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Review

# Understanding the function of bacterial and eukaryotic thiolases II by integrating evolutionary and functional approaches

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ABSTRACT

Acetoacetyl-CoA thiolase (EC 2.3.1.9), commonly named thiolase II, condenses two molecules of acetyl-CoA to give acetoacetyl-CoA and CoA. This enzyme acts in anabolic processes as the first step in the biosynthesis of isoprenoids and polyhydroxybutyrate in eukaryotes and bacteria, respectively. We have recently reported the evolutionary and functional equivalence of these enzymes, suggesting that thiolase II could be the rate limiting enzyme in these pathways and presented evidence indicating that this enzyme modulates the availability of reducing equivalents during abiotic stress adaptation in bacteria and plants. However, these results are not sufficient to clarify why thiolase II was evolutionary selected as a critical enzyme in the production of antioxidant compounds. Regarding this intriguing topic, we propose that thiolase II could sense changes in the acetyl-CoA/CoA ratio induced by the inhibition of the tricarboxylic acid cycle under abiotic stress. Thus, the high level of evolutionary and functional constraint of thiolase II may be due to the connection of this enzyme with an ancient and conserved metabolic route.

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**Contents**



## 1. Thiolase II background

Thiolase is a conserved enzyme present in the three domains of life (Bacteria, Archaea and Eukarya). This ubiquitous enzyme catalyzes the reversible thiolytic cleavage of 3-ketoacyl-CoA into acyl-CoA and acetyl-CoA, a two-step reaction involving a covalent intermediate formed with a catalytic cysteine. There are two major types of thiolases, (i) acyl-CoA:acetyl-CoA C-acyltransferase (EC 2.3.1.16), also named thiolase I or 3-oxoacyl-CoA thiolase, and (ii) acetyl-CoA:acetyl-CoA C-acetyltransferase (EC 2.3.1.9), also called thiolase II or acetoacetyl-

*Abbreviations:* AACT, Acetoacetyl-CoA thiolase; ABC, Polyhydroxybutyrate biosynthesis; DXS, 1-deoxy-D-xylulose-5-phosphate synthase; HMGR, 3-hydroxy-3-methylglutaryl-CoA reductase; HMG-CoA, 3-hydroxy-3-methylglutaryl-CoA; IPP, Isopentenyl diphosphate; MEP, Plastidic methylerythritol phosphate; MVA, Mevalonate; NJ, Neighbor-joining; PCR, Polymerase chain reaction; PHB, Polyhydroxybutyrate; PPP, Pentose phosphate pathway; TCA, Tricarboxylic acid.

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CoA thiolase. While both classes of thiolase catalyze reversible reactions, thiolase I is associated with catabolic processes, whereas thiolase II usually shows anabolic functions under physiological conditions. Contrary to thiolase I, which shows broad chain-length specificity for its substrates (C4–C22) (Yang et al., 1990), thiolase II has high substrate specificity (Merilainen et al., 2008). Thus, thiolase II is specific for C4 chains and catalyzes the condensation of two molecules of acetyl-CoA to give acetoacetyl-CoA and CoA.

Thiolase II is involved in the biosynthesis of various highly reduced compounds, depending on their genomic background. For instance, in bacteria, thiolase II catalyzes the first step in the production of the polyhydroxybutyrate (PHB) via the ABC pathway (Steinbuchel and Hein, 2001), whereas in eukaryotes it catalyzes the production of isoprenoids through the mevalonate (MVA) pathway (Kirby and Keasling, 2009) (Fig. 1). In this article, we review and present new data suggesting that thiolase II is a conserved enzyme that catalyzes the rate-limiting step in the biosynthesis of PHB and isoprenoids during abiotic stress adaptation, and propose an integrative scenario to explain the conserved sequence and function of thiolase II, where this enzyme senses the tricarboxylic acid (TCA) cycle for the maintenance of the redox balance.

## 2. Evolutionary equivalence of thiolase II

Despite their functional similarity, at least in terms of enzyme activity, bacterial and eukaryotic thiolases II are not classically described as orthologous genes. In fact, phylogenetic trees constructed using thiolases I and II from the three domains of life are incongruent with rRNA data (Pereto et al., 2005). These incongruent phylogenetic trees may be explained by multiple transfer events, suggesting that thiolase II cannot be assigned as orthologous (Pereto et al., 2005). Consequently, the functional and evolutionary equivalence of thiolase II from the ABC and MVA pathways is not typically considered. In this context, studies based on bacterial and eukaryotic thiolases II have been regarded as two distant and unrelated worlds. However, thiolases I and II have low amino acid identity (<25%), and as it is well known for evolution researchers, large distances generate aberrant phylogenetic trees (Hughes et al., 2005; Phillips, 2006).

We have recently proposed a novel phylogenetic framework of thiolase II evolution (Soto et al., 2011) (Fig. 2). The main methodological difference between our study and previous works was that we excluded proteins with extremely low identity (specifically thiolases I) from the analysis, using a more stringent selection criterion (Soto et al., 2011). This allowed us to find congruence between thiolase II and organismal trees (Fig. 2) (Soto et al., 2011). Because the probability of obtaining a congruent pattern by chance is virtually null (Li, 1997), our evolutionary analysis constitutes strong evidence supporting the hypothesis that the bacterial and eukaryotic thiolases II associated with PHB and isoprenoid biosynthesis are truly orthologous (Soto et al., 2011). As orthologous proteins in different organisms are likely to share a same or similar function, the data presented in our previous work could be used as a starting point in the integration of experimental data regarding thiolase II.

## 3. Bacterial thiolase II and PHB production

The PHB polymer is a highly reduced bacterial storage compound, whose production was firstly associated with nutrient storage (Dawes and Senior, 1973). The most widely distributed PHB biosynthetic pathway, named the ABC pathway, includes a thiolase II (PhbA), which condenses two molecules of acetyl-CoA to give acetoacetyl-CoA, a NADPH- or NADH-dependent reductase (PhbB), which reduces this compound to give D(−)-3-hydroxybutyryl- CoA, and a PHB polymerase (PhbC), which uses this monomer as a substrate for polymerization (Fig. 1) (Steinbuchel and Hein, 2001). Contrary to that observed for the

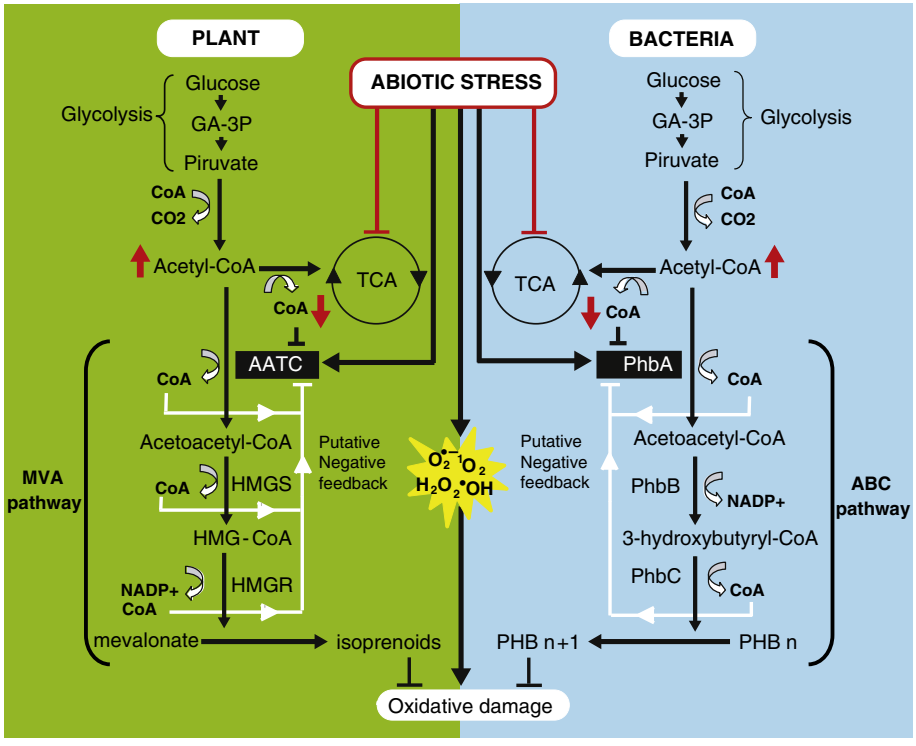


**Fig. 1.** Thiolases II probably play a key role in the interpretation of metabolic state in response to abiotic stress in bacteria and plants. Proposed model for the rerouting of metabolites from the TCA cycle to antioxidants biosynthetic pathways in response to an abiotic stress in plant (left) and bacteria (right). Red lines show the inhibitory effect of abiotic stress over the TCA cycle, the concomitant availability of acetyl-CoA (AACT substrate) and the decreased of CoA levels (AACT inhibitor). The increase of AACT activity promoted the biosynthesis of isoprenoids and PHB that help to maintain the cell redox balance opposing the oxidative effect of the stress. Abbreviations: AACT, acetoacetyl-CoA thiolase; ABC, phbA, phbB, phbC; GA-3P, glyceraldehyde 3-phosphate; HMG-CoA, 3-hydroxy-3-methylglutaryl-CoA; HMGR, HMG-CoA reductase; HMGS, HMG-CoA synthase gene; $H_2O_2$, hydrogen peroxide; MVA, mevalonate; NADP+, nicotinamide adenine dinucleotide phosphate; $^1O_2$, singlet oxygen; $O^{\bullet-}$, superoxide; $^{\bullet}OH$, hydroxyl radical; PHB, polyhydroxybutyrate; TCA, tricarboxylic acid.

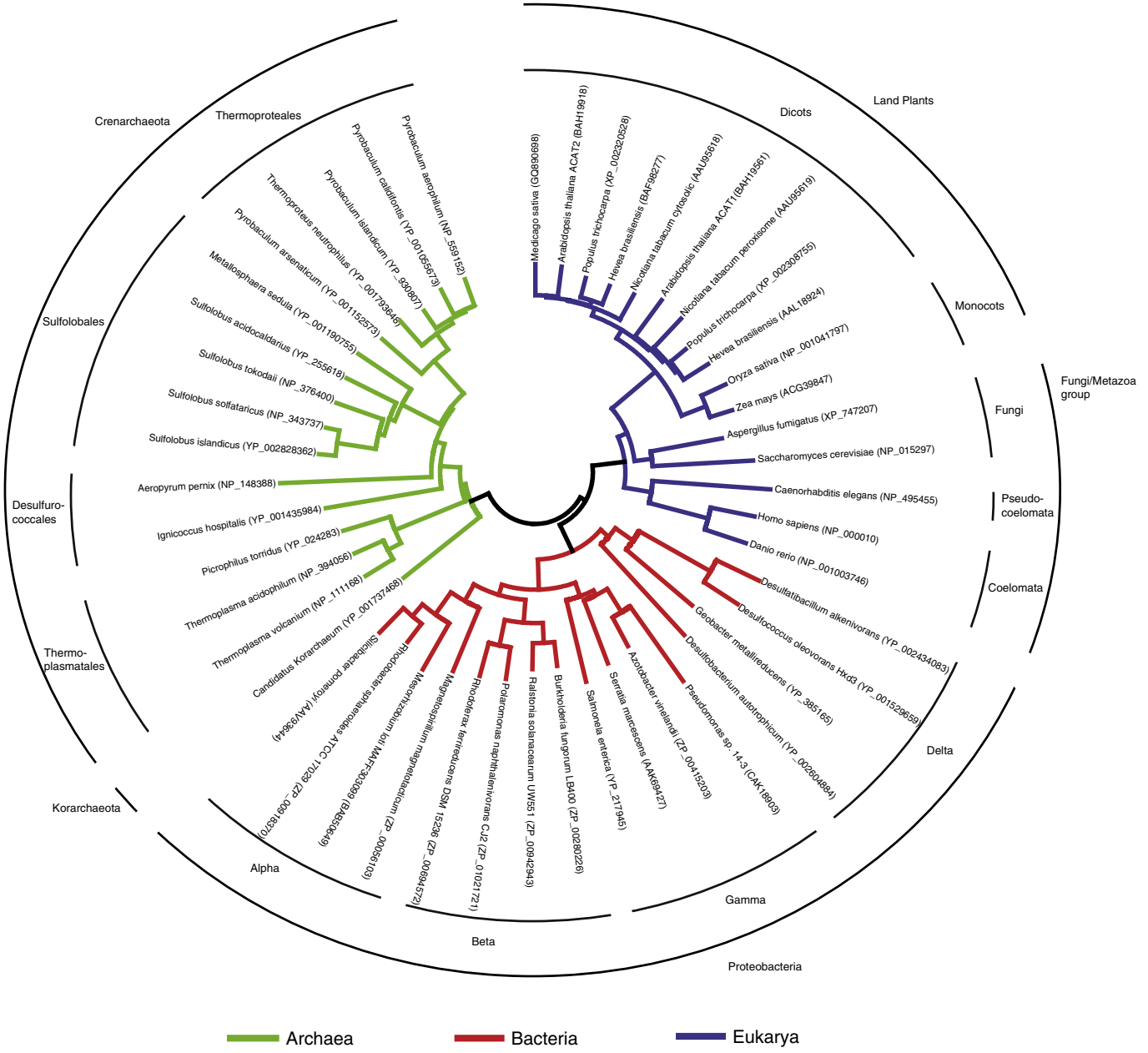


**Fig. 2.** Phylogenetic analysis of thiolase II protein sequences using the neighbor-joining method. Genetic distances computed using Poisson correction model by using the following parameters: substitutions to include = all, gaps/missing data = pair-wise deletion, phylogeny test = bootstrap 500 replicates and root on midpoint (Cuyeu et al., 2013).

*phbA* gene, several studies have demonstrated the feasibility to construct *phbB* and *phbC* mutants derived from natural PHB producing-strains (Steinbuchel and Hein, 2001). These results have been interpreted as supporting the hypothesis that the gene encoding for thiolase II is an essential housekeeping gene in bacteria. Nevertheless, the concept of bacterial thiolase II function and the role of bacterial PHB biosynthesis have changed radically in the last years.

Initially, we described the first thiolase II mutant, the Antarctic bacterium *Pseudomonas* sp. 14–3 (Ayub et al., 2004). Sequence analysis of the *Pseudomonas* sp. 14–3 *phbA* gene suggested that this gene had suffered a deletion, giving rise to a defective thiolase II (Ayub et al., 2006). We confirmed that this bacterial isolate was unable to produce PHB via the ABC pathway, by using gas chromatography and demonstrated that the incorporation of a functional thiolase II from *Pseudomonas putida* KT2440 into *Pseudomonas* sp. 14–3 is sufficient to reactivate the ABC pathway (Ayub et al., 2006). This result is a strong counterexample that rejects the hypothesis that thiolase II is an essential gene for the functioning of the bacterial cell. In addition, our phylogenetic studies and genome-sequencing analyses suggest that bacterial thiolase II can be transferred between different *Pseudomonas* strains via large mobile genetic elements called genomic islands (Ayub et al., 2007; Soto et al., 2012). We also demonstrated that acquisition of bacterial thiolase II gives the host cell the ability to produce PHB, and therefore, improved the fitness under multiple abiotic stresses, such as freezing, high temperature, oxidizing agents and salinity (Fig. 1) (Ayub et al., 2009; Soto et al., 2012). Most recently, we have constructed and analyzed different PHB-deficient mutants derived from extremophile bacterial isolates and found that PHB can protect bacteria by at least two different but probably complementary molecular mechanisms: one where the PHB monomer (β-hydroxybutyrate) can act as a chemical chaperone preventing the protein aggregation induced by abiotic stress (Soto et al., 2012), and another, where PHB degradation could supply the reductive power necessary to mitigate the oxidative stress induced during abiotic stress adaptation (Fig. 1)

(Ayub et al., 2009). Thus, the bacterial thiolase II gene could be now considered as an "accessory" gene involved in the tolerance to abiotic stress rather than a bacterial core gene.

## 4. Eukaryotic thiolase II and isoprenoid production

The condensation reaction of thiolase II from eukaryotes, usually termed acetyl-CoA acetyltransferase (AACT), has been recognized as the first step in the isoprenoid biosynthesis via the MVA pathway. However, the relevance of this reaction in this anabolic process has not been classically considered. In contrast, 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR), the enzyme that catalyzes the irreversible conversion of 3-hydroxy-3-methylglutaryl-CoA to MVA (Fig. 1), has been considered a key regulatory step controlling isoprenoid metabolism in mammals, fungi, insects and plants (Bach and Lichtenthaler, 1983; Chappell and Nable, 1987; Chappell et al., 1989, 1995; Goldstein and Brown, 1990; Kuzuyama and Seto, 2012; Miziorko, 2011; Morgen et al., 1982). Thus, the reaction catalyzed by HMGR is the rate limiting step of isoprenoid biosynthesis in eukaryotes, at least under unstressed growth conditions.

In plants, there are two alternative pathways to produce isoprenoids: the conserved cytosolic MVA pathway and the plastidic methylerythritol phosphate (MEP) pathway (Fig. S1) (Cordoba et al., 2009; Kirby and Keasling, 2009; Kuzuyama and Seto, 2012). These two pathways are connected by the exchange of the metabolic precursor isopentenyl diphosphate (IPP) across the chloroplast membrane (Fig. S1) (Laule et al., 2003). However, this exchange seems to be so small that the MVA and MEP pathways could be considered two independent routes (Suzuki et al., 2009). Supporting the idea of two independent pathways, these routes have different final products, and more importantly, are regulated by different enzymes (Chappell et al., 1995; Cordoba et al., 2009; Kirby and Keasling, 2009). More specifically, HMGR, which catalyzes the third step of the MVA pathway, and 1-deoxy-D-xylulose-5-phosphate synthase (DXS), the first enzyme of the MEP pathway, have been described as the rate-limiting steps in the isoprenoid biosynthesis via the MVA and MEP pathways in plants, respectively (Fig. S1) (Vranová et al., 2013).

The regulatory function of the enzymes involved in isoprenoid production in plants, such as HMGR and DXS, has been analyzed during optimum growth conditions. This is completely reasonable considering that some isoprenoids, such as phytohormones (e.g. abscisic acid), pigments (e.g. chlorophyll) or sterols (e.g. brassinosteroid), are essential for plant growth (Clouse, 2011; Suzuki et al., 2009). Similar to the characterization of the role of HMGR in the regulation of the MVA pathway in mammals because this metabolic route is responsible for the biosynthesis of essential molecules such as cholesterol (Miziorko, 2011). However, experimental data also indicate that some isoprenoids have a critical antioxidant function under biotic and abiotic stress in plants (Chappell and Nable, 1987; Chappell et al., 1989, 1991; Vickers et al., 2009; Vogeli and Chappell, 1988). Regarding this, we have recently reported a critical role for thiolase II during abiotic stress and taking into account that the critical step within a metabolic pathway can change depending on the physiological state, we believe that it is necessary to reassess which enzymes are key to the MVA pathway in plants exposed or not to abiotic stress.

## 5. Structural and functional equivalence of thiolases II

Because of their simplicity, heterologous expression assays have been used to experimentally demonstrate the activity of plant thiolases II. For example, thiolases II from *Arabidopsis thaliana* (*acat2*) and *Hevea brasiliensis* (*HbAACT1*) have been shown to complement the growth deficit of *Saccharomyces cerevisiae*-derived mutant (Δ*ERG10*), and alfalfa thiolase II (*MsAACT1*) was able to restore PHB production in the Antarctic bacterium *Pseudomonas* sp. 14–3 (Carrie et al., 2007; Jin et al., 2012; Soto et al., 2011).

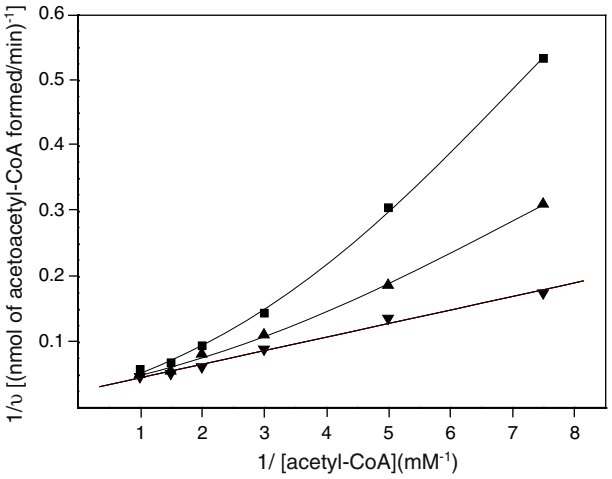


**Fig. 3.** Effect of CoA on the condensation reaction catalyzed by MsAACT1. The reaction mixture was supplemented with none (inverted triangle), 25 μM (triangle) and 100 μM (rectangle) of CoA and measured according to Soto et al. (2011). Shown is one representative experiment of 3 replicates.

In concordance with the enzymatic characterization of bacterial thiolase II (Senior and Dawes, 1973), we have recently shown that the condensation reaction of MsAACT1 is further inhibited by increasing concentrations of free CoA (Fig. 1) (Soto et al., 2011). In addition, here we show that the condensation reaction performed by MsAACT1 results in Michaelis–Menten kinetics in the absence of CoA but becomes sigmoidal in the presence of CoA (Fig. 3). This is consistent with the previous characterization of bacterial thiolase II involved in PHB production and the proposed regulation of the activity of this enzyme by the formation of homotetramers (Kursula et al., 2002; Senior and Dawes, 1973).

To better understand the conservation of thiolase II activity and structure, we compared the three-dimensional conformations of bacterial (PhbA) and homology modeled plant (MsAACT1) thiolases II as well as their affinities for CoA. In line with their evolutionary and functionality equivalence, these enzymes showed almost identical

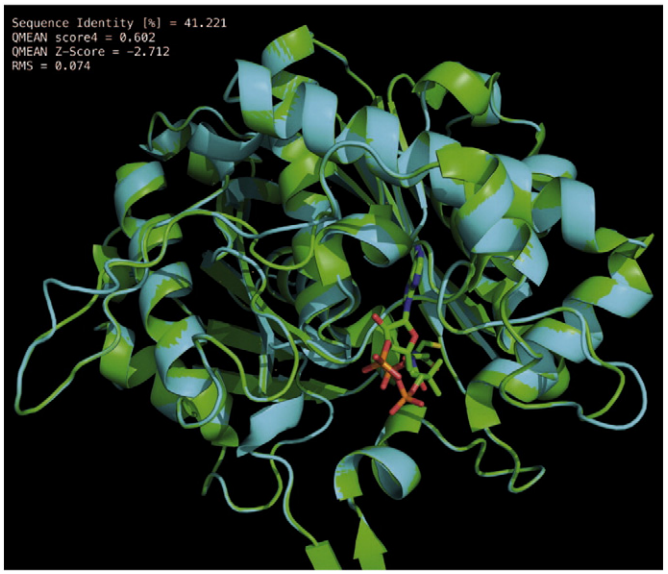


**Fig. 4.** Superimposition of the predicted structure of plant thiolase II to the corresponding bacterial template. Cartoon representation of the three dimensional structures of homology modeled acetoacetyl-CoA thiolase from *Medicago sativa* (cyan) and biosynthetic thiolase from *Zoogloea ramigera* in complex with CoA (green, pdb: 1DLV (Modis and Wierenga, 2000)). Modeling details are also reported.

three-dimensional structure, mainly on the basis of sequence identity and results of homology modeling analysis (Fig. 4). Additionally, docking analysis showed an equilibrium dissociation constant of 4.4 μM for the MsAACT1-CoA complex, in good agreement with the experimentally measured value reported for bacterial thiolase II (9 μM) (Modis and Wierenga, 2000). Furthermore, the molecular docking of CoA on bacterial and plant thiolases II predicted analogous binding modes in the active site, establishing the same number of hydrogen bonds and similar type of molecular interactions with the enzyme (Fig. 5).

All these results further demonstrate the structural and activity equivalence of thiolases II among highly distant organisms. However, they do not provide information about the conservation of the thiolase II critical role in response to abiotic stress.

## 6. Functional equivalence of thiolases II under abiotic stress

The first approach to analyze the functional equivalence of bacterial and plant thiolases II during abiotic stress adaptation was the characterization of *Pseudomonas* sp. 14–3 overexpressing the *MsAACT1* gene. This recombinant bacterium not only restored PHB production but also regained its freezing and salinity resistance (Soto et al., 2011). Moreover, we showed that overexpression of the *MsAACT1* gene in transgenic alfalfa roots increased thiolase II activity, isoprenoid production (squalene biosynthesis) and abiotic stress tolerance (salinity resistance) without altering HMGR activity (Soto et al., 2011). More importantly, transgenic alfalfa roots showed high antioxidant capacity and the sensitive phenotype of wild-type alfalfa was completely reverted by the addition of a non-isoprenoid reducing compound (vitamin C) (Soto et al., 2011). Taken together, these results strongly suggest that plant thiolase II catalyzes the rate-limiting step in the biosynthesis of isoprenoid via the MVA pathway during abiotic stress adaptation and that the MVA pathway plays a critical role in the production of reducing equivalents to mitigate the oxidative stress induced by abiotic stress (Fig. 1). Thus, the regulatory roles of HMGR and thiolase II in the MVA pathway are not mutually exclusive and could depend on the physiological background.

## 7. A hypothetical interpretation of the metabolic state by thiolase II

The hypothesis that thiolase II is a conserved enzyme that catalyzes the rate-limiting step in the biosynthesis of PHB and isoprenoids via the ABC and MVA pathways during abiotic stress adaptation is supported by several evidences (Fig. 1): (i) bacterial and plant thiolases II catalyze the first step and the highest endergonic reaction ($\Delta G = +25$ kJ/mol) in these anabolic processes (Fig. S1) (Kadouri et al., 2005), (ii) plant and

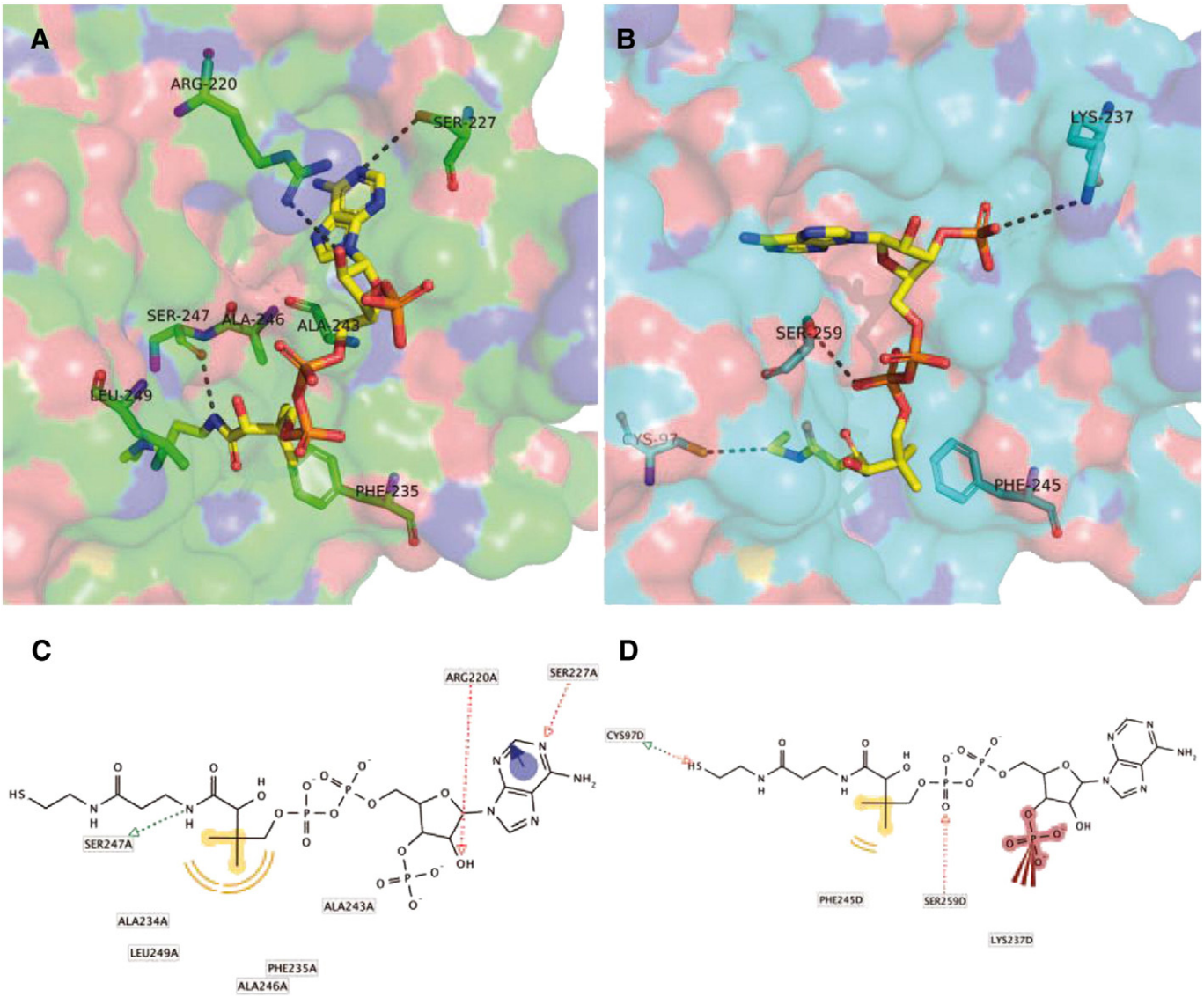


**Fig. 5.** Molecular docking between plant thiolase II and CoA. Comparison between thiolase/CoA complex resulting from docking analysis (B and D) and that obtained from bacterial crystallographic structure (A and C, see Fig. 3 legend for details). H-bonds and all molecular interactions between CoA and amino acids are also shown.

bacterial thiolases II are transcriptionally regulated in response to adverse environmental conditions (Fig. 1) (Kadouri et al., 2005; Soto et al., 2011) and (iii) the activity of bacterial and plant thiolases II is post-transcriptionally regulated by CoA (Figs. 1 and 3) (Senior and Dawes, 1973; Soto et al., 2011). This negative regulation of thiolase II activity by CoA could be the result of a negative feedback regulation and/or the metabolic state of bacterial and eukaryotic cells (Fig. 1). However, these evidences are not enough to explain why thiolase II was evolutionarily selected as a key enzyme in the biosynthesis of antioxidant compounds during abiotic stress adaptation in so divergent organisms such as *Pseudomonas* and alfalfa.

To explain the conserved function of thiolase II, we propose to analyze an integrative scenario, where this enzyme senses the tricarboxylic acid (TCA) cycle for the maintenance of the redox balance during abiotic stress adaptation (Fig. 1). The first argument supporting this hypothesis is related to the ancient origin of the TCA cycle. In fact, this metabolic pathway is a truly ancestral cycle present in the three domains of life. The second argument is related to the impact of the regulation of the TCA cycle on the metabolic status of the cell. Acetyl-CoA (the thiolase II substrate) is oxidized by the TCA cycle, whereas CoA (a thiolase II inhibitor) is released by this pathway during optimal growth conditions (Fig. 1). Thus, the production of antioxidant compounds (PHB and isoprenoids) by thiolase II is repressed under favorable conditions, characterized by high respiratory rates (Fig. 1) (Ayub et al., 2009; Soto et al., 2011). On the other hand, several evidences indicate that the TCA cycle is inhibited by the oxidative stress induced under abiotic stress exposure in highly divergent organisms such as yeast, animals, plants and bacteria (Fig. 1) (Baxter et al., 2007; Godon et al., 1998; Grant, 2008; Liu et al., 2005; Pomposiello and Demple, 2002). Therefore, the levels of acetyl-CoA would be increased and the amount of free CoA would be decreased under abiotic stress conditions (Fig. 1). Thus, bacteria and plants can redirect the flow of reducing power from the TCA cycle to the ABC and MVA pathways by thiolases II, and then, mitigate the oxidative stress induced by abiotic stress (Fig. 1).

## 8. Future perspectives

Further studies involving bacterial, archaeal and eukaryotic thiolases II will show whether thiolases II indeed have a conserved and ancestral function in abiotic stress adaptation such as the sensing of the TCA cycle for the maintenance of the redox balance. Finally, the results discussed in this article clearly show that the regulation of anabolic pathways for the biosynthesis of antioxidant compounds could be more complex than at first thought and would prompt the scientific community to study its analyzing different metabolic sceneries.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.gene.2013.09.096.

## Conflict of interest

None.

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