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Proteome analysis reveals differential expression of proteins involved in triacylglycerol accumulation by *Rhodococcus jostii* RHA1 after addition of methyl viologen

José Sebastián Dávila Costa,[1,2] Roxana A. Silva,[1] Lars Leichert[3] and Héctor M. Alvarez[1,*]

Abstract

Rhodococcus jostii RHA1 is able to degrade toxic compounds and accumulate high amounts of triacylglycerols (TAG) upon nitrogen starvation. These NADPH-dependent processes are essential for the adaptation of rhodococci to fluctuating environmental conditions. In this study, we used an MS-based, label-free and quantitative proteomic approach to better understand the integral response of *R. jostii* RHA1 to the presence of methyl viologen (MV) in relation to the synthesis and accumulation of TAG. The addition of MV promoted a decrease of TAG accumulation in comparison to cells cultivated under nitrogen-limiting conditions in the absence of this pro-oxidant. Proteomic analyses revealed that the abundance of key proteins of fatty acid biosynthesis, the Kennedy pathway, glyceroneogenesis and methylmalonyl-CoA pathway, among others, decreased in the presence of MV. In contrast, some proteins involved in lipolysis and β-oxidation of fatty acids were upregulated. Some metabolic pathways linked to the synthesis of NADPH remained activated during oxidative stress as well as under nitrogen starvation conditions. Additionally, exposure to MV resulted in the activation of complete antioxidant machinery comprising superoxide dismutases, catalases, mycothiol biosynthesis, mycothione reductase and alkyl hydroperoxide reductases, among others. Our study suggests that oxidative stress response affects TAG accumulation under nitrogen-limiting conditions through programmed molecular mechanisms when both stresses occur simultaneously.

INTRODUCTION

Currently, two of the biggest environmental challenges are the development of strategies for cleaning up polluted environments and the search for alternative renewable energy sources. Over the past years, several studies demonstrated that the soil-residing actinobacterium *Rhodococcus jostii* RHA1 is an interesting model for both purposes. For instance, *R. jostii* RHA1 is able to degrade organic compounds such as polychlorinated biphenyls, 1,4-dioxane and lignin [1–3]. Biodegradation of toxic compounds may produce a cellular redox imbalance; thus, micro-organisms able to carry out this process often possess an efficient NADPH-dependent antioxidant mechanism. *R. jostii* RHA1 is also an oleaginous bacterium able to accumulate high amounts of triacylglycerols (TAGs) [4]. Oleagenicity requires a special metabolic network involving concerted reactions and pathways, including those providing key metabolic intermediates and cofactors [5]. Generation of acyl-CoA for TAG biosynthesis, which is performed by the FAS-I system in rhodococci, demands high levels of NADPH. Thus, the oleagenic biosynthesis of TAG and the oxidative stress response could be considered antagonistic processes, both competing for the limited pool of NADPH in the cell. Previously, we investigated the effect of pro-oxidants, such as methyl viologen (MV) and H_2O_2, on the growth, synthesis and mobilization of TAG in diverse *Rhodococcus* strains [6]. We then studied redox-regulated proteins involved in the TAG biosynthesis in *R. jostii* RHA1 that respond to MV [7]. These studies demonstrated that exposure of oleaginous rhodococcal cells to pro-oxidants causes a depletion of the TAG synthesis capacity and decreases the accumulation of TAG.

The response of actinobacteria to oxidative agents and the role of antioxidant systems in the defence against these agents have been investigated in *Mycobacterium tuberculosis*, *Streptomyces coelicolor*, *Amycolatopsis tucumanensis* and *Corynebacterium glutamicum* [8–11]. Organisms in the

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Author affiliations: [1]Instituto de Biociencias de la Patagonia (INBIOP), Universidad Nacional de la Patagonia San Juan Bosco y CONICET, Km 4-Ciudad Universitaria 9000, Comodoro Rivadavia (Chubut), Argentina; [2]Planta Piloto de Procesos Industriales Microbiológicos (PROIMI), CONICET, Av. Belgrano y Pasaje Caseros, 4000 Tucumán, Argentina; [3]Ruhr-Universität Bochum, Medizinisches Proteom-Center, Redox Proteomics Group, Bochum, Germany.
***Correspondence:** Héctor M. Alvarez, halvarez@unpata.edu.ar

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Abbreviations: AGPAT, 1-acylglycerol-3-phosphate *O*-acyltransferase; CAT, catalase; CSP, cold shock protein; DGAT, diacylglycerol acyltransferase; LC, liquid chromatography; MSH, mycothiol; MV, methyl viologen; SOD, superoxide dismutase; TAG, triacylglycerol.

Four supplementary figures and three supplementary tables are available with the online Supplementary Material.

Rhodococcus genus seem to be particularly resistant to oxidative stress [6, 12–14]; however, the components of antioxidant systems in these micro-organisms remain to be investigated in detail. Often, nitrogen starvation and oxidative stress occur simultaneously in natural environments, e.g. in semi-arid soils of Patagonia, Argentina (oligotrophic and dryness conditions). We therefore performed a global analysis to study the integration of the cellular responses against both stress conditions.

MS-based proteomics is a powerful tool to identify proteins that are regulated in response to specific physiological and stress conditions and thus increase or decrease their abundance. Often, these MS-based approaches rely on *in vivo* or *in vitro* labelling of proteins with isotopes. Recent advances in liquid chromatography (LC) as well as MS and, most importantly, in the available evaluation software now make label-free proteomic approaches feasible. Label-free proteomics is conceptually an ideal method for a comparative quantification of proteins, particularly for large cohorts of samples [15]. It has been successfully applied in bacterial proteome studies determining the response of bacteria to different stimuli and examining metabolic pathways [16, 17].

In the present work, we used an MS-based, label-free and quantitative proteomic approach as a starting point for understanding the response of *R. jostii* RHA1 to MV with respect to the synthesis and accumulation of TAG. Comparing cells grown in the presence of MV to untreated cells, we identified key proteins involved in the TAG biosynthesis that were affected by the addition of the pro-oxidant. In addition, our study showed that some metabolic pathways remain active during both oxidative stress and nitrogen starvation conditions. To our knowledge, this is the first integrative study performed in oleaginous rhodococci to clarify the antagonism of two processes that are essentials in the adaptation of these bacteria to environmental conditions.

METHODS

Culture media, growth conditions and lipid analyses

R. jostii RHA1 was grown in mineral salts medium [18] supplemented with 1 g l^{-1} ammonium chloride (MSM1) in order to promote cellular growth. MSM lacking ammonium chloride (MSM0) was used to induce lipid accumulation [19]. Sodium gluconate (1 %, w/v) was used as a carbon source. MSM0 supplemented with 10 mM MV (MSM0+MV) was used to evaluate oxidative stress response. All incubations were performed at 28 °C and 200 r.p.m. The qualitative and semiquantitative analyses of intracellular lipids in *R. jostii* RHA1 were performed as described elsewhere [7].

Preparation of protein extracts for MS-based label-free quantitative proteomic analysis

MSM1 culture medium was inoculated with *R. jostii* RHA1 and incubated overnight. Afterwards, fresh MSM0 medium was inoculated (initial OD_{600} ~0.2) and incubated for 8 h at 28 °C. At that point, the MSM0 culture was split into subcultures. One subculture was then supplemented with MV (10 mM) to induce oxidative stress. After 1 h, for each sample, 2 ml of cell culture was harvested by centrifugation (16 100 ***g***, 4 °C, 10 min) and washed twice with washing buffer (25 mM Tris buffer, pH 7, 2 mM EDTA). The cell pellet was then resuspended in 100 µl of urea solution (6 M urea, 100 mM Tris, pH 7.8) and 5 µl of reducing solution (200 mM DTT, 100 mM Tris, pH 7.8). Cells were disrupted by sonication and proteins were prepared according to Dávila Costa *et al.* [7].

LC-MS/MS analysis of protein extracts, data analysis and protein quantification

LC-MS/MS experiments of at least three biologically independent replicates were performed. A sample (1.2 ml) containing 600 ng of peptides was dissolved in 30 ml of 0.1 % trifluoroacetic acid and the methodology described by Dávila Costa *et al.* [7] was followed. Analysis of the data from free-label proteomic analyses was performed using MaxQuant version 1.4.1.2 [20]. MSM0 samples were compared to the corresponding MSM0+MV samples. Andromeda was used as peptide search engine and the protein database of *R. jostii* RHA1 from NCBI was used for peptide identification [21]. Proteins were considered significantly regulated when (1) they showed, on average, an increase >2-fold in their abundance in MSM0, (2) the fold change was at least 1.5-fold in each of the individual biological replicates and (3) Student's *t*-test showed a *P* value below 0.05. *P* value was calculated using the T.TEST function of Excel version 2007 (Microsoft). Upregulated proteins in MSM0+MV were expressed with a positive sign in order to differentiate from those upregulated in MSM0 (negative sign). To estimate the limit of detection of our instrument on the protein level, we considered the ninety-ninth percentile of the intensity of all proteins identified by MaxQuant passing the identification score threshold with a false discovery rate of 0.01. This value was 5579. We then used this value to impute missing intensity values in our label-free quantitation. Values of intensity in Table 1 are expressed as $\log_2$.

Bioinformatic analyses

RVBD_2855 (from *M. tuberculosis* H37Rv; GenBank accession number YP_177910) was used as query for the searching using the BLASTP program (NCBI website) in order to find homologous proteins in several species of *Rhodococcus*, using the default parameters and considering as homologous proteins those with identities higher than 50 %.

Multiple alignments and consensus sequence were performed using T-Coffee Sequence Alignment method [22]. To detect conserved motifs and Rossmann fold sequence domains (and predict the union of cofactor FAD, NAD or NADP), we used Cofactory v.1.0 Server (www.cbs.dtu.dk/services/Cofactory/) and ScanProsite tool (http://prosite.expasy.org/scanprosite/), respectively. To predict a secondary structure from RHA1_RO06610 sequence, PSIPRED secondary structure prediction method (version 3.3) was

used [23]. Besides, I-TASSER and Phyre2 servers were used for an *ab initio* modelling of the tertiary structure [24, 25]. The global accuracy in I-TASSER model is estimated using a C-score where values between 0 and 2 indicate a high confidence prediction, while Phyre2 considers the coverage of sequence to calculate the confidence. The resulting model was generated using the program PyMOL (DeLano Scientific; www.pymol.org).

RESULTS AND DISCUSSION

Cultivation of *R. jostii* RHA1 cells under lipid-accumulating conditions in the presence/absence of MV

In a previous study, we determined that MV negatively affected TAG biosynthesis and accumulation in *Rhodococcus opacus* PD630. In these cells, addition of MV to nitrogen-limited medium resulted in a dramatic decrease of the TAG accumulation typical in this medium [6]. To analyse the interaction between lipid metabolism and the response to MV-induced oxidative stress in rhodococci, we analysed the expression of proteins in *R. jostii* RHA1 in medium that promotes lipid synthesis and accumulation (MSM0), both in the presence and in the absence of MV. Cells were grown in nitrogen-containing MSM1 medium to the mid-exponential growth phase and then transferred to nitrogen-free MSM0. In this medium, which still contains the carbon source gluconate, the culture exhibited a slight increase in cellular biomass between 2 and 10 h of incubation due to carry over of the nitrogen source. The oxidant MV was added to MSM0 after 8 h of incubation, before the cell culture reached the stationary growth phase, which occurred after 11 h of incubation (Fig. 1). A slight inhibition of growth after the addition of the pro-oxidant was observed (Fig. 1).

Effect of MV on lipid metabolism

Depletion of TAG accumulation in *R. jostii* RHA1 by MV is caused by downregulation of Kennedy pathway enzymes, the FAS system and glyceroneogenesis

For proteomics studies, cells were harvested from MSM0 and MSM0 with added MV 9 h after inoculation, with *R. jostii* RHA1 being in late exponential growth phase at this point (Fig. 1). Similar to *R. opacus* PD630 [6], a decrease in the TAG accumulation by *R. jostii* RHA1 cultivated in MSM0 +MV compared to MSM0 was observed at this time point (Fig. 1). TAG are synthesized in rhodococci through the Kennedy pathway. This pathway is composed of sequential reactions catalysed by four different enzymes. Our proteomic data revealed a significant decrease in the abundance of some enzymes of this pathway. Four different isoenzymes of 1-acylglycerol-3-phosphate *O*-acyltransferase (AGPAT) were significantly downregulated in the presence of MV (RHA1_RS05380, RHA1_RS19670, RHA1_RS09865 and RHA1_RS10035) (Table 1). These AGPAT isoenzymes have been found previously to be highly synthesized during TAG accumulation in *R. jostii* RHA1 [7]. Of these, RHA1_RS05380 was the AGPAT most affected by the presence of MV. Amara *et al.* [26] reported that RHA1_RS19670 occurs in an operon with RHA1_RS19675 and RHA1_RS19680, which encode another AGPAT enzyme and a haloacid dehalogenase type hydrolase (predicted as a potential phosphatase). In our study, RHA1_RS19675 and RHA1_RS19680 were also detected, but they did not pass our test to be considered significantly regulated (see Methods and Table S1, available in the online Supplementary Material). These proteins may have been affected by the presence of MV to some extent, but under the strict parameter of significance that we used for the analysis of our proteomes, they were not considered as significantly affected proteins. In addition, two diacylglycerol acyltransferase (DGAT) isoenzymes were significantly downregulated in the presence of MV (Atf6 and Atf8) (Table 1). Atf6 is the orthologue of the well-characterized DGAT Atf2 from *R. opacus* PD630, which contributes significantly to TAG synthesis and accumulation [27]. On the other hand, the gene coding for Atf8 isoenzyme was the most upregulated gene in *R. jostii* RHA1 during cultivation of cells with benzoate under nitrogen-limiting conditions [26]. The remaining DGAT isoenzymes from strain RHA1 did not significantly changed their abundances after addition of MV to the culture medium.

Although the Kennedy pathway is necessary for the production of TAG, it is not sufficient, as TAG synthesis requires glycerol-3-phosphate and acyl-CoA produced by the FAS systems. MV also led to a significant decrease in the abundance of FAS-I and FAS-II proteins, which could lead to a depletion of the acyl-CoA pool in cells (Table 1). Downregulation of protein components of FAS-I and FAS-II in the presence of MV correlated with the decrease of phosphoenolpyruvate carboxykinase, the first enzyme of the glyceroneogenesis pathway, and glycerol-3-phosphate dehydrogenase $NAD(P)^+$, which catalyses the conversion of DHAP to glycerol-3-phosphate in the same pathway. Interestingly, enzymes involved in the methylmalonyl-CoA pathway, such as methylmalonyl-CoA mutase and propionyl-CoA carboxylase, which provides propionyl-CoA as precursor for odd-numbered fatty acid synthesis, were significantly downregulated in the presence of MV in comparison to MSM0 (Table 1). Chemical analyses of lipid fractions extracted from preparative TLCs revealed that significant amounts of odd-numbered fatty acids (approximately 30 % of the total fatty acids, w/w) occurred in the TAG fraction, whereas the phospholipid fraction contained only even-numbered fatty acids. This result and the concomitant downregulation of the methylmalonyl-CoA route and fatty acid and TAG biosynthesis pathways (FAS-I, FAS-II, AGPAT, DGAT, etc.) in RHA1 cells during cultivation under TAG-accumulating conditions with the addition of MV (Table 1) suggested that the methylmalonyl-CoA pathway is part, to some extent, of the regulatory network controlling TAG synthesis and accumulation in strain RHA1.

MV induces enzymes involved in the degradation of TAG

The observed decrease in TAG accumulation upon exposure to MV can be explained, in part, by a decrease in synthesis capacity. However, several enzymes involved in TAG mobilization, β-oxidation and glycerol assimilation were

Table 1. Proteins of *R. jostii* RHA1 affected by the presence of MV

Gene†	NCBI accession no.	*t*-test (*P*)‡	Protein name	Metabolic pathway§/ description	Intensity* MSM0 MV	Intensity* MSM0	Fold change‖
Central metabolism							
Proteins downregulated in the presence of MV							
RHA1_RS19765	ABG95863.1	0.001	Propionyl-CoA carboxylase	Methylmalonyl-CoA	16.87	17.89	-2
RHA1_RS35295	ABG98997.1	0.01	Methylmalonyl-CoA mutase small subunit	Methylmalonyl-CoA	19.03	20.69	-3
RHA1_RS35300	ABG98998.1	0.006	Methylmalonyl-CoA mutase large subunit	Methylmalonyl-CoA	18.44	20.50	-4.1
Fatty acid metabolism							
Proteins downregulated in the presence of MV							
RHA1_RS16605	ABG95225.1	0.0006	Probable fatty acid desaturase	Biosynthesis of unsaturated fatty acids	12.44	17.14	-26.5
RHA1_RS06915	ABG93245.1	0.01	Fatty acid synthase type I (FAS-I)	Fatty acid biosynthesis	20.90	24.92	-16.2
RHA1_RS05810	ABG93026.1	0.00004	Acyl carrier protein	Fatty acid biosynthesis. FAS-II	21.48	25.55	-16.8
RHA1_RS05805	ABG93025.1	0.01	Malonyl-CoA-[ACP] transferase	Fatty acid biosynthesis. FAS-II. 'FabD'	17.95	20.09	-4.4
RHA1_RS05815	ABG93027.1	0.04	β-Ketoacyl-[ACP] synthase	Fatty acid biosynthesis. FAS-II. 'FabF'	19.40	21.01	-3.6
RHA1_RS25440	ABG96980.1	0.004	β-Ketoacyl-[ACP] reductase	Fatty acid biosynthesis. FAS-II. 'FabG'	15.96	17.25	-2.4
RHA1_RS11425	ABG93315.1	0.007	β-Ketoacyl-[ACP] reductase	Fatty acid biosynthesis. FAS-II. 'FabG'	15.09	17.52	-5.3
RHA1_RS25350	ABG96961.1	0.001	Phosphoenolpyruvate carboxykinase	Glyceroneogenesis	17.74	20.16	-7.5
RHA1_RS31815	ABG98278.1	0.002	Glycerol-3-phosphate dehydrogenase $NAD(P)^+$	Glyceroneogenesis. Gene: *gpsA*	16.61	18.82	-4.6
RHA1_RS07790	ABG93414.1	0.00003	Diacylglycerol acyltransferase (DGAT) *atf6*	Kennedy pathway	12.44	15.98	-11.59
RHA1_RS26160	ABG97136.1	0.01	Diacylglycerol acyltransferase (DGAT) *atf8*	Kennedy pathway	12.44	15.11	-6.34
RHA1_RS05380	ABG92942.1	0.00002	1-Acylglycerol-3-phosphate *O*-acyltransferase (AGPAT)	Kennedy pathway	12.44	18.11	-50.88
RHA1_RS19670	ABG95844.1	0.0001	1-Acylglycerol-3-phosphate *O*-acyltransferase (AGPAT)	Kennedy pathway	16.86	18.67	-3.4
RHA1_RS09865	ABG93832.1	0.005	1-Acylglycerol-3-phosphate *O*-acyltransferase (AGPAT)	Kennedy pathway	14.43	15.86	-2.6
RHA1_RS10035	ABG93864.1	0.007	1-Acylglycerol-3-phosphate *O*-acyltransferase (AGPAT)	Kennedy pathway	14.02	17.25	-9.4
RHA1_RS23635	ABG96624.1	0.006	Cyclopropane fatty acyl phospholipid synthase	Phospholipid cyclopropane biosynthesis	14.11	16.29	-4.5
RHA1_RS28630	ABG97640.1	0.04	Acyl-[acyl-carrier protein] desaturase	Polyunsaturated fatty acid biosynthesis	17.42	18.42	-2
RHA1_RS06825	ABG93229.1	0.01	Linoleoyl-CoA desaturase	Polyunsaturated fatty acid biosynthesis	16.05	18.41	-5.1
Proteins upregulated in the presence of MV							
RHA1_RS30630	ABG98042.1	0.02	FAD-dependent glycerol-3-phosphate	Conversion of glycerol-3-	17.76	15.64	4.3

Table 1. cont.

Gene†	NCBI accession no.	*t*-test (*P*)‡	Protein name	Metabolic pathway§/ description	Intensity* MSM0 MV	Intensity* MSM0	Fold change‖
			dehydrogenase	phosphate to dihydroxyacetone phosphate. Gene: *glpD*			
RHA1_RS30625	ABG98041.1	0.01	Glycerol kinase	Conversion of glycerol to glycerol-3-phosphate. Gene: *glpK*	17.32	15.72	3.1
RHA1_RS24955	ABG96881.1	0.003	Acyl-CoA dehydrogenase	β-Oxidation	19.24	18.24	2
RHA1_RS04015	ABG92675.1	0.001	Possible diacylglycerol kinase	Converwion of diacylglycerol to phosphatidic acid	14.62	12.44	4.5
RHA1_RS31160	ABG98149.1	0.006	Probable triacylglycerol lipase	Triacylglycerol degradation	17.29	15.40	3.7
RHA1_RS09240	ABG93710.1	0.001	Probable long-chain fatty acid CoA ligase	Breakdown of complex fatty acids	14.72	12.44	4.8
Oxidative stress response							
Proteins upregulated in the presence of MV							
RHA1_RS15870	ABG95078.1	0.03	Alkyl hydroperoxide reductase (AhpC)	Antioxidant protein. Reduction of organics hydroperoxides	21.58	19.92	3.1
RHA1_RS15875	ABG95079.1	0.001	Alkyl hydroperoxide reductase (AhpF)	Catalyses the NAD(P)H-dependent reduction of the peroxiredoxin AhpC	18.48	13.65	28.3
RHA1_RS27680	ABG97452.1	0.001	Cold shock protein (CSP)	DNA binding, regulation of transcription	22.04	19.52	5.7
RHA1_RS02860	ABG92423.1	0.01	Cold shock protein (CSP)	DNA binding, regulation of transcription	18.26	16.34	3.7
RHA1_RS20960	ABG96100.1	0.04	Catalase (CAT)	Antioxidant protein. *katE* gene	18.33	16.95	2.6
RHA1_RS25800	ABG97056.1	0.00009	Catalase (CAT)	Antioxidant protein. *katG* gene	21.43	17.91	11.5
RHA1_RS28495	ABG97613.1	0.0008	Catalase (CAT)	Antioxidant protein. *katE* gene	18.38	17.36	2
RHA1_RS00535	ABG91937.1	0.04	DNA protection during starvation protein	Stress response	22.23	20.97	2.4
RHA1_RS31490	ABG98214.1	0.0001	Glutaredoxin-like protein NrdH	Oxidative stress response	17.07	12.44	24.8
RHA1_RS26805	ABG97277.1	0.003	Heat shock protein DnaK	Hsp70 family, chaperone protein, stress response	18.39	17.01	2.6
RHA1_RS26810	ABG97278.1	0.0004	Heat shock protein GrpE	Oxidative stress response. Molecular chaperone GrpE	19.41	16.94	5.5
RHA1_RS26815	ABG97279.1	0.01	Heat shock protein DnaJ	Hsp70 family, chaperone protein, stress response	16.05	14.30	3.3
RHA1_RS16730	ABG95250.1	0.007	Possible *myo*-inositol-1-phosphate synthase	Mycothiol biosynthesis. Ino1 protein. It generates Ins-P	22.08	20.04	4.1
RHA1_RS28995	ABG97712.1	0.00004	(MshB) Possible deacetylase	Mycothiol biosynthesis	18.02	12.44	47.7
RHA1_RS04190	ABG92710.1	0.005	(MshC) Cysteine tRNA ligase	Mycothiol biosynthesis	19.13	17.26	3.6
RHA1_RS23745	ABG96646.1	0.0001	(MshD) Possible acetyltransferase	Mycothiol biosynthesis	19.14	17.70	2.7
RHA1_RS12655	ABG94392.1	0.00003	Mycothiol-dependent formaldehyde dehydrogenase	Production of *S*-formylmycothiol and	19.67	16.36	9.8

Table 1. cont.

Gene†	NCBI accession no.	*t*-test (*P*)‡	Protein name	Metabolic pathway§/ description	Intensity* MSM0 MV	Intensity* MSM0	Fold change‖
				NADPH. EC: 1.1.1.306			
RHA1_RS32310	ABG98383.1	0.002	Mycothiol disulfide reductase	Reduction of oxidized mycothiol	19.55	18.10	2.7
RHA1_RS23930	ABG96679.1	0.006	Organic hydroperoxide resistance protein	Antioxidant protein	20.25	17.99	4.8
RHA1_RS31200	ABG98157.1	0.00009	Glutaredoxin	Antioxidant protein	17.95	12.44	45.47
RHA1_RS29420	ABG97797.1	0.03	GSH *S*-transferase	Antioxidant protein	14.78	13.34	2.7
RHA1_RS19485	ABG95808.1	0.02	Protein methionine *S*-oxide reductase		17.97	15.64	5
RHA1_RS23555	ABG96608.1	0.002	GSH peroxidase	Antioxidant protein	20.51	17.21	9.8
RHA1_RS11035	ABG94069.1	0.01	Probable oxidoreductase		20.56	18.50	4.1
RHA1_RS07925	ABG93444.1	0.01	Riboflavin synthase ß-subunit		17.76	16.20	2.9
RHA1_RS11940	ABG94247.1	0.02	Stress-related protein		20.40	18.83	2.9
RHA1_RS07605	ABG93377.1	0.0002	Shikimate 5-dehydrogenase		17.68	15.99	3.2
RHA1_RS10605	ABG93980.1	0.01	Superoxide dismutase (SOD)	Fe-Mn superoxide dismutase (SOD), *sodA*	20.46	18.71	3.3
RHA1_RS19475	ABG95806.1	0.0002	Superoxide dismutase (SOD)	Fe-Mn superoxide dismutase (SOD), *sodA*	21.97	18.20	13.7
RHA1_RS11970	ABG94253.1	0.03	Tellurium resistance protein		22.63	19.43	9.1
RHA1_RS08150	ABG93492.1	0.02	Tellurium resistance protein		19.27	18.49	2
RHA1_RS08235	ABG93509.1	0.008	Tellurium resistance protein		15.38	13.36	6.2
RHA1_RS04745	ABG92817.1	0.0005	Thioredoxin	Antioxidant protein	16.87	12.44	21.5
RHA1_RS05780	ABG93020.1	0.01	Thioredoxin-dependent thiol peroxidase	Antioxidant protein	20.54	18.13	5.3
RHA1_RS07050	ABG93271.1	0.007	Thioredoxin	Antioxidant protein	20.46	18.57	3.7
RHA1_RS17710	ABG95453.1	0.04	Thioredoxin disulfide reductase	Antioxidant protein	19.62	18.14	2.7
RHA1_RS17715	ABG95454.1	0.03	Thioredoxin	Antioxidant protein	21.71	20.14	2.5
RHA1_RS10490	ABG93956.1	0.03	Universal stress protein	Oxidative stress response	16.40	15.57	3.5
RHA1_RS11030	ABG94068.1	0.01	Universal stress protein	Oxidative stress response	17.78	15.47	4.9
RHA1_RS11040	ABG94070.1	0.01	Universal stress protein	Oxidative stress response	17.41	15.12	4.8

*Average of intensity label-free quantitation values calculated by MaxQuant version 1.4.1.2 [20]. Intensity values are expressed as $\log_2$.

†According to the NCBI database. New annotation for *R. jostii* RHA1.

‡*P* value calculated using the T.TEST function of Excel version 2007 (Microsoft).

§According to the KEGG pathways database.

‖Positive fold change means higher abundance in MSM0 MV while negative fold change means higher abundance in MSM0.

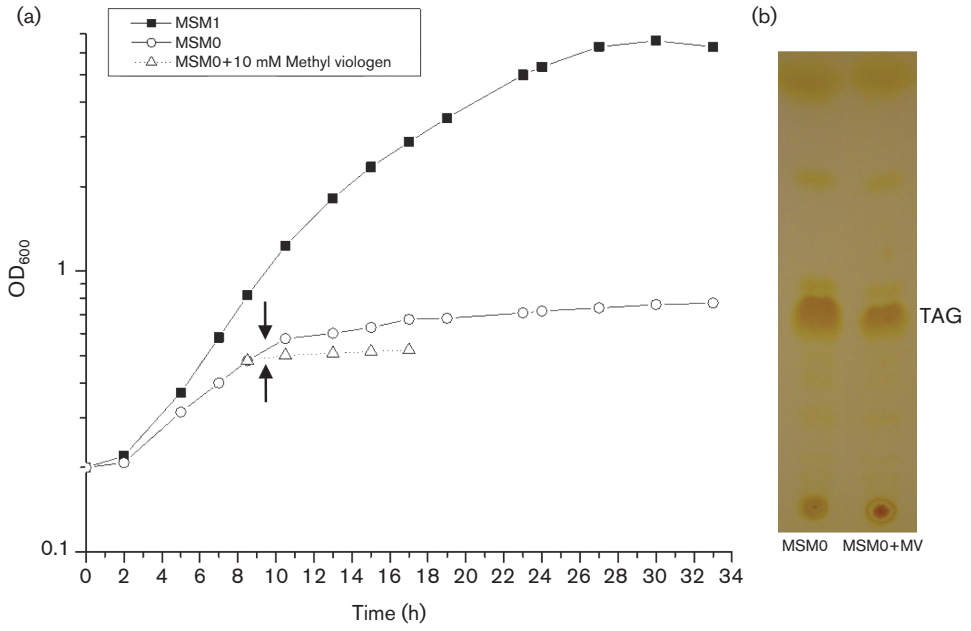

Fig. 1. (a) Cultivation of *R. jostii* RHA1 in mineral salts medium supplemented with sodium gluconate (1 %, w/v) without any nitrogen source (MSM0) and MSM0 supplemented with 10 mM MV (MSM0+MV). Vertical arrows represent sampling point for proteomic studies. (b) Total TAG content using TLC.

significantly upregulated during cultivation of cells in the presence of MV (Table 1). The abundance of a TAG lipase, a long-chain fatty acid CoA ligase and an acyl-CoA dehydrogenase (β-oxidation pathway), as well as the glycerol kinase (GlpK) and FAD-dependent glycerol-3-phosphate dehydrogenase (GlpD) (conversion of glycerol-3-phosphate in dihydroxyacetone phosphate), increased more than two-fold in the presence of MV (Table 1, Fig. 2).

There is some evidence that the degradation and biosynthesis of TAG can occur simultaneously in rhodococci, leading to a steady turnover of glycerol-3-phosphate and fatty acids [7]. However, under nitrogen-limiting conditions, lipogenesis dominates; thus, an increase of TAG content is usually observed under these conditions [7]. In the presence of MV, the balance between these two processes seems to switch and the decrease in TAG accumulation may be the combined result of an inhibition of lipogenesis and an induction of lipolysis (Fig. 1, Table 1). In addition, we could hypothesize that the increase in the abundance of GlpK and GlpD promotes the flux of dihydroxyacetone phosphate to the central metabolic reactions. This hypothesis may be supported by the downregulation of GpsA (glycerol-3-phosphate dehydrogenase), an enzyme that consumes dihydroxyacetone phosphate. Overall, this may contribute to the production of NADPH principally by the Entner–Doudoroff and pentose phosphate pathways, which is needed for antioxidant responses in the presence of MV in the culture medium (Fig. 2). However, metabolomics or metabolic flux studies would be required in order to demonstrate it.

Oxidative stress response caused by MV in *R. jostii* RHA1

Classical enzymatic scavengers were upregulated in the presence of MV

The first line of defence is composed by enzymes that detoxify reactive species, such as superoxide dismutases (SODs) and catalases (CATs). Exposure to MV led to significant upregulation of two Fe/Mn SODs and three CATs in *R. jostii* RHA1 (Table 1, Fig. 3). This is similar to the observation by Bequer Urbano *et al.* [6] that Mn SODs are activated in several *Rhodococcus* strains when exposed to pro-oxidants. The protein most affected by MV was CAT RHA1_RS25800, which showed a fold change of 11.5 (Table 1). Previously, four CATs (KatA, KatB, KatC and KatD) were identified in the pathogen *Rhodococcus equi* [28], but only KatA was determinant in the resistance to exogenous H_2O_2 exposure and crucial for surviving in mouse peritoneal macrophages [28]. RHA1_RS25800 exhibited 30 % of similarity with KatA of *R. equi*, whereas the reciprocal best hits of KatA (RHA1_RS20960) with 88 % of similarity exhibited a fold change of 2.6 (Table 1). In addition, Goordial *et al.* [13] also reported the presence of SODs and several CATs in the cold-adapted *Rhodococcus* sp. JG3.

While SODs and CATs directly detoxify the insulting agents of oxidative stress, bacteria also count on NADPH-dependent antioxidant mechanisms that can repair damage caused by oxidants and contribute to maintain the cellular integrity.

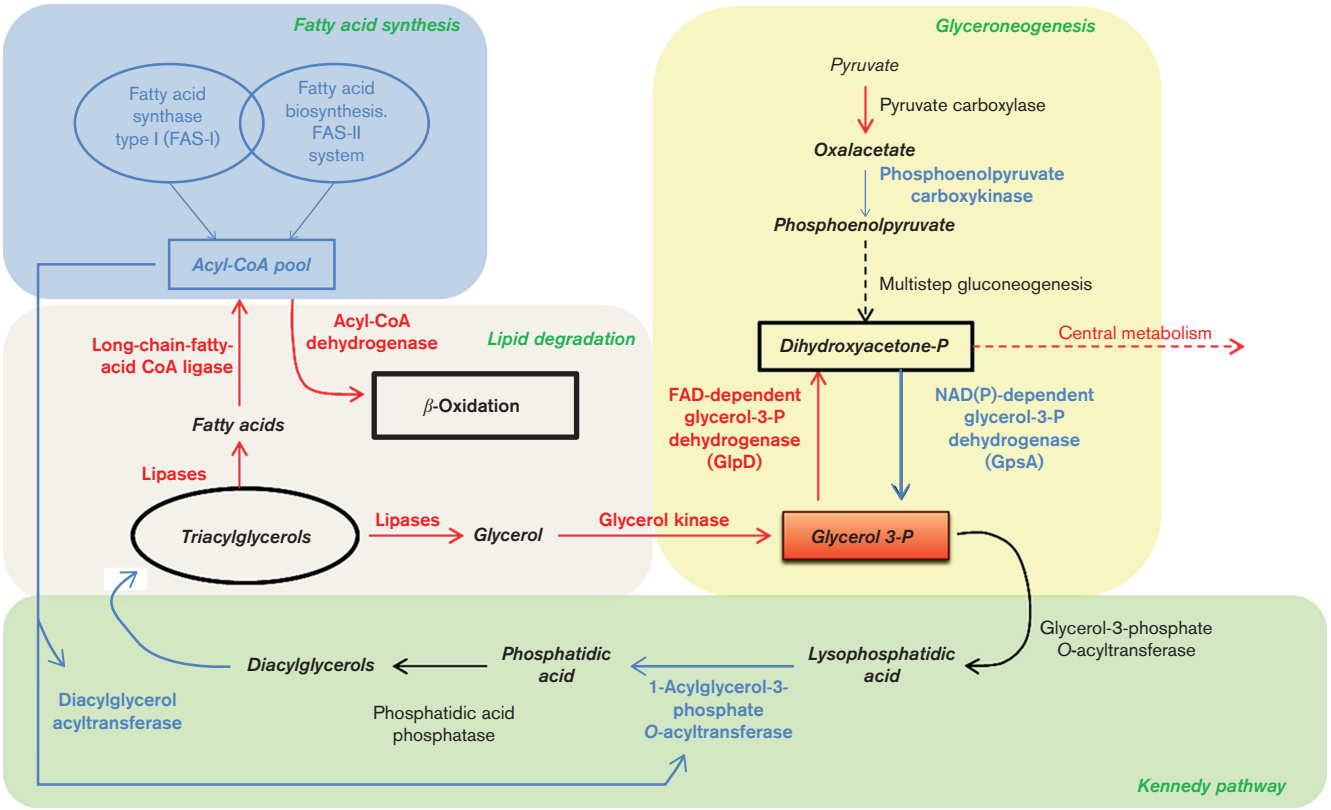

Fig. 2. Overview of reactions involved in lipid metabolism and abundance of proteins under exposure of cells to MV. Metabolites are shown in black. Enzymes in red were upregulated, whereas enzymes in blue were downregulated in the presence of MV.

MV activated the NADPH-dependent mycothiol antioxidant system

Low-molecular-weight thiols, including GSH, are important antioxidants. GSH depends on NADPH to keep its reduced, active form. Instead of gluthatione, some Gram-positive bacteria, including rhodococci, use mycothiol (MSH), a conjugate of *N*-acetylcysteine with 1-D-*myo*-inosityl-2-amino-2-deoxy-α-D-glucopyranoside, as their major low-molecular-weight thiol [29, 30]. Biosynthesis of MSH consists of sequential reactions catalysed by six enzymes [29]. Four of these enzymes, Ino1, MshB, MshC and MshD, were upregulated in the proteome of *R. jostii* RHA1 in response to MV (Table 1, Fig. 3). The antioxidant property of MSH relies on the sulfur atom of the amino acid cysteine, functional only in its reduced (–SH) form. Oxidized MSH (MSSM) is then reduced by a specific NADPH-dependent mycothione disulfide reductase Mtr [31]. While genes involved in the synthesis of MSH were previously identified in *R. jostii* RHA1 [32], a mycothione reductase (Mtr) has not been identified yet. Mtr has been mainly studied in *M. tuberculosis* because of its protective role against macrophage and antibiotics [33, 34]. The first identified Mtr, RVBD_2855 from *M. tuberculosis* H37Rv, is a homodimer that belongs to the group of *F*lavoprotein *D*isulfide *R*eductases enzymes (FDR enzymes [35]) and uses NADPH as cofactor. A protein with 64 % of amino acid identity to the known Mtr RVBD_2855, RHA1_RS32310 (the reciprocal best hits), was upregulated in the presence of MV in *R. jostii* RHA1 with a fold change of 2.7 (Table 1, Fig. 3). Combined with further bioinformatic analyses, we suggest that RHA1_RS32310 is indeed *R. jostii* RHA1's Mtr. High similarities were observed with Mtrs in other *Rhodococcus* species, while other bacterial genera showed lower identities (Table S2). Conserved domains and amino acids described for RVBD_2855 were also found in RHA1_RS32310 (Fig. S1), including the probable binding site for NADPH (YYGXGXXA, where Y is apolar; amino acids 173–180 in this protein sequence) [33]. The predicted secondary and tertiary structures of RHA1_RS32310 are shown in Figs. S2–S4.

Other NADPH-dependent antioxidant systems were activated by MV

The NADPH-dependent thioredoxin/thioredoxin reductase system was also upregulated in *R. jostii* RHA1 in the presence of MV (Table 1, Fig. 3). The classical system is formed by the small redox-active protein thioredoxin whose reduction is attended by the NADPH-dependent thioredoxin reductase. Thioredoxins are ubiquitous proteins that serve

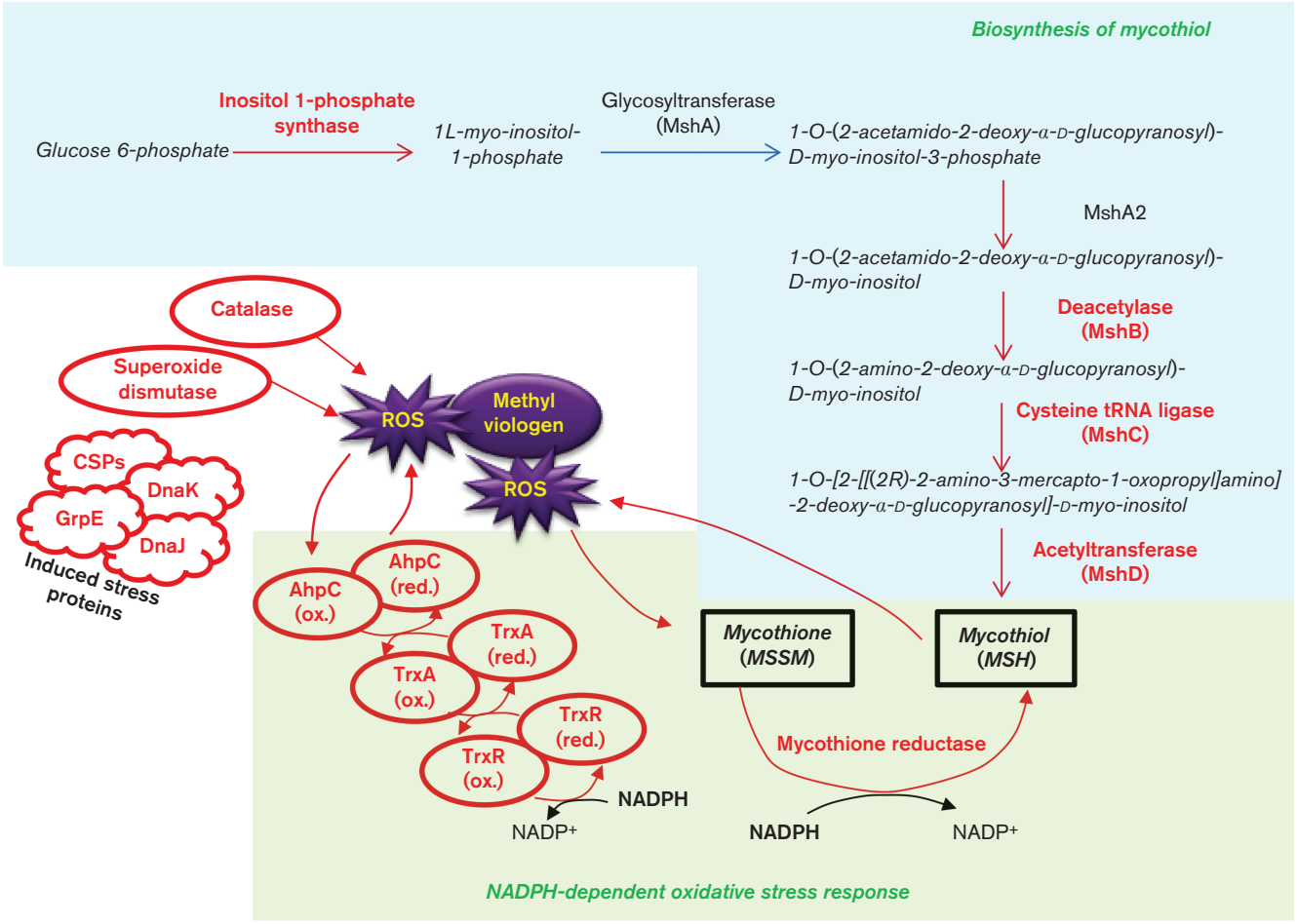

Fig. 3. Responsive mechanisms to oxidative stress proposed for *R. jostii* RHA1. Enzymes in red were upregulated in the presence of MV. Abbreviations: AhpC, alkyl hydroperoxide reductase; TrxA, thioredoxin; TrxR, thioredoxin reductase; MSH, mycothiol; MSSM, mycothione (oxidized MSH); Mtr, mycothione reductase; CSP, cold shock protein; DnaJ, DnaK, GrpE, heat shock proteins.

as a source of electrons in numerous metabolic processes, including the oxidative stress response [36].

Alkyl hydroperoxide reductases (Ahps) are also central elements of the antioxidant defence mechanisms by reducing hydroperoxides. This leads to the formation of a disulfide bond. The formed disulfide bond is then reduced by designated reductases, thioredoxin or MSH [37, 38]. An AhpCF system of *R. jostii* RHA1 was upregulated. Fold changes for AhpC and AhpF were 3.1 and 28.3 under MV exposure, respectively (Table 1).

The high abundance of the enzymes mentioned above would support the presumption that thioredoxin and MSH were active during MV exposure of *R. jostii* RHA1 cells (Fig. 3).

General stress proteins complemented the antioxidant defence of *R. jostii* RHA1

Additionally, cold shock protein (CSP), heat shock protein, heavy metal resistance protein and universal stress protein were identified upregulated in response to MV (Table 1). These proteins are often upregulated upon stress conditions generated by anoxia, ethanol, certain heavy metal ions and antibiotics [39–41]. The proteome of *R. jostii* RHA1 showed two CSPs with high abundance in the presence of MV (Table 1). CSPs were extensively studied; however, their precise function in normal and cold shock conditions is still unknown in many genera, including *Rhodococcus*. CSPs from *R. jostii* RHA1 possess the typical conserved residues of these proteins (Fig. S1) [39]. The elevated fold change for CSPs in the presence of MV (3.7 and 5.7) suggests a more important role of these proteins during the oxidative stress response than under normal growth conditions.

DnaK, DnaJ and GrpE are heat shock proteins whose synthesis is induced upon exposure to high temperatures. Similar to CSPs, these proteins are involved in a variety of cellular processes [42–44]. The proteome of *R. jostii* RHA1 also showed induction of this chaperone system (Table 1).

There are no previous reports on these proteins in *Rhodococcus*; thus, their role in the rhodococcal cells remains to be investigated.

Reactions and pathways which remained active under both stress conditions

NADPH-producing pathways of the central metabolism remain active during TAG accumulation and oxidative stress response

In a previous proteomic study, we demonstrated that central metabolic routes, such as the pentose phosphate and Entner–Doudoroff pathways and the amino acid degradation, among others, were induced during TAG-accumulating conditions [7]. Several of these pathways generate reducing equivalents in the form of NADPH, which is a required cofactor not only for the biosynthesis of fatty acids but also for the antioxidant response of cells.

In cells exposed to 10 mM MV, no significant differences in the abundance of enzymes involved in these NADPH-generating pathways were found when compared to MSM0 (Table S3a). These results indicated that NADPH-generating reactions need to be active under TAG-accumulating conditions as well as oxidant conditions.

Overall, our results suggested that NADPH may be redirected from lipogenesis pathways to antioxidant reactions when RHA1 cells are cultivated in the presence of MV.

Amino acid degradation is active under nitrogen-limited conditions in the absence as well as in the presence of MV

Previously, we demonstrated that several enzymes involved in the degradation of proteins and amino acids significantly increase their abundance during cultivation of *R. jostii* RHA1 under nitrogen-limiting, TAG-accumulating conditions [7]. Degradation of amino acids significantly contributes to the generation of metabolic energy and reducing equivalents (NADPH) [45], as well as in the release of bioavailable nitrogen. The addition of MV during cultivation of cells in MSM0 did not significantly change the level of proteins involved in the degradation of amino acids. Enzymes involved in the degradation of L-isoleucine, L-glutamate and L-valine were synthesized in similar amounts in both conditions (Table S3b). The degradation of L-glutamate and L-valine directly generates NADPH [7]. Since TAG biosynthesis decreased in the presence of MV (Fig. 1), we again propose that the produced NADPH is needed for antioxidant systems, which were upregulated during oxidative stress. The release of nitrogen under nitrogen starvation conditions through amino acid degradation and the upregulation of specific amino acid transporters in MSM0 in the absence/presence of MV (Table S3b) may help the cells to maintain nitrogen homeostasis.

Cells produce proteins to access alternative nitrogen sources in ammonium-chloride-lacking medium MSM0 in the presence of MV

In addition to the amino acid transports, the bacterial cells try to tap alternative nitrogen sources. Key proteins of nitrogen metabolism were not affected by the absence or presence of MV in MSM0. Cells synthesized enzymes for nitrate or nitrite reduction, cleavage of nitrogen sources (urease complex) and proteins responsible for nitrogen uptake, among others (Table S3b). Our previous work demonstrated that the abundance of amino-acid-degrading enzymes, transporters and proteins of nitrogen metabolism were upregulated in the absence of nitrogen [7]. These results suggest that the downregulation of lipid accumulation is a specific response towards oxidative stress, whereas *R. jostii* RHA1's response to nitrogen starvation is not affected by MV in the culture medium and thus not regulated by oxidative stress.

Conclusions

Our label-free proteomic analysis performed in the oleaginous *R. jostii* RHA1 strain indicates that the decrease of TAG biosynthesis and accumulation by cells after adding MV to the culture medium is the result of a programmed physiological mechanism. The main changes in protein expression induced by MV affecting TAG accumulation included (1) downregulation of glyceroneogenesis enzymes and GpsA, which decrease the availability of glycerol-3-phosphate; (2) decrease of the abundance of enzymes involved in the synthesis of methylmalonyl-CoA, which provide propionyl-CoA as precursor for the synthesis of odd-numbered fatty acids; (3) decrease in the abundance of FAS-I and FAS-II components, which generate the acyl-CoA pool available for TAG biosynthesis; (4) downregulation of several acyltransferase enzymes of the Kennedy pathway involved in TAG synthesis; (5) upregulation of proteins involved in lipolysis and β-oxidation of fatty acids and (6) activation of GlpK and GlpD involved in the degradation and assimilation of glycerol. In contrast to proteins related to TAG accumulation, those involved in NADPH-generating pathways, such as ED, PPP and amino acid degradation, did not change their abundance significantly in the presence of MV. Additionally, the expression of proteins involved in nitrogen metabolism and the molecular responses of cells to nitrogen starvation were not affected by the presence of the pro-oxidant.

Exposure to MV resulted in the upregulation of the antioxidant machinery composed of SODs and CATs as first line of defence. Additionally, NADPH-dependent antioxidant mechanisms, mainly governed by thioredoxin, MSH and Mtr, as well as Ahp, were also upregulated. Our study suggested that *R. jostii* RHA1 counters combined nitrogen starvation and oxidative stress with a directed cellular response, affecting specifically TAG biosynthesis, which may be through a redirection of NADPH fluxes towards antioxidant systems, while keeping systems that maintain nitrogen homeostasis unaffected.

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Conflicts of interest
The authors declare that there are no conflicts of interest.

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