies were generated by multiple point mutations of the target genes), while integration is randomly directed in cells of the BY background.

Yeast cultures were grown in minimal synthetic medium (0.67% w/v YNB Biolife without amino acids) with 2% w/v of D-glucose as carbon source. When required, supplements such as leucine, uracil, lysine and histidine were added to a final concentration as carbon source. When required, supplements such as leucine, uracil, lysine and histidine were added to a final concentration (50 mg/l, while the antibiotic nourseotricine sulphate (cloNAT) was added to a final concentration of 100 mg/l. Yeast cultures were grown in shake flasks at 30°C with an orbital speed of 150 rpm and in 250 ml Erlenmeyer flasks with 50 ml of medium. All strains were grown in shake flasks at 30°C and 200 rpm, and in 250 ml Erlenmeyer flasks with 50 ml of medium. The experiment was repeated at least three times.

Gene amplification and expression plasmid construction

All genes for the reconstruction of the L-Asp pathway in yeast were PCR amplified on a GeneAmp PCR System 9700 (PE Appl. Biosystems, Inc.) using Pwo DNA Polymerase (Roche Diagnostics) and following the manufacturer’s instructions. Template DNA for AtLGDH, AtME, and AtMIP was a cDNA library from A. thaliana (ATCC 77500) [33]; template DNA for ScAL01 consisted of 50 ng of genomic DNA from S. cerevisiae GRF18U, extracted with a standard method (according to [34], slightly modified) RunGTF was PCR amplified from a kidney cDNA library (Origene, CR-1003). The following primer pairs were used: for ALO1: ALO1rev for TTT CAC CAT ATG TCT ACT ATC C and ALO1rev rev AAG GAT CCT AGT CGG ACA ACT C; for LGDH: LGDHfor for ATG ACG AAA ATA GAG CTT CGA GC and LGDHrev TTA GTT CTG ATG GAT TCT AGC ACT TGG; for ME: MEfor for GGC CCA TGG GAA GTA CCA ATG GAA CA and MErev for GGC CTC GAG TCA CTC TTT TCC ATT ATC A; for MIP: MIPfor for ATC CAT GCC GGA CAA TGA TTC TC and MIPrev for AAT CAT GAT CCT GCT GTA AGC CGC; for FGT: FGTfor for TAG GAC ATG GAG ACT TCT CGG GAA and FGTrev for CTC AAT TAA GAT TCT TCT AAA TCA GAT GTG TTT TTA TTA GGT. The PCR fragments were sub-cloned into the pSTBlue-1 vector using the Perfectly Blunt Cloning kit (Novagen) and checked by sequence analysis. The obtained sequences were the same as those reported in Genebank except for MIP in which two silent point substitutions (A271T and T685G) were detected. Finally, the coding sequences were EcoRI cut and sub-cloned into S. cerevisiae expression vectors of the YX series (R&D Systems, Inc.) or derivatives (pYX102/T and pYX602, generated for this study, see below) and into the centromeric expression vectors pZ3 μ pZ4 μ [35] and pZ5 (this study, see below). In detail, ALO1 was sub-cloned into pYX042 (integrative, LEU2 auxotrophic marker, S/TPi promoter); LGDH was sub-cloned into pYX022 (integrative, HIS3 auxotrophic marker, S/TPi promoter), and ME and MIP into pYX602 (integrative, LYS2 auxotrophic marker, PCR amplified with oligonucleotides LYS2for TGC CAG CGG AAT TCC ACT TGC and LYS2rev for AAT CTT TGT GAA GCT TCG CAA GTA TTC ATT from S. cerevisiae genome and substituted to the URA3 auxotrophic marker in the plasmid pYX012 DelIII/NotI cut and blunt ended, S/TPi promoter) and pYX012 (integrative, URA3 auxotrophic marker, S/TPi promoter) respectively. ME EcoRI cut and blunt ended was also sub-cloned into pZah∩ (centromeric, G4188 dominant marker, 2/TPi promoter) BstI cut, blunt ended: the expression cassette bT promoter-AIME-polyA terminator was then PCR amplified with oligonucleotides bTPIfor ATC GTA TTC GAT CAA TTC TTC TTC TTT GTGA and polyAmprev GGG GTA CCC CAG CTG GAG GTA CAC AAA GAC which has a recognition sequence for kpnI at the 5’ site; the cassette was then subcloned into pSTBlue-1 vector, and, after sequencing, kpnI excised and sub-cloned into pYX102-MIP kpnI cut, resulting in the pYX102 bTME-MIP expression vector. This construct allowed the integration of two heterologous genes into the yeast genome at the same time by the use of single marker of selection. Finally, RunGTF EcoRI cut was sub-cloned into pZ5 µ. This is a centromeric vector derived from pBR1 [pYX022 in which the ARS-CEN fragment from Yeplac33 has been inserted [35]]; pBR1 was cut kpnI and blunt ended, and the nourseotricine cassette (NatII) PshaI/SstI cut and blunt ended from pAgA5 [36], has been inserted. All the restriction and modification enzymes used were from New England Biolabs (Hitchin, Herts, UK) or from Roche Diagnostics (Mannheim, Germany). Standard procedures [37] were employed for all cloning purposes.

All plasmids generated and utilized for the present study are described in Table 1.

Determination of L-ascorbic acid

For intracellular L-ascorbic acid determinations yeast cells were inoculated at an initial optical density of 0.05 in minimal medium and grown for about 18 hours in order to reach a mid-exponential phase of growth. Cells were then harvested by centrifugation at 4000 rpm for 3 min at 4°C, washed once with ice-cold distilled water and then resuspended in about three times volume of ice-cold 10% (w/v) trichloroacetic acid, vortexed vigorously, and kept in ice for 20 minutes. The supernatant was then cleared from cell debris by centrifugation. Ascorbic acid was determined spectro-
photonically following a method adapted from that of Sullivan et Clarke [16,19]. We previously confirmed the identity of ascorbic acid by HPLC using yeast cells functionally transformed to produce ascorbic acid from L-galactose [16].

Flow cytometric analyses

Reactive oxygen species (ROS) were detected by Dihydrorhodamine 123 as described in [20]. Cells were incubated with Dihydrorhodamine 123 (DHR 123, Sigma Chemical Co., St. Louis, MO, USA) for 2 h, washed twice with PBS buffer and subsequently resuspended in propidium iodide solution 0.46 mM for the identification of dead or severely compromised cells.

Samples were then analyzed using a Cell Lab Quanta SC flow cytometer (Beckman Coulter, Fullerton, CA, USA) equipped with a diode laser (excitation wavelength 488 nm, laser power 22 mW). The fluorescence emission was measured through a 525–550 nm band pass filter (FL1 parameter) for DHR signal and through a 670 nm long pass filter (FL3 parameter) for PI signal. The sample flow rate during analysis did not exceed 600–700 cells/s. A total of 20,000 cells was measured for each sample. Data analysis was performed afterwards with WinMDI 2.8 software, build 2-13 01-19-2000 (Purdue University, Cytometry Laboratories [http://facsc.scripps.edu/software.html]).

Fluorescence microscopy

Samples of the same yeast cultures treated and FACS analyzed for ROS content were also directly analysed using a Nikon Eclipse 90i fluorescence microscope (excitation, 488 nm) using standard FITC filters. Images were taken using the software Metaphorm 2.2.

ACKNOWLEDGMENTS

The authors thank Marta Papini and Simone Passolunghi for technical contribution, Daniel Darabán and Minoska Valli for critically reading the manuscript and Carla Smeraldì for critically revising the manuscript and the language.

Author Contributions

Conceived and designed the experiments: DP PB MS DM. Performed the experiments: PB TF RP. Analyzed the data: DP PB TF MS DM. Wrote the paper: DP PB TF MS DM.

REFERENCES

1. Bai Z, Harvey LM, McNeal B (2003) Oxidative stress in submerged cultures of fungi. Crit Rev Biotechnol 23: 267–302.
2. Mattanovich D, Gasser B, Hohenblum H, Sauer M (2004) Stress in recombinant protein producing yeasts. J Biotechnol 113: 121–135.
3. Ryter SW, Kim HP, Hertzog A, Park JW, Nakahira K, et al. (2007) Mechanisms of cell death in oxidative stress. Annu Rev Biochem 76: 49–89.
4. Temple MD, Perrone GG, Davess JW (2005) Complex cellular responses to reactive oxygen species. Trends Cell Biol 15: 319–326.
5. Apel K, Hirt H (2004) Reactive oxygen species: metabolism, oxidative stress, and signal transduction. Annu Rev Plant Biol 55: 373–399.
6. Padh H (1991) Vitamin C: new insights into its biochemical functions. Nutr Rev 49: 65–70.
7. Padh H (1990) Cellular functions of ascorbic acid. Biochem Cell Biol 68: 1166–1173.
8. Levine M (1996) New concepts in the biology and biochemistry of ascorbic acid. N Engl J Med 314: 892–902.
9. Huh WK, Lee BH, Kim ST, Kim YR, Rhee GE, et al. (1998) D-Erythroascorbic acid is an important antioxidant molecule in Saccharomyces cerevisiae. Mol Microbiol 30: 895–903.
10. Scipit et al. SM, Smimof N, Pitt AR (2000) The biosynthesis of erythroascorbate in Saccharomyces cerevisiae and its role as an antioxidant. Free Radic Biol Med 28: 103–192.
11. Huh WK, Kim ST, Kim H, Jeong G, Kang SO (2001) Deficiency of D-erythroascorbic acid attenuates hyphal growth and virulence of Candida albicans. Infect Immun 69: 3939–3946.
12. Wheeler GL, Jones MA, Smirnoff N (1998) The biosynthetic pathway of vitamin C in higher plants. Nature 393: 365–369.
13. Banhegyi G, Braun L, Csaia M, Puiska F, Mandi J (1997) Ascorbate metabolism and its regulation in animals. Free Radic Biol Med 23: 793–803.
14. Kili EM (1994) Review: cell wall assembly in yeast. Yeast 10: 851–869.
15. Hancock RD, Galquin JR, Vida R (2000) Biosynthesis of L-ascorbic acid (vitamin C) by Saccharomyces cerevisiae. FEMS Microbiol Lett 186: 493–504.
16. Sauer M, Branduardi P, Valli M, Alberghina L, et al. (2004) The last step in the Smirnoff-Wheeler pathway to ascorbic acid in Arabidopsis VTC2 encodes a GDP-L-Fucose phosphorolylase. Purification, cDNA cloning, and properties of the enzyme. J Biol Chem 279: 133–140.
17. Linster CL, Gomez TA, Christensen KC, Adler LN, Young BD, et al. (2007) Arabidopsis VTC2 Encodes a GDP-L-Galactose Phosphorolylase, the Last Unknown Enzyme in the Smirnoff-Wheeler Pathway to Ascorbic Acid in Plants. J Biol Chem 282: 18079–18085.
18. Pastuzak I, Ketchum C, Hermanson G, Sjoberg EJ, Drake R, et al. (1998) GDP-L-Fucose phosphorolylase. Purification, cDNA cloning, and properties of the enzyme. J Biol Chem 273: 30162–30174.
19. Sullivan MX, Clarke HCN (1953) A highly specific procedure for ascorbic acid. Assoc Off Agr Chem 36: 514–518.
20. Medford F, Frolich E, Lagram M, Grey M, Sigrist SJ, et al. (1999) Oxygen stress: a regulator of apoptosis in yeast. J Cell Biol 145: 757–767.
21. Sasaki DT, Dumas SE, Engleman EG (1987) Discrimination of viable and non-viable cells using propidium iodide in two color immunofluorescence. Cytometry 8: 413–420.
22. Xiao A, Zhou X, Zhou L, Zhang Y (2006) Improvement of cell viability and hirudin production by ascorbic acid in Pichia pastoris fermentation. Appl Microbiol Biotechnol 72: 837–844.
23. Hancock RD, Vida R (2002) Biotechnological approaches for L-ascorbic acid production. Trends Biotechnol 20: 299–305.
24. Bremus C, Herrmann U, Bringer-Meyer S, Sahn H (2006) The use of microorganisms in L-ascorbic acid production. J Biotechnol 124: 196–205.
25. Survase SA, Bajaj IB, Singhal RS (2006) Biotechnological production of vitamins. Food Technology and Biotechnology 44: 381–396.
26. Valko M, Rhodes CJ, Moncol J, Izakovic M, Mazur M (2006) Free radicals, metals and antioxidants in oxidative stress-induced cancer. Chem Biol Interact 160: 1–40.
27. Knelt P, Jarvinen R, Sepjanen R, Rissanen A, Aromaa A, et al. (1991) Dietary antioxidants and the risk of lung cancer. Am J Epidemiol 134: 471–479.
28. Naidu KA (2003) Vitamin C in human health and disease is still a mystery? An overview. Nutr J 2: 7.
29. O’Brien TJ, Ceryak S, Patierno SR (2003) Complexities of chromium carcinogenesis: role of cellular response, repair and recovery mechanisms. Mutat Res 533: 3–36.
30. Trommer H, Bottcher R, Popp A, Hoentsch J, Wartewig S, et al. (2002) Role of ascorbic acid in stratum corneum lipid models exposed to UV irradiation. Pharm Res 19: 982–990.
31. Branduardi P, Bolzani D, Compagnol C, Carrera Y, van Dijken JP, et al. (1999) NADH reoxidation does not control glycolytic flux during exposure of respiring Saccharomyces cerevisiae cultures to glucose excess. FEMS Microbiol Lett 171: 133–140.
32. Giez RD, Woods RA (2002) Transformation of yeast by lithium acetate/single-stranded carrier DNA/polyethylene glycol method. Methods Enzymol 350: 87–96.
33. Minet M, Dufour ME, Lacroute F (1992) Complementation of Saccharomyces cerevisiae auxotrophic mutants by Arabidopsis thaliana cDNAs. Plant J 2: 413–420.
34. Hoffman CS, Winston F (1987) A ten-minute DNA preparation from yeast cells. Anal Biochem 160: 1–40.
35. Banhegyi G, Braun L, Csaia M, Puiska F, Mandi J (1997) Ascorbate metabolism and its regulation in animals. Free Radic Biol Med 23: 793–803.
36. Goldstein AL, McCusker JH (1999) Three new dominant drug resistance markers in Saccharomyces cerevisiae: PB TF RP. Analyzed the data: DP PB MS DM. Performed the experiments: PB TF RP. Analyzed the data: DP PB TF MS DM.