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RESEARCH PAPER

Acetoacetyl-CoA thiolase regulates the mevalonate pathway during abiotic stress adaptation

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Abstract

Acetoacetyl-CoA thiolase (EC 2.3.1.9), also called thiolase II, condenses two molecules of acetyl-CoA to give acetoacetyl-CoA. This is the first enzymatic step in the biosynthesis of isoprenoids via mevalonate (MVA). In this work, thiolase II from alfalfa (*MsAACT1*) was identified and cloned. The enzymatic activity was experimentally demonstrated *in planta* and in heterologous systems. The condensation reaction by *MsAACT1* was proved to be inhibited by CoA suggesting a negative feedback regulation of isoprenoid production. Real-time RT-PCR analysis indicated that *MsAACT1* expression is highly increased in roots and leaves under cold and salinity stress. Treatment with mevastatin, a specific inhibitor of the MVA pathway, resulted in a decrease in squalene production, antioxidant activity, and the survival of stressed plants. As expected, the presence of mevastatin did not change chlorophyll and carotenoid levels, isoprenoids synthesized via the plastidial MVA-independent pathway. The addition of vitamin C suppressed the sensitive phenotype of plants challenged with mevastatin, suggesting a critical function of the MVA pathway in abiotic stress-inducible antioxidant defence. *MsAACT1* over-expressing transgenic plants showed salinity tolerance comparable with empty vector transformed plants and enhanced production of squalene without altering the 3-hydroxy-3-methylglutaryl-CoA reductase (HMGR) activity in salt-stress conditions. Thus, acetoacetyl-CoA thiolase is a regulatory enzyme in isoprenoid biosynthesis involved in abiotic stress adaptation.

Key words: Abiotic stress, acetoacetyl-CoA thiolase, isoprenoid, MVA pathway, thiolase II.

Introduction

Acetoacetyl-CoA thiolase (EC 2.3.1.9), also called thiolase II, is a condensing enzyme that catalyses the production of acetoacetyl-CoA from two molecules of acetyl-CoA. Although thiolase II catalyses reversible reactions, most of these thiolases act in anabolic processes as the first step in the biosynthesis of polyhydroxybutyrate (PHB) via the thiolase II pathway and isoprenoids via the mevalonate (MVA) pathway in bacteria and eukarya, respectively (Steinbüchel and Hein, 2001; Kirby and Keasling, 2009; Fig. 1). However, some thiolases II are involved in catabolic pathways such as the last step of fatty acid β-oxidation.

PHB is a highly reduced bacterial storage compound. Biosynthesis of this polymer is induced under abiotic stress and thiolase II occupies a key position in this production (Senior and Dawes, 1973; Wu *et al.*, 2001; Peralta-Gil *et al.*, 2002, Kadouri *et al.*, 2005). It has recently been

Abbreviations: AACT, acetoacetyl-CoA thiolase; HMG-CoA, 3-hydroxy-3-methylglutaryl-CoA; IPP, isopentenyl diphosphate; MEP, plastidic methylerythritol phosphate; MS, Murashige and Skoog; MVA, mevalonate; NJ, Neighbor–Joining; PCR, polymerase chain reaction; PHB, polyhydroxybutyrate; PPP, pentose phosphate pathway; ROS, reactive oxygen species; RT, reverse transcriptase; TAC, total antioxidant capacity; TCA, tricarboxylic acid.

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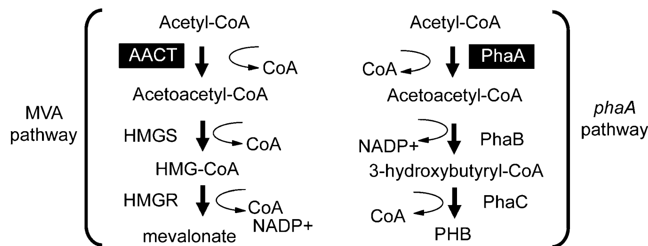

Fig. 1. Biosynthesis of isoprenoids via mevalonate (MVA) and polyhydroxybutyrate (PHB) via the thiolase II pathway in eukarya and bacteria, respectively. Thiolase II products involved in these pathways are boxed.

demonstrated that PHB is essential to maintain the redox balance during low temperature adaptation in *Pseudomonas* sp. 14-3 (Ayub *et al.*, 2007, 2009). This strain is a natural thiolase II mutant (Ayub *et al.*, 2006), where PHB biosynthesis only occurs through the β-oxidation pathway (Ayub *et al.*, 2004), a *phaA*-independent pathway (Aldor and Keasling, 2003).

In plants, isoprenoid biosynthesis takes place via two pathways: the cytosolic MVA pathway and the plastidic methylerythritol phosphate (MEP) pathway. These two pathways are connected by the metabolic precursor isopentenyl diphosphate (IPP) exchange across the chloroplast membrane (Laule *et al.*, 2003). However, the native cross-talk has remained unclear (Suzuki *et al.*, 2009). The 3-hydroxy-3-methylglutaryl-CoA reductase (HMGR), the enzyme that catalyses the third step of the MVA pathway, has been described as the critical step. Several experiments suggest that HMGR activity is post-transcriptionally and post-translationally regulated in plants (Kirby and Keasling, 2009). The 1-deoxy-D-xylulose-5-phosphate synthase (DXS), the first enzyme of the MEP pathway, plays a major role in the overall regulation of this route (Cordoba *et al.*, 2009).

Experimental data suggest that isoprenoids have an antioxidant function under biotic and abiotic stress *in planta* (Maeda *et al.*, 2006; Abbasi *et al.*, 2007; Matringe *et al.*, 2008; Bajda *et al.*, 2009; Vickers *et al.*, 2009). However, many molecular aspects of this function remain unclear. Interestingly, the condensation reaction of thiolase II from eukaryotes (named AACT) has not been recognized as an important regulatory point in anabolic processes. In *Arabidopsis thaliana*, a member of the AACT family called *acat2*, has been characterized with cytosolic location (Carrie *et al.*, 2007; Ahumada *et al.*, 2008). The expression level of the gene encoding this protein is strongly positively correlated with the expression levels of the HMG-CoA synthase gene, which codes for the MVA pathway enzyme. In *Hevea brasiliensis*, this gene has been identified using yeast as a heterologous expression system (Sando *et al.*, 2008). Heterozygous yeast lacking an allele of the thiolase II gene involved in the MVA pathway and expressing *Hb*AACT1 is able to grow in minimum medium (Sando *et al.*, 2008). In *Helianthus annuus*, the active enzyme form of a thiolase II has been purified (Dyer *et al.*, 2009). This enzyme showed the same sequence similarity with *acat1* (β-oxidation pathway) and *acat2* (MVA pathway) thiolases II from *Arabidopsis thaliana* (Dyer *et al.*, 2009).

It must be taken into account that the genetic study of the biosynthetic cytoplasmic thiolases II is considerably complicated due to several issues, for example (i) the over-expression of these enzymes during the transformation/regeneration procedure is sufficient to avoid generation of stable transgenic plants (Bohmert *et al.*, 2002), (ii) degradative (β-oxidation pathway) and biosynthetic (MVA pathway) thiolases II from dicots have an extremely high nucleotide identity making the RNAi strategy difficult to design with the certainty of ensuring its specificity for plants with non-sequenced genome such as alfalfa, and (iii) the study of the *acat2* homozygous mutant form *Arabidopsis thaliana* (T-DNA mutant) as an alternative approach is non-viable since *Arabidopsis thaliana* has only one copy of *acat2* and the *hmg1-1 hmg2-1* double mutant that completely lacks MVA pathway activity was not obtained (Suzuki *et al.*, 2009). So, given the complexity of the investigation of the thiolases activity by means of genetic studies, the metabolic approaches became even more relevant.

In this study, the role of alfalfa (*Medicago sativa* L.) thiolase II in isoprenoid biosynthesis and in physiological adaptation to low temperature and high salinity was investigated. Alfalfa in one of the most cultivated forage legumes in the world, for that reason it has a great economic importance. Alfalfa is also a particularly abiotic stress-sensitive crop. In the case of osmotic stress, alfalfa yield is reduced about 50% at 100 mM NaCl (10 dS m^{-1}) (Smith, 1994). So, studying putative stress-related genes would have a substantial biotechnological importance. The information presented is especially relevant, not only for biotechnological applications with potential economic impact such as isoprenoid production but also for the improvement of crop performances under stress conditions.

Materials and methods

RNA isolation

Total RNA was extracted by using an RNeasy Plant Mini Kit (QIAGEN Cat# 74903, Germany) following the manufacturer's instructions. Samples of 2 μg total RNA isolated from roots and leaves were reverse-transcribed in a 25 μl reaction using MMLV-RT (Promega Cat# M1701, Madison, WI, USA) following the manufacturer's instructions. For PCR amplification, 1 μl of RT reaction was used. The PCR reactions were carried out in 25 μl with 0.5 μM of each primer (Soto *et al.*, 2008).

Isolation of cDNA encoding the MsAACT1 *gene and plasmid construction*

The *MsAACT1* gene (available at Genbank, accession number GQ890698) was isolated using a pair of primers designed against the main motif of thiolase II (5′-AGAGATGTTTGTATTGTTG-GTGTTGC-3′ and 5′-AGCTCTACAACAAGAGCGGATGCA-CC-3′) and thermal asymmetric interlaced polymerase chain reaction (TAIL-PCR) and a nested set of gene-derived primers (Liu and Whittier, 1995). Full-length cDNA was amplified by PCR

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(5′-TTACAGGAGCTCCACAACAAG-3′ and 5′-ATGGCAGCATCAGACTCCACTATA-3′) and this fragment was cloned into a pGEM-t easy vector. The sequencing reactions were performed at INTA-CICVyA (www.inta.gov.ar/biotec). Sequences were aligned, assembled, and analysed by using Bioedit Sequence Alignment Editor CAP (Hall, 1999). The cDNA sequence was named *MsAACT1* (*M. sativa* acetyl-CoA acetotransferase 1). *MsAACT1* was amplified by PCR using the primers: 5′-CCGAATTCTTACAGGAGCTCCACAACAAG-3′ and 5′-CCGAATTCATGGCAGCATCAGACTCCACTATA-3′ (*Eco*RI restriction sites are underlined). This amplification fragment was digested with *Eco*RI and cloned into the bacterial broad host range expression vector pSJ33 (Jaenecke and Díaz, 1999), producing the new plasmid labelled pINTA2, and afterward introduced in *Pseudomonas* 14-3 and *Escherichia coli*. A second PCR was carried out to amplify a fragment containing the entire *MsAACT1* gene using the primers 5′-CCTCTAGATTACAGGAGCTCCACAACAAG-3′ and 5′-CCGAGCTCATGGCAGCATCAGACTCCACTATA-3′ (*Xba*I and *Sac*I restriction sites are underlined). This amplification fragment was digested with *Xba*I and *Sac*I, and cloned into pBI121 (AF485783). The resulting plasmid was named pMsAACT1.

Plant transformation

The vectors of pMsAACT1 and pBI121 were transformed into alfalfa through the mediation of *Agrobacterium rhizogenes* MSU440 as described previously (Limpens *et al.*, 2004). *A. rhizogenes*-transformed alfalfa roots were excised and transferred to 25 ml MS medium containing 300 μg ml^{-1} cefotaxime in glass flasks. The glass flasks were incubated in the dark at 23 °C for 4 weeks. All transformation events were checked by PCR analysis. Alternatively, plants transformed by *A. rhizogenes* were transferred to soil in a growth room maintained at 23±2 °C and a relative humidity of 80% with a 14 h photoperiod at 50 μE m^{-2} s^{-1}. Transgenic plants were grown for 1, 2 or 3 weeks and then irrigated with 200 mM NaCl for 1 month. Three sets of primer pairs, *virC*for (5′-ATCATTTGTAGCGACT-3′) and *virC*rev (5′-AGCTCAAACCTGCTTC-3′), *AAT*for (5′-CAATTTCGCATCTCATTAAGATCG-3′) and *AAT*rev (5′-ACCACATCCCAAATAAATAAGATTCTAAC-3′) (AAB46610), and 35S*MsAACT1*for (5′-GATGCCTCTGCCGACAGTGGTCCC-3′) and 35S*MsAACT1*rev (5′-TGTCTAGCAGGAGCTTGCCCCAAAT-3′) were evaluated for *virC* from *Agrobacterium rhizogenes* (Sawada *et al.*, 1995), aspartate aminotransferase (AAB46610), and 35S*MsAACT1* (GQ890698) detection by PCR.

Analysis of MsAACT1 expression in alfalfa

Real-time quantitative PCR (qPCR) was performed by using 1 U *Taq* Platinum ADN polymerase (Invitrogen), buffer *Taq* Platinum 10×, 0.25 mM dNTP, 0.4 mM primers, and 2 mM $MgCl_2$ with Opticon2 (MJ Research), according to the manufacturer's instructions. Primers for the real-time qPCR were 5′-ATTGGGCATAAATGACGTTGTTG-3′ and 5′-GGGCAGAAATTCGACGTTCAAAA-3′. Real-time qPCR conditions comprised: 1 cycle at 94 °C for 3 min, 34 cycles of 94 °C for 45 s, 59.1 °C for 1 min, and 72 °C for 1 min. At each cycle, accumulation of PCR products was detected by the reporter dye SybrGreen I (1:50 000, Molecular Probes). The amplification fragment was sequenced and it was identical (100% nucleotide identity) to the *MsAACT1* gene. The expression level of *MsAACT1* was normalized to that of 18S rRNA, which was used as a reference, using primers 5′-GTGACGGGTGACGGAGAATT-3′ and 5′-GACTCAATGAGCCCGGTATTG-3′. The efficiency of primer binding was determined by linear regression by plotting the cycle threshold (C_T) value versus the log of the cDNA dilution (Soto *et al.*, 2010). qPCR experiments were independently performed three times, with comparable results. The three PCR reactions were carried out in duplicate.

Phylogenetic analysis of sequence data

A sequence search was performed using BLASTP tools at (http://www.ncbi.nlm.nih.gov). Protein identity calculations were performed using MatGAT v2.02 (Campanella *et al.*, 2003). Phylogenetic and molecular evolutionary analyses were conducted by using MEGA version 4.0 (Tamura *et al.*, 2007). Protein sequences were aligned using the ClustalW program. Phylogenetic trees were constructed using the Neighbor–Joining (NJ) method with genetic distances computed using the p-distance model and bootstrap analysis of 1000 re-samples and root on midpoint.

PHB and isoprenoid content determinations

To quantify PHB accumulation, cultures were carried out in 0.5 NE_2 medium (Huisman *et al.*, 1992) plus sodium octanoate (0.25% wt/vol), glucose (3% wt/vol) or sodium gluconate (3% wt/vol) at 30 °C for 24 h (Ayub *et al.*, 2006). The PHB content was measured as described earlier by Ayub *et al.* (2004). The content of squalene in roots was determined by the chromatographic method as described previously by Seo *et al.* (2005). For an estimation of pigment content, leaves were ground in 80% acetone. Total carotenoid and total chlorophyll (Chlorophyll *a*+Chlorophyll *b*) contents were determined according to Lichtenthaler (1987).

Enzyme assays

Escherichia coli strain (wild type) and its recombinant strains containing pINTA2 were grown overnight at 30 °C with shaking (250 rpm) in LB medium supplemented with 1 mM isopropyl-β-D-thiogalactopyranoside in 250 ml Erlenmeyer flasks containing 50 ml of medium. Samples of 10 ml culture broth were centrifuged at 5000 *g* and 4 °C for 10 min. The pellets were washed three times with thiolase II buffer (100 mM TRIS-HCl, pH 7.3) and re-suspended in 200 μl of lysis buffer (Roche protease inhibitor cocktail tablet). Crude cell lysates were achieved by five consecutive cycles of freezing in liquid nitrogen followed by thawing at 37 °C. After centrifugation at 14 000 *g* and 4 °C for 20 min, the supernatant was retained for enzyme assays. The total protein concentration of this supernatant was determined by the Bradford method (Bradford, 1976) using bovine serum albumin as standard.

Thiolase activity was assayed using acetoacetyl-CoA and CoA as substrates by measuring the decrease in acetoacetyl-CoA concentration at 303 nm (Inui *et al.*, 2008). Hydroxyacyl-CoA dehydrogenase activity was measured using acetoacetyl-CoA as substrate and NADPH as cofactor. The decrease in NADPH concentration resulting from 3HB-CoA formation from acetoacetyl-CoA was analysed at 340 nm (Schubert *et al.*, 1988). One enzyme unit was defined as the conversion of 1 μmol of substrate to product min^{-1} at 25 °C. Condensation reaction of thiolase was assayed by the method of Senior and Dawes (1973) performed in the presence of 0.13 mM of acetyl-CoA. The preparation of the microsomal fraction was performed as described by Chappell *et al.* (1995). HMGR and thiolase activities *in planta* were measured as described by Mozzicafreddo *et al.* (2010) and Dyer *et al.* (2009), respectively.

Stress experiments in bacteria

Bacteria were exposed to thermal and salinity stresses. The culture medium used was NBG, which consisted of nutrient broth with 3% glucose and 1 mM isopropyl-β-D-thiogalactopyranoside. NBG medium was supplemented with 50 μM glutathione in control experiments. To assay bacterial survival to freezing, 150 μl of exponentially growing (OD_{580} nm=0.5) cell cultures were transferred to 850 μl of NBG medium, cooled down to 4 °C and exposed to –20 °C. The frozen suspension was thawed, mixed using a vortex, and the number of viable cells after different times of exposure to –20 °C was measured by plating aliquots on nutrient agar (NA). To investigate resistance to salinity, 0.5 ml of each diluted culture was added to tubes containing 15 ml of NBG

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medium either with or without 1 M NaCl. Aliquots were taken at specific times and plated in NA to estimate the number of colony forming units. In both stress treatments, survival was expressed as a percentage of the number of colony forming units used as controls.

Plant material, growth conditions, and addition of inhibitors (inhibitor assay)

For *MsAACT1* induction assay, alfalfa seeds were surface-sterilized and sown on MS medium in glass flasks. The glass flasks were incubated for 21 d at 23 °C under a 10/14 h (day/night) photoperiod and 100 μE m^{-2} s^{-1} of light intensity provided by fluorescent lamps. Seedlings were transferred either to fresh MS medium (controls) or to MS medium containing 100 mM NaCl and further incubated at 23 °C or 4 °C in darkness for 5 h. Root and leaf samples were immediately frozen in liquid nitrogen and stored at –80 °C until RNA extractions.

For the inhibitor (mevastatin) assay, alfalfa seeds were surface-sterilized and germinated in a growth chamber at 23 °C, in hydroponic cultures containing vermiculite 100% and MS medium. This solution was replaced with fresh solution every 2 d. Twenty-eight days later, plants were transferred either to fresh MS medium (controls) or to MS medium containing 300 mM NaCl. In some experiments, the culture medium was supplied with the inhibitor and vitamin C (4 mM). Media containing the inhibitors were prepared by adding aliquots from the inhibitor stock solution to a final concentration of 50 μM. Plants were incubated for 16 h in the growth chamber at 23 °C or 4 °C. Finally, survival and antioxidant capacity was evaluated. Antioxidant capability was measured as described previously (Cook *et al.*, 1998) using the Total Antioxidant Assay Kit (Sigma-Aldrich, CS0790).

Results

Identification and phylogenetic analysis of alfalfa thiolase II

To study the function of alfalfa thiolase II, a 1175 bp cDNA fragment was isolated from roots by RT-PCR with a major motif of the thiolase II specific primers. To obtain the full-length DNA sequence encoding this gene, TAIL-PCR was performed using sequence-specific primers. The PCR product was cloned into a pGEM-t easy vector (pINTA1) and sequenced. This cDNA fragment shares 95.6% identity with thiolase II (ACJ84564) from *Medicago truncatula* (Fig. 2), hence it was named *MsAACT1* (*M. sativa* acetoacetyl-CoA thiolase 1). The phylogenetic analysis was restricted to thiolase II genes with high protein identity (>30%). The tree included bacterial thiolases involved in PHB biosynthesis, such as the PhbA protein from *Azotobacter vinelandii*. The thiolases II tree showed a complete congruence with the organismal tree in the three domains of life (Fig. 2). Thus, phylogenetic analyses suggest a common ancestor between bacterial and eukaryotic thiolase II. In addition, the phylogenetic tree showed that *MsAACT1* is the orthologue of the *Arabidopsis thaliana* MVA cytosolic thiolase (BAH19918-AT5G48230-*acat2*) and the *Physcomitrella patens* thiolase II (XP_001773218) and revealed that a gene duplication event occurred in dicots after their divergence from the common ancestor with monocots with a high bootstrap value (99%) (Fig. 2). This duplication event gave rise to the thiolases II of the β-oxidation and MVA pathways in dicots (Fig. 2). The evolutionary framework allows, for example, the classification of the thiolase II from *Helianthus annuus* as a degradative enzyme from the β-oxidation pathway (Fig. 2).

Functional complementation of alfalfa thiolase II in bacteria

In order to characterize the activity of the alfalfa thiolase II, *MsAACT1* was amplified by PCR and ligated into the broad host range vector pSJ33 (pINTA2) for expression in *Escherichia coli* and *Pseudomonas* 14-3. *E. coli* has previously been reported to be a suitable model to evaluate thiolase activity (Tseng *et al.*, 2009). Thiolase II activity in wild-type *E. coli* was almost undetectable (<0.1 U mg^{-1} protein), whereas *E. coli* expressing *MsAACT1* showed a high thiolase II activity (21±2.6 U mg^{-1} protein). To study the condensation activity of *MsAACT1*, *Pseudomonas* 14-3 was used as a heterologous expression system. This strain is a natural thiolase II defective mutant. Complementation assays were performed by growing *Pseudomonas* 14-3 with carbohydrates (glucose and gluconate) as the carbon source. The PHB content was measured by chromatography, as the product of condensation activity of MsAACT1. Table 1 shows that *Pseudomonas* 14-3 regained the ability to accumulate PHB from glucose and gluconate upon expression of alfalfa thiolase II. Taken together, these results strongly suggest that MsAACT1 is a functional thiolase II.

To investigate whether alfalfa thiolase II is involved in abiotic stress tolerance, wild type and recombinant *Pseudomonas* 14-3 stationary cultures growing at 30 °C were transferred to –20 °C (cold stress) or 1 M NaCl (salinity stress). The recombinant bacteria showed a better performance in response to low temperature (Fig. 3A) and high osmolarity (Fig. 3B) than their parental strain. The stress resistance of *Pseudomonas* 14-3 was then analysed in a glutathione-supplemented medium. The reduced compound was able to suppress the lethal effects of abiotic stress (Fig. 3). Thus, heterologous expression of alfalfa thiolase II could be associated with redox homeostasis.

Regulation of MsAACT1 by CoA

To understand the activity of alfalfa thiolase II better, the inhibition of the condensation reaction by CoA was analysed using a recombinant *Escherichia coli* pINTA2 strain. Due to background thiolase II and hydroxyacyl-CoA dehydrogenase activities in wild-type *E. coli* that were almost undetectable (<0.1 U mg^{-1} protein) *E. coli* was a suitable model to study the condensation reaction by MsAACT1 enzyme. As mentioned before, *E. coli* expressing MsAACT1 showed a high thiolase II activity (21±2.6 U mg^{-1} protein) showing that the enzyme is well expressed. The condensation reaction of MsAACT1 in the absence or presence of CoA was then assayed. The results obtained suggest that MsAACT1 is inhibited by CoA (Fig. 4).

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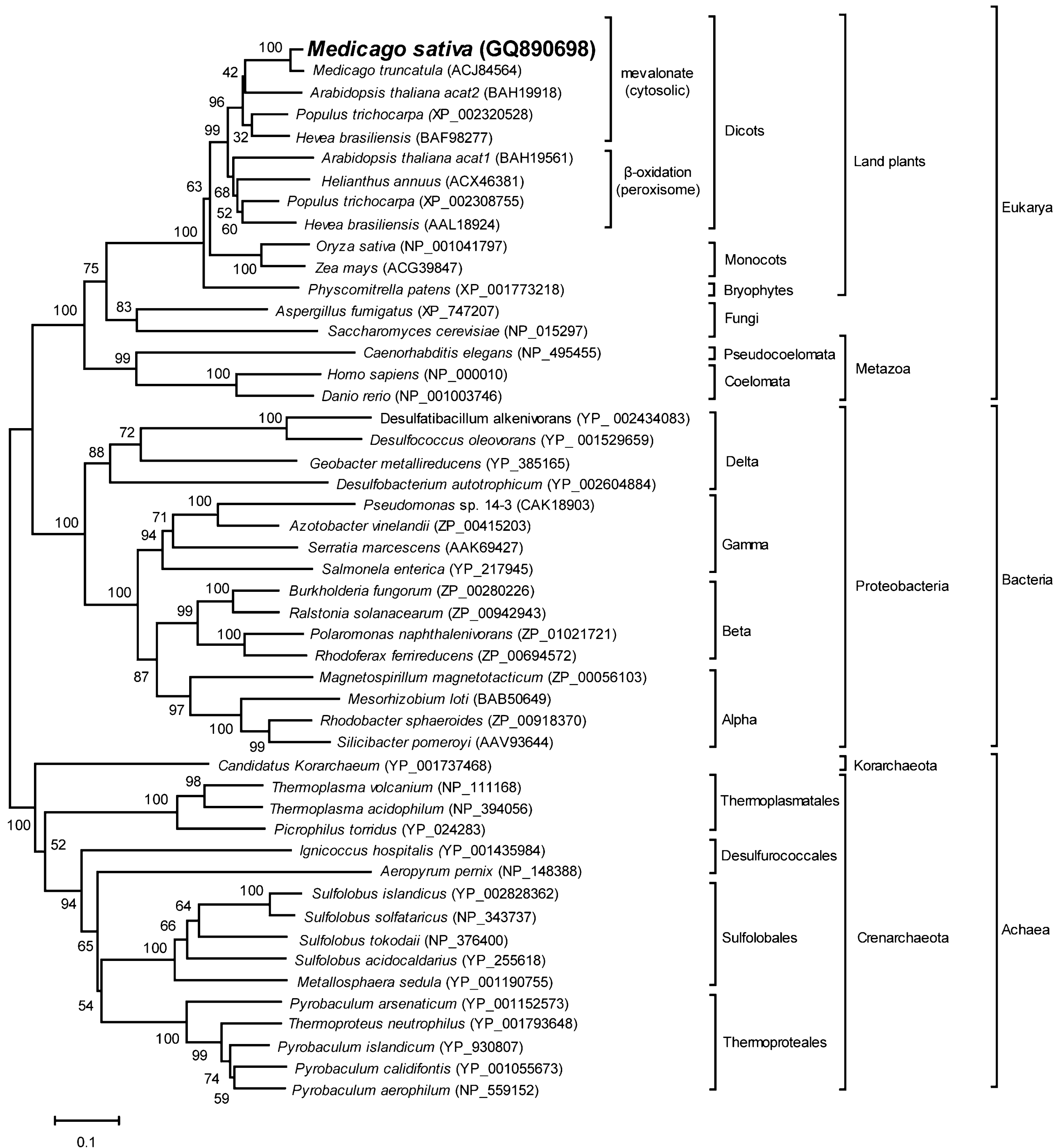

Fig. 2. Phylogenetic tree of thiolase II protein sequences. Functional classification (anabolic-MVA pathway and catabolic β-oxidation pathway) of thiolases is shown. Bootstrap percentages are indicated at the branch points. In all cases, tree topologies were obtained using the NJ method, Minimum evolution and Maximum parsimony methods were identical.

Alfalfa thiolase II expression is enhanced under abiotic stress

Alfalfa is one of the most abiotic stress-sensitive crop species and salinity and low temperature would reduce its production substantially. To evaluate whether alfalfa thiolase II expression is modified under non-lethal salt stress, real-time RT-PCR assays were performed to quantify changes in transcript levels. Total RNA was extracted from 28-d-old wild-type alfalfa seedlings growing in axenic medium. *MsAACT1* transcripts were about 200-fold higher in roots than in leaves when seedlings were grown under control conditions (Fig. 5). Interestingly, after a 5 h treatment with low temperature (4 °C) or salinity (100 mM NaCl), *MsAACT1* expression was increased both in leaves and roots (Fig. 5). Thus, alfalfa thiolase II transcripts are notably up-regulated by abiotic stress.

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Table 1. Production of PHB by wild-type *Pseudomonas* sp. 14-3 and recombinant *Pseudomonas* sp. 14-3 pINTA2

PHB content is expressed as a percentage of total cell dry weight. Values represent media ±SD of triplicate experiments.

Strain	Carbon source	PHB content (wt %)
Pseudomonas sp. 14-3	Glucose	<0.05
	Gluconate	<0.05
Pseudomonas sp. 14-3 pINTA2	Glucose	36±5
	Gluconate	57±4

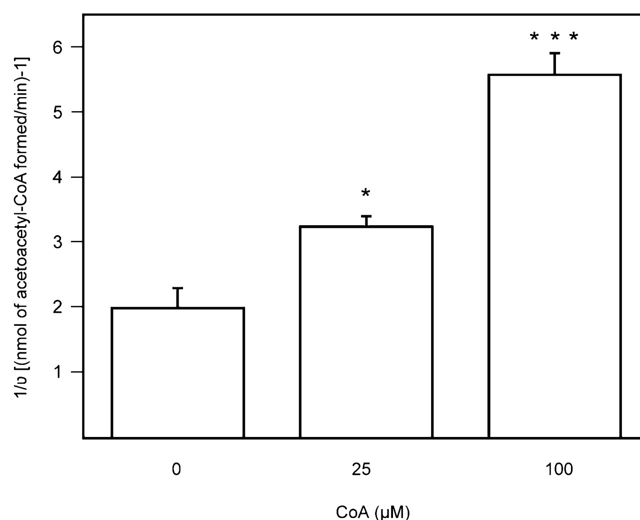

Fig. 4. Effect of CoA on the condensation reaction catalysed by MsAACT1. The reaction mixture was supplemented with 25 μM or 100 μM of CoA. The values are the mean ±SEM of three independent replicates. Significant differences between treatments were calculated using Tukey contrast comparing 25 or 100 μM with the absence of CoA. *P <0.05, ***P <0.001.

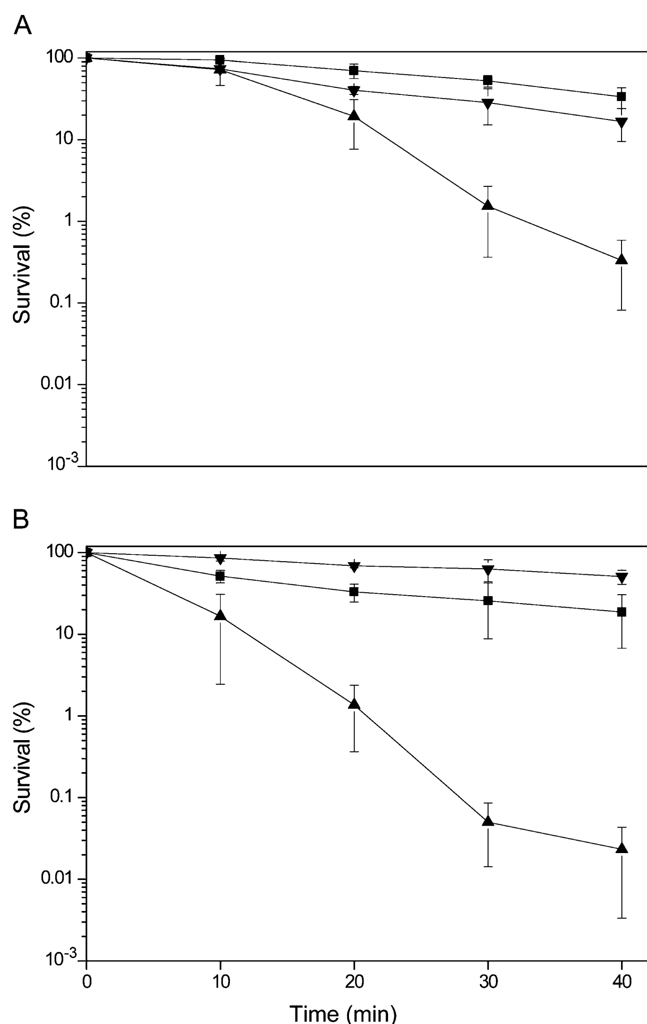

Fig. 3. Behaviour of *Pseudomonas* 14-3 and its recombinant strains expressing thiolases II under abiotic stress. Exponentially growing cells of *Pseudomonas* 14-3 (triangles) and *Pseudomonas* 14-3 pINTA2 (rectangles) strains in NBG medium were shifted to –20 °C (A) or 1 M NaCl (B) for 40 min. Similar experiments were performed with *Pseudomonas* 14-3 in NBG medium supplied with glutathione 50 μM (inverted triangle). Survival is expressed as a percentage of colony forming units (cfu) before the onset of the experiments. Values represent media ±SD of triplicate experiments, n=3 independent experiments.

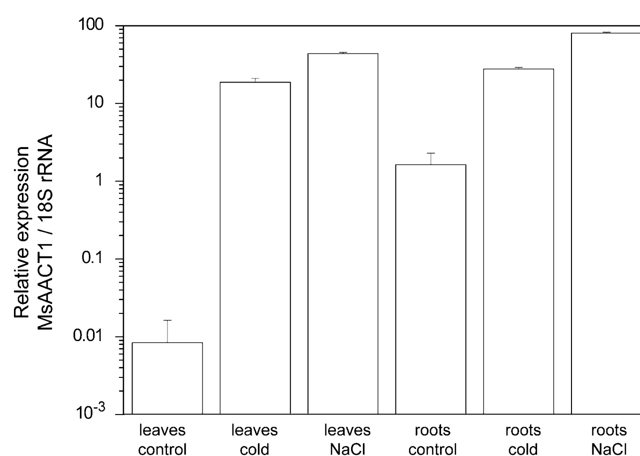

Fig. 5. *MsAACT1* expression pattern. Real-time RT-PCR analysis of *MsAACT1* expression in roots and leaves of alfalfa after treatment with low temperature (4 °C) or salinity (100 mM NaCl) for 5 h. CT18s rRNA values were stable in the tissues examined and used to normalize data. Values represent media ±SEM, n=3 independent experiments.

MVA pathway is involved in alfalfa adaptation to abiotic stress

In order to evaluate the relevance of the MVA pathway *in planta*, an alfalfa-based model system was developed. Alfalfa plants were subjected to mevastatin, a specific inhibitor of the HMGR enzyme. Contrary to thiolase inhibitors based on CoA esters (e.g. 4-bromocrotonyl-CoA), the mevastatin would be taken up intact and transported through the plant. As expected, in the presence of mevastatin (50 μM) the levels of MVA-derived squalene decreased significantly and no change was observed in the levels of chlorophyll, which is synthesized via the plastidial

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MVA-independent pathway (Table 2). Alfalfa plants were then subjected to low temperature (4 °C) or high salinity (300 mM NaCl) for 16 h in hydroponic medium supplied with mevastatin (50 μM) and the reducing compound vitamin C (4 mM). After 7 d of re-acclimation at 23 °C, plants were analysed for their survival ability and/or their vigour. Controls (untreated) and plants treated with mevastatin and vitamin C remained green and turgid, whereas inhibitor-treated alfalfa plants lost turgor and became chlorotic under abiotic stress (Fig. 6). To test whether this increased fitness conferred by the ability to synthesize isoprenoids was associated with its antioxidant activity, total antioxidant capacity (TAC) was evaluated as an indicator of the intracellular redox state. This assay was carried out in plants 16 h after the abiotic stress challenges. The antioxidant content ranged from 2.5–16.7 μmol Troxol equivalent g^{-1} dry dwt (Fig. 7). At low temperature or high salinity, the antioxidant capacity was drastically reduced with inhibitor treatment and the decrease in antioxidant capacity was overcome by hte addition of vitamin C in both stress treatments (Fig. 7). Thus, these results suggest that the biosynthesis of isoprenoids provide plants with a better fitness to abiotic stress and is related to their antioxidant activity.

Table 2. Effect of mevastatin in alfalfa isoprenoid production

Values are average of triplicate experiments ±SD. Significant differences between treatments were calculated using Tukey contrast (**P* <0.05).

Mevastatin (mM)	Compound (mg g^{-1} dry weight)		
	Squalene	Chlorophylls	Carotenoids
0	*0.064±0.003*	1320±141	253±65
50	*0.006±0.005*	1423±165	276±42

Over-expression of alfalfa thiolase II in alfalfa roots increases squalene production and stress tolerance

Alfalfa roots were stably transformed by *Agrobacterium rhizogenes* (Fig. 8A). Gel-electrophoresis of PCR products from transformed and non-transformed root cultures as well as from *Agrobacterium* strains was carried out to show the presence or absence of 35S-*MsAACT1* and the *virC* gene (Fig. 8B). The *virC* gene is present in *Agrobacterium* Ti and Ri plasmids. The amplification of this gene indicates the presence of *Agrobacterium.* 35S-*MsAACT1* PCR products were present in L1, L2, and L3 transgenic root lines but not in non-transformed root cultures, whereas a PCR product corresponding to a fragment of the *virC* gene was absent in all root lines. Thus, roots were not contaminated with *A. rhizogenes* and were actually transformed. As expected, over-expression of *MsAACT1* increases thiolase activity, and any alteration of HMGR activity was detected under stress and control conditions (Fig. 8C, D). Then, the

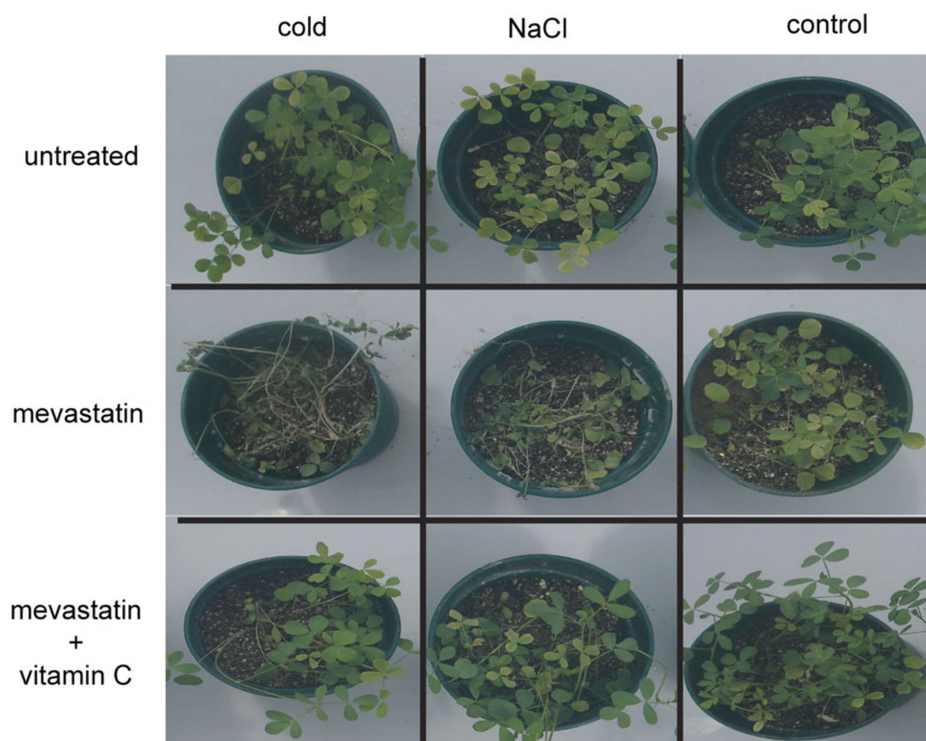

Fig. 6. Effects of inhibitor treatments on alfalfa plants. Phenotypic characteristics of untreated control plants and plants treated with mevastatin (50 μM) and vitamin C (4 mM) during incubation at 4 °C and exposure to 23 °C either with or without NaCl (300 mM). Plants were exposed to abiotic stress for 16 h in hydroponic medium. After 7 d re-acclimation at 23 °C in hydroponic medium without inhibitor and vitamin C, plants were analysed for survival. Each experiment was performed six times and typical results are presented. Mev, mevastatin; vitC, vitamin C.

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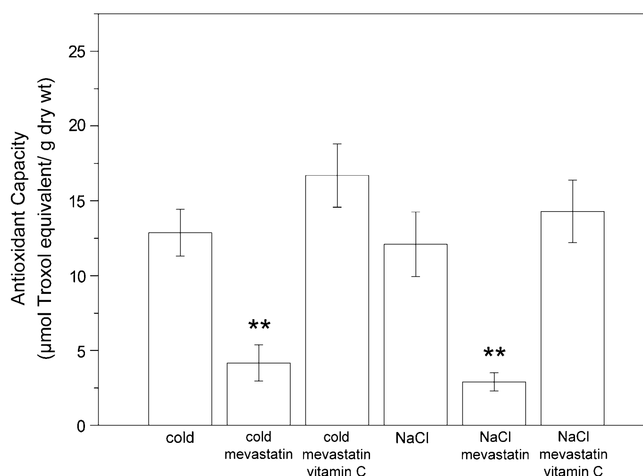

Fig. 7. Total antioxidant capacity of aqueous extracts of alfalfa. Plants were exposed to abiotic stress and supplied with inhibitors and antioxidant (vitamin C) according to Fig. 4. The antioxidant content was measured after exposure to abiotic stress. Values represent media ±SEM of three independent replicates. Significant differences between treatments were calculated using tukey contrast. ***P* <0.001 compared with the wild-type line treated with abiotic stress and mevastatin. Mev, mevastatin; vitC, vitamin C.

squalene levels in wild-type and transformed root lines were analysed by GC-MS. In the absence of salt stress conditions, squalene levels of wild-type and transformed root lines were similar (Fig. 8E). However, over-expressing transgenic roots showed significantly enhanced levels of squalene when they were exposed to saline stress. These results confirmed *in planta*, the thiolysis activity of *MsAACT1* previously observed using bacteria as a heterologous system. In addition, these results demonstrated that *MsAACT1* over-expression does not stimulate an up-regulation of HMGR activity under abiotic stress and suggest a critical function of alfalfa thiolase II in isoprenoid biosynthesis via the mevalonate pathway under abiotic stress adaptation.

In order to evaluate the role of alfalfa thiolase II in the saline stress response *in planta, A. rhizogenes*-transformed plants were transferred from *in vitro* to compost conditions. Mosaic plants were grown for 1, 2 or 3 weeks and then irrigated with 200 mM NaCl for 1 month. The salinity-induced chlorotic phenotype and loss of turgor was notably lower in thiolase II-over-expressing roots lines compared with controls consisting of mosaic plants with roots transformed with the empty vector (Fig. 9). Thus, these results strongly suggest that thiolase II is involved in alfalfa salinity tolerance.

Discussion

In the present work, the role of alfalfa (*Medicago sativa* L.) thiolase II in isoprenoid biosynthesis and in physiological adaptation to low temperature and high salinity were studied. The cDNA was isolated from roots by RT-PCR and named after the corresponding phylogenetic analysis *MsAACT1* (*M. sativa* Acetoacetyl-CoA thiolase 1). The phylogenetic analysis was restricted to thiolase II proteins and, interestingly, the thiolases II tree showed a complete congruence with the organismal tree in the three domains of life (Fig. 2). In previous phylogenetic analyses of thiolase proteins, trees constructed using thiolases I and II from the three domains of life were incongruent with rRNA data (Peretó *et al.*, 2005). Based on these results, it has been argued that high plant thiolases II have a δ-*Proteobacteria* origin (Peretó *et al.*, 2005). However, thiolases I and II have less than 25% of amino acid identity (data not shown), and it is known that the evolutionarily large distances generate problems in the alignment of the sequences analysed producing aberrant phylogenetic trees (Hughes *et al.*, 2005; Phillips *et al.*, 2006). The fact that, in our analysis, it was possible to reconstruct the thiolase II phylogenetic history, not only does it not support the horizontal transfer from δ-*Proteobacteria* hypothesis, but it also suggests that high plant thiolases II have been acquired by vertical transfer. In further support of the vertical origin of high plant thiolases II, this gene is present in *Physcomitrella patens* (Fig. 2); if an ancestor of higher plants had acquired the thiolase II gene by horizontal transfer, then this gene would not be present in Bryophytes.

In addition, the phylogenetic analysis of thiolase II was consistent with rRNA data even within each domain (Fig. 2), demonstrating that orthologous thiolase II assignment is possible in any living organism. This congruent pattern showed that thiolase II from high plants is the orthologue of bacterial thiolase (PhaA) (Fig. 2). This evolutionary finding suggests that, as orthologues, PhaA and MsAACT1 may have the same function.

The thiolase activity of MsAACT1 was confirmed and not only using bacteria as heterologous system but also *in planta* (Table 1; Figs 3, 8C). As expected, for the bacterial thiolase II orthologue, it was found that the expression of alfalfa thiolase II increased abiotic stress resistance in bacteria (Fig. 3) and *in planta* (Fig. 9), these results strongly hint at an important role of *MsAACT1* in abiotic stress tolerance.

The experiments *in planta* also showed that the expression levels of *MsAACT1* were higher in roots than in leaves under optimal growth conditions (Fig. 5). A similar expression pattern had previously been observed for *Arabidopsis thaliana* cytosolic thiolase II (At5g48230, BAH19918) involved in the MVA pathway (Carrie *et al.*, 2007) (www.geneinvestigators.com). Regarding this observation, the constitutive biosynthesis of isoprenoids in roots has been suggested to be part of the plant strategy to interact with rhizosphere bacteria and fungal pathogens (Kirby and Keasling, 2009).

It was reported that the bacterial thiolase II (PhaA) enzyme was inhibited by CoA (Senior and Dawes, 1973). So, given the evolutionary and functional equivalence between thiolase II enzymes mentioned above, it was evaluated whether eukaryotic thiolase II can also be inhibited by CoA. Using *E. coli* as a heterologous expression system, it was shown that the condensation reaction of

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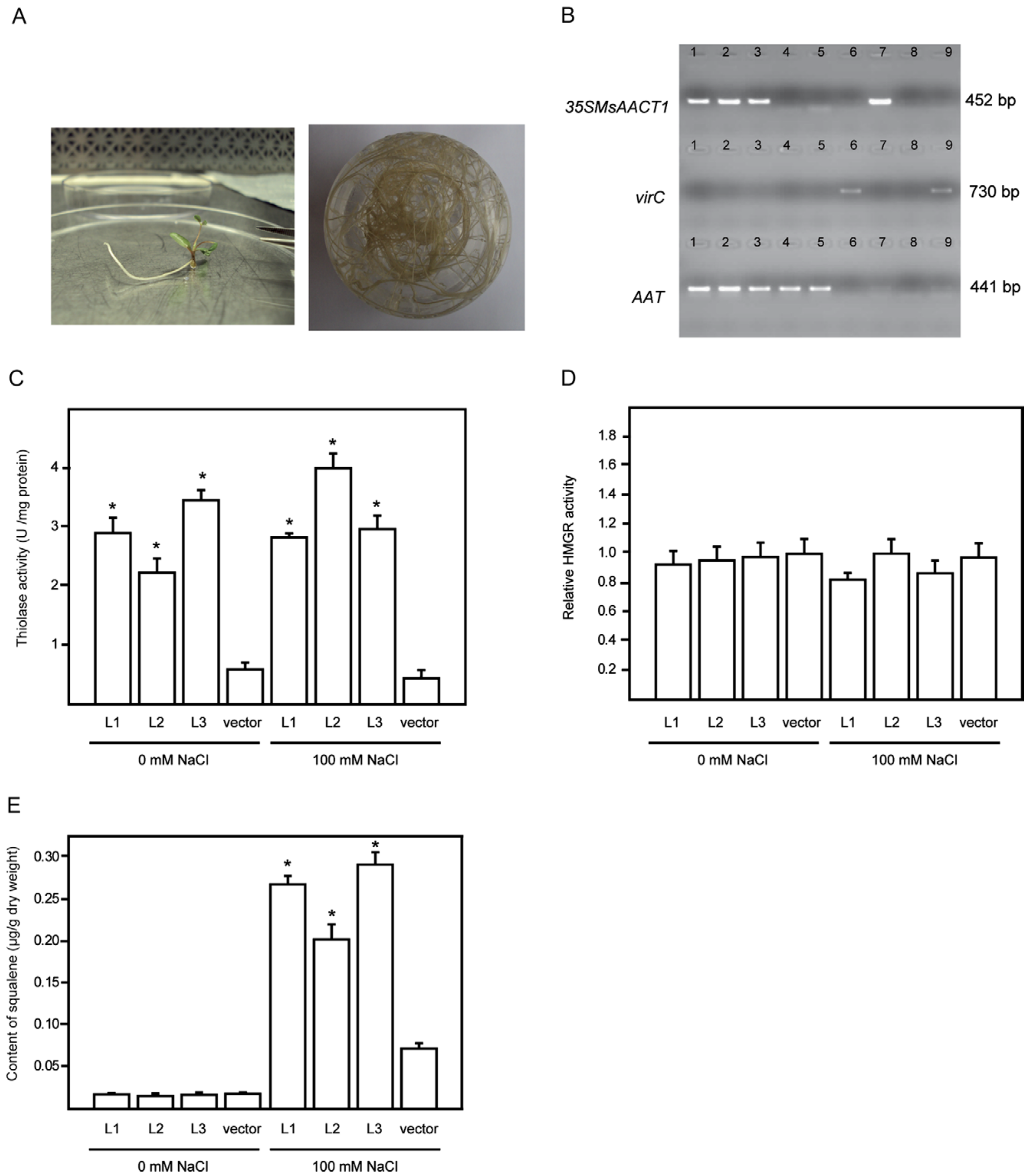

Fig. 8. Characterization of transgenic roots over-expressing the *MsAACT* gene. (A) A representative response of explants 3 weeks after inoculation by *A. rhizogenes* MSU440 (left) and cultured transformed alfalfa roots until squalene quantification (right). (B) PCR of *35SMsAACT1*, *virC*, and *AAT* (alfalfa house-keeping gene) genes of transgenic root lines A4 (1), A6 (2), A22 (3), and vector (4) and wild-type roots (5). *Agrobacterium rhizogenes* MSU440 (6), *A. rhizogenes* MSU440 transformed with *35SMsAACT1* (7), water (8), and *Agrobacterium tumefaciens* LB4404 (9) were used as control. (C) Thiolysis activity *in planta*. (D) HMGR activity *in planta*. (E) The content of squalene in transgenic root lines over-expressing the *MsAACT* gene (L1–L3) and empty vector transgenic roots (vector), in the presence and absence of salinity treatment (100 mM NaCl) for 30 d. In the study of HMGR activity, the expression in empty vector-transformed plants without saline stress was given a value of 1. The values are the mean ±SEM of three or four independent measurements. Significant differences between treatments were calculated using ANOVA test, α <0.05. In thiolysis activity and squalene production analysis, significant differences comparing L1–L3 lines with vector line were calculated using Tukey contrast. **P* <0.05.

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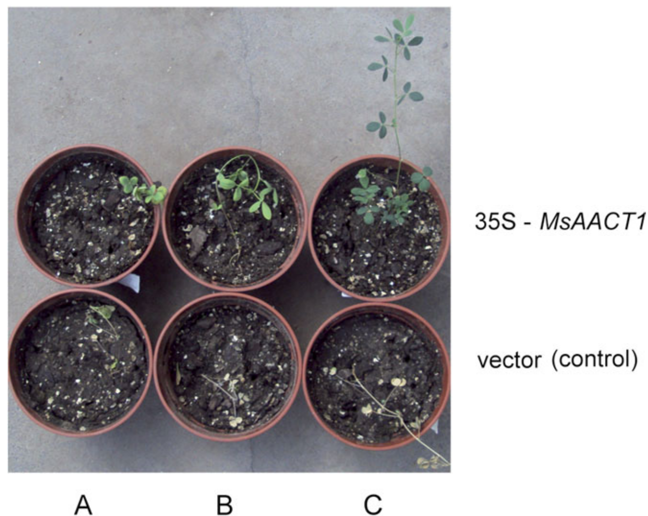

Fig. 9. Role of thiolase II in abiotic stress adaptation. Plants transformed by *A. rhizogenes* maintained at 23±2 °C and a relative humidity of 80% with a 14 h photoperiod at 50 μE m^{-2} s^{-1} for 1 (A), 2 (B) or 3 (C) weeks and were irrigated with 200 mM NaCl for 1 month.

the MsAACT1 enzyme is further inhibited by increasing concentrations of free CoA (Fig. 4). In mevalonate production, CoA is released at three points downstream of the action of thiolase II. Therefore, CoA could act as a negative feedback regulator. Moreover, the CoA pool may depend on the metabolic change that occurs when a plant adapts to abiotic stress.

Abiotic stress limits crop productivity and plays a key role in determining the adaptation of plant species to diverse environments. The present work focuses on two stress factors: salinity and cold. The positive association between the *MsAACT1* gene and the abiotic stress response could be inferred from the induction of the *MsAACT1* gene by low temperature and high salinity in alfalfa found here (Fig. 5). It is interesting to remark that, in bacteria; it was shown that thiolasa II (*pha*A) gene transcription was induced under adverse conditions (Wu *et al.*, 2001; Peralta-Gil *et al.*, 2002).

In addition, using mevastatin, the capability to synthesize isoprenoids via the MVA pathway was observed to be essential for the adaptation to stress conditions in alfalfa (Fig. 6). Vitamin C is not an isoprenoid but plays important roles in scavenging ROS during exposure to abiotic stress in plants (Noctor and Foyer, 1998; Huang *et al.*, 2005). Treatment with vitamin C completely reversed the abiotic stress-sensitive phenotype in the plants exposed to the inhibitor (mevastatin) (Fig. 6). This result suggests that the MVA pathway could be important for producing antioxidant molecules capable of maintaining the redox state and increasing fitness under abiotic stress. This hypothesis was supported by the measurement of antioxidant capacity (Fig. 7). Unfortunately, the effect of the lack of thiolase II in alfalfa could not be evaluated in this work as no silenced roots were obtained. *MsAACT1* silencing was studied using a RNAi approach, three plant expression vectors were constructed and assayed, a 200 bp *MsAACT1* 5′ terminal and the entire *MsAACT1* sequence were cloned in the pHANIBAL vector both in the sense and the antisense orientation and the pHANIBAL empty vector was used as control (Wesley *et al.*, 2001). One hundred transformations for each construct were performed. No viable transformation event for the two MsAACT1 silencing constructs was observed, while 19 transgenic events of the empty vector were obtained. These results probably indicate that the silencing of thiolase II has detrimental effects on transformation/regeneration efficiency. It is not surprising considering that dicots have only one biosynthetic thiolase II (Fig. 2) and that the complete lack of a MVA pathway was previously reported as not being possible to obtain, possibly because MVA is an essential pathway (Suzuki *et al.*, 2009).

Regarding the importance of thiolases II in the isoprenoid biosynthesis pathways, it was mentioned that bacterial thiolase II (PhaA) catalyses the rate-limiting step in the PHB pathway (Kadouri *et al.*, 2005). The expression of the *phaA* gene is induced by stress conditions (Wu *et al.*, 2001; Peralta-Gil *et al.*, 2002) but the production of the PHB polymer ultimately depends on the metabolic status because the PhaA enzyme is inhibited by CoA (Senior and Dawes, 1973). This molecule is released by the tricarboxylic acid (TCA) cycle during optimal growth conditions. Under stress conditions, the TCA cycle is inhibited; producing high acetyl-CoA (thiolase II substrate) and low CoA (thiolase II inhibitor) levels (Senior and Dawes, 1973). Thus, bacterial thiolase II activity is controlled by a universal pathway (the TCA cycle). Two important points are usually considered to explain why this enzyme is the rate-limiting step: (i) the bacterial thiolase II enzyme catalyses the first step in PHB biosynthesis; and (ii) the condensation reaction produced by bacterial thiolase II is highly endergonic and irreversible under the physiological state (Senior and Dawes, 1973; Modis and Wierenga, 1999; Kadouri *et al.*, 2005).

HMGR has been described as the rate-limiting step in the isoprenoid biosynthesis via the mevalonate pathway (Kirby and Keasling, 2009). In fact, the transcriptional levels of the HMGR gene are directly associated with isoprenoid production *in planta* (Chappell *et al.*, 1995; Dai *et al.*, 2010). Recently, an alternative biosynthetic thiolase II from *Streptomyces* sp. strain CL190 (called nphT7) was described as a critical regulatory enzyme in the MVA pathway (Okamura *et al.*, 2010). Co-expression of nphT7 with the HMG-CoA synthase gene (HMGS) and the HMGR gene in *Escherichia coli* showed 3.5-fold higher production of mevalonate than when only the HMGS and HMGR genes were expressed (Okamura *et al.*, 2010). It is shown here that over-expression of the *MsAACT1* gene in transgenic roots increased thiolase activity without altering HMGR activity (Fig. 8). Under abiotic stress, the squalene production and salinity tolerance, were significantly enhanced (Figs 8, 9). Taking these results together and considering the inhibition of MsAACT1 by CoA, its seems to be that, as in bacteria, thiolase II could also be considered as the rate-limiting step in an anabolic process (MVA pathway) under abiotic stress condition in eukaryotes. Further evidence that support the

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hypothesis of AACT (both bacterial and eukaritic) as a limiting step in the MVA pathway are: (i) it is the first step in an anabolic process and (ii) it catalyses the highest endergonic reaction in IPP biosynthesis by the MVA and MEP pathways (Blum, 2009, PhD thesis).

It is interesting to highlight that the increase in thiolase activity in transgenic roots over-expressing the *MsAACT1* gene did not produce an increase in squalene production under optimal growth conditions (Fig. 8C), which may be due to CoA post-translational regulation. So, probably the isoprenoids production in eukaryotes, like in bacteria, ultimately depends on the intracellular CoA concentration as MsAAT1 is inhibited by CoA.

Under this scenario, thiolase II can be a considered to be a conserved key element in the abiotic stress response in highly divergent organisms. Usually, the tricarboxylic acid (TCA) cycle is inhibited under abiotic stress in most organisms such as bacteria, yeast, animals, and plants (Godon *et al.*, 1998; Pomposiello and Demple, 2002; Liu *et al.*, 2005; Baxter *et al.*, 2007; Grant, 2008). In addition, organisms can redirect their metabolic flux from the TCA cycle to the *phaA* and *AACT* pathways to provide reductant for the antioxidant response (Pomposiello and Demple, 2002; Baxter *et al.*, 2007). Moreover, it was shown that the glycolysis and pentose phosphate pathways (PPP), two alternative pathways to oxidize glucose via TCA, have been involved in the biosynthesis of isoprenoids via the MVA pathway (Nomura *et al.*, 2009).

Further studies involving other eukaryotic thiolase II enzymes will show whether the association between biosynthetic thiolase II and abiotic stress resistance found in this work is a general characteristic of eukaryotes. As the over-expression of alfalfa thiolase II possibly leads to a better performance of transgenic cultivars under abiotic stress conditions, the results of this work may have a great impact on biotechnology technics focused on agriculture and livestock.

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