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L-ascorbic acid producing yeasts learn from plants how to recycle it

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ABSTRACT

Microorganisms employed in industrial fermentation processes are often subjected to a variety of stresses that negatively affect growth, production and productivity. Therefore, stress robustness is an important property for their application. Reactive Oxygen Species (ROS) accumulation is a common denominator to a lot of these stress factors. Ascorbic acid (L-AA) acts as ROS scavenger, thus potentially protecting cells from harmful oxidative products. We have previously reported the development of *Saccharomyces cerevisiae* strains able to produce L-AA. This was obtained by expressing the known plant pathway genes and by complementing the missing step with an animal activity. Here, we show that L-AA accumulation inside yeast cells can be improved by expressing the complete biosynthetic plant pathway and, even further, by recycling its oxidation products.

These new strains can be seen in a double perspective of exploitation: as novel organisms for vitamin C production and as novel cell factories for industrial processes.

1. Introduction

Reactive Oxygen Species (ROS) such as hydrogen peroxide, the superoxide anion and hydroxyl radicals are normally produced through incomplete reduction of O_2 during respiration (Jamieson, 1998). Moreover a variety of stressful agents, of metabolic or environmental origin, can indirectly lead to ROS generation (Allen et al., 2010; Du et al., 2008; Kitanovic et al., 2009; Kotchoni and Gachomo 2006; Miller et al., 2008 to cite a few). ROS are generally considered as key intermediates among the common stress factors (Apel and Hirt, 2004; Ryter et al., 2007; Temple et al., 2005) and their involvement in lipid, protein and nucleic acid oxidative damages has been demonstrated (Halliwell and Gutteridge, 1990; Khan and Wilson, 1995). Consequences of such cellular damages include lowered metabolic activity, lowered growth rate and even decreased viability (reviewed in Kim et al., 2006 and Skulachev 2006).

Cells detoxify ROS through various defence mechanisms, including the expression of protective enzymes (such as superoxide dismutase, catalase, several peroxidases and thioredoxin) and the production of antioxidants (such as glutathione (GSH), uric acid, carotenoids, tocopherol (vitamin E) and ascorbic acid (L-AA or vitamin C), (Herrero et al., 2008; Limón-Pacheco and Gonsebatt, 2009; Pukacka and Ratajczak, 2006).

Among the main antioxidants, L-AA is produced by most eukaryotic organisms, even if with different biosynthetic pathways between animals and plants (Bánhegyi et al., 1997; Wheeler et al., 1998). In plants L-AA represents the major redox buffer, being found in all cellular compartments as well as in the apoplast (Pignocchi et al., 2003). L-AA is synthesised from D-glucose via GDP-D mannose and GDP-L galactose, Fig. 1, and serves as an electron donor, reacting with ROS (Pignocchi et al., 2003). In this process, L-AA is oxidised to MonoDeHydroAscorbate (MDHA), which can further non-enzymatically disproportionate to DeHydroAscorbate (DHA) (Noctor and Foyer, 1998). DHA can in turn irreversibly be hydrolysed to 2,3 diketogulonic acid. MonoDeHydroAscorbate Reductase (MDHAR) and DeHydroAscorbate Reductase (DHAR), respectively, regenerate L-AA from its oxidation products, thus, recapturing it before it is irreversibly lost (Fig. 1). Consistently, MDHAR and DHAR activities increase in plants in response to various environmental stresses promoting ROS generation (Romero-Puertas et al., 2007; Stevens et al., 2008; Urano et al., 2000; Ushimaru et al., 1992).

Because of its antioxidant properties, L-AA is abundantly used as an additive in food and beverages (Borenstein, 1987). In addition, it is an indispensable nutrient for humans because of its vital role in a variety of cellular functions (Padh, 1990; Padh, 1991).

L-AA is nowadays produced with a variety of methods which simplified the original Reichstein process by including fermentative steps (Reichstein, 1940; Reichstein, 1941). These are still multistep processes and most of them still imply the use of chemicals. For said reasons, a process converting D-glucose into

Abbreviations: L-AA, L-ascorbic acid; EAA, eritro ascorbic acid; ROS, reactive oxygen species; MDHA, monodehydroascorbate; DHA, dehydroascorbate; GSH, glutathione; EGTA, ethylene glycol tetra-acetic acid; DHR123, di hydrorhodamine 123; PI, propidium iodide

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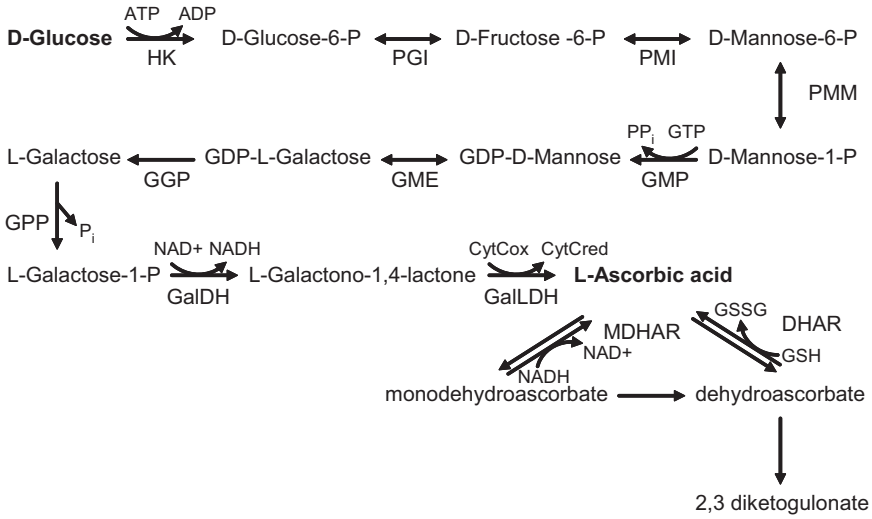

Fig. 1. Ascorbic acid biosynthesis and recycling in plants. Schematic representation of the pathways for L-AA production and recycle in plants. The following enzymes are involved: biosynthetic pathway: HK, hexokinase (2.7.1.1); PGI, glucose-6-phosphate isomerase (5.3.1.9); PMI, mannose-6-phosphate isomerase (5.3.1.8); PMM, phosphomannomutase (5.4.2.8); GMP, mannose-1-phosphate guanylyltransferase (2.7.7.22); GME, GDP-mannose-3,5-epimerase (5.1.3.18), GGP, GDP-L-galactose phosphorylase/L-galactose guanylyltranferase (E.C.C. not assigned); GPP, L-galactose 1-phosphate phosphatase (3.1.3.25); GaLDH, L-galactose dehydrogenase (1.1.1.122); GaLLDH L-galactono-1,4-lactone dehydrogenase (1.3.2.3). Recycling pathway: MDHAR, monodehydroascorbate reductase (1.6.5.4); DHAR, dehydroascorbate reductase (2.5.1.18).

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

Expression vector	Promoter	Expressed protein(s)	Plasmid status	Selection	Transformed strain
pZ_5 VTC2	*Sc*TPI	*At L-galactose guanylyltransferase*	CEN	Nat^R	GRF18U
pZ_5 MDHAR	*Sc*TPI	*At monodehydroascorbate reductase*	CEN	Nat^R	GRF18U/CEN.PK
pZ_4 DHAR	*Sc*TPI	*At dehydroascorbate reductase*	CEN	Hph^R	GRF18U/CEN.PK
p012*b*T VTC2-MIP	*Zb*TPI/ *Sc*TPI	*At L-galactose guanylyltransferase/At myo-inositol phosphatase/L-galactose-1 p phosphatase*	INT	*Sc URA*3	GRF18U/CEN.PK
p022*b*T ME-LGDH	*Zb*TPI/ *Sc*TPI	*At GDP-Mannose-3′,5′-epimerase/At L-galactose dehydrogenase*	INT	*Sc HIS*3	GRF18U/CEN.PK

Abbreviations: *Sc*: *Saccharomyces cerevisiae*; *Zb*: *Zygosaccharomyces bailii*; *At*: *Arabidopsis thaliana*; *Rn*: *Rattus norvegicus*; TPI: triose phosphate isomerise; CEN: centromeric; INT: integrative; Nat^R: cassette conferring resistance to nourseotricine; Hph^R: cassette conferring resistance to hygromycin.

L-AA in one step could be desirable. Intracellular L-AA accumulation could be also positively seen as a required step to create the ideal superbug. Indeed, when used for biotechnological productions, microorganisms are particularly exposed to a number of environmental stresses, which can limit production, productivity and yield of the desired product. Therefore, stress is a highly undesirable phenomenon and the development of microorganisms more tolerant (also said "robust") to harmful conditions is a main goal.

Based on the fact that the addition of L-AA to the growth medium has beneficial effects for heterologous protein production in yeast (Xiao et al., 2006), we developed *Saccharomyces cerevisiae* strains able to convert D-glucose into L-AA (that naturally lacks in yeast), by engineering them with the necessary heterologous activities coming from plant and animal biosynthetic pathways (Branduardi et al., 2007). As a consequence, the recombinant yeasts became more tolerant to a number of stressful conditions, and displayed a clear reduction in ROS intracellular content coupled with increased cell viability.

The goal of the present work was to improve the L-AA production in the previously developed yeasts by further manipulating the production pathway and by inserting the plant recycle system. Results with respect to the improvement in L-AA production and in oxidative stress tolerance will be presented and commented in view of a potential industrial utilisation.

2. Materials and methods

2.1. Yeast strains, transformation, media and cultivation

The *S. cerevisiae* parental strains used in this study were GRF18U (*MATa*; *ura3*; *his3-11,15*; *leu2-3,112*; cir^+) (Brambilla et al., 1999) and CEN.PK 102-5B (*MATa*; *ura3-52*; *his3-11*; *leu2-3/112*; *TRP1*; *MAL2-8c*; *SUC2*—Dr. P. Kötter, Institute of Microbiology, Johann Wolfgang Goethe-University, Frankfurt, Germany) (van Dijken et al., 2000).

Yeast transformations were performed according to the LiAc/PEG/ss-DNA protocol (Gietz and Woods, 2002) and the strains were transformed with one or more of the constructs described below (Table 1): in parallel, parental strains were transformed with the corresponding empty plasmid(s). The presence of the heterologous genes was confirmed by PCR analysis. For each set of transformation at least three independent clones were 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.

Yeast cultures were grown in minimal synthetic medium (0.67% [w/v] YNB medium [catalogue no. 919-15 Difco Laboratories, Detroit, Mich.] with 2% [w/v] D-glucose). Amino acids histidine, uracil, leucine, lysine, isoleucine and valine were added to a final concentration of 50 mg/l, while the antibiotics nourseotricine sulphate (cloNAT, WERNER BioAgents, Germany) and