RHIZOPHAGUS INTRARADICES OR ITS ASSOCIATED BACTERIA AFFECT GENE EXPRESSION OF KEY ENZYMES INVOLVED IN THE ROSMARINIC ACID BIOSYNTHETIC PATHWAY OF BASIL

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RUNNING HEAD: Differential gene expression of rosmarinic acid pathway by AMF and bacteria

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Abstract

In recent years arbuscular mycorrhizal fungi (AMF) have been reported to enhance plant biosynthesis of secondary metabolites with health-promoting activities, such as polyphenols, carotenoids, vitamins, anthocyanins, flavonoids and lycopene. In addition, plant growth promoting (PGP) bacteria were shown to modulate the concentration of nutraceutical compounds in different plant species. This study investigated for the first time whether genes encoding key enzymes of the biochemical pathways leading to the production of rosmarinic acid (RA), a bioactive compound showing antioxidant, antibacterial, antiviral and anti-inflammatory properties, were differentially expressed in *Ocimum basilicum* (sweet basil) inoculated with AMF or selected PGP bacteria, by

using quantitative real-time reverse transcription-PCR. *O. basilicum* plants were inoculated with either the AMF species *Rhizophagus intraradices* or a combination of two PGP bacteria isolated from its sporosphere, *Sinorhizobium meliloti* TSA41 and *Streptomyces* sp. W43N. Present data show that the selected PGP bacteria were able to trigger the overexpression of *tyrosine amino-transferase (TAT)*, *hydroxyphenylpyruvate reductase (HPPR)* and *p-coumaroyl shikimate 3'-hydroxylase isoform 1 (CS3'H iso1)* genes, 5.7-fold, 2-fold and 2.4-fold, respectively, in *O. basilicum* leaves. By contrast, inoculation with *R. intraradices* triggered *TAT* upregulation and *HPPR* and *CS3'H iso1* downregulation. Our data suggest that inoculation with the two selected strains of PGP bacteria utilised here could represent a suitable biotechnological tool to be implemented for the production of *O. basilicum* plants with increased levels of key enzymes for the biosynthesis of RA, a compound showing important functional properties as related to human health.

Keywords Arbuscular mycorrhizal fungi; *Rhizophagus intraradices*; Spore associated bacteria; Rosmarinic acid; *Tyrosine amino-transferase* expression levels; *Hydroxyphenylpyruvate reductase* expression levels.

Introduction

In recent years consumers, producers and scientists have shown a rising interest in plant food content in secondary metabolites, called phytochemicals, which play a fundamental role in promoting human health by reducing oxidative damages, modulating detoxifying enzymes, stimulating the immune system, and showing chemopreventive actions (Duthie 2000; Johnson 2007). Phytochemicals may be constitutively expressed and also induced by diverse environmental factors, plant genotype, harvest season, soil quality and fungal/bacterial attacks. In particular, the

establishment of mycorrhizal symbioses by a group of beneficial microorganisms, arbuscular mycorrhizal fungi (AMF), may enhance plant biosynthesis of secondary metabolites with health-promoting activities, such as polyphenols, carotenoids, anthocyanins, flavonoids, phytoestrogens, lycopene, and total antioxidant activity and antiradical power (Giovannetti et al. 2013). AMF live with the roots of most crop plants, including major important agricultural fodder and grain crops, such as cereals, legumes, fruit trees, vegetables and herbal plants. They are obligately biotrophic symbionts belonging to the phylum Glomeromycota and play a key role in plant growth and nutrition, as they enhance the uptake and transfer of soil mineral nutrients, such as phosphorus (P), nitrogen (N), sulfur (S), potassium (K), calcium (Ca), iron (Fe), copper (Cu), and zinc (Zn), by means of a large network of extraradical hyphae spreading from colonized roots into the soil (Smith and Read 2008). Moreover, AMF improve plant tolerance to root pathogens, promoting plant performance and health and reducing the need of chemical fertilizers and pesticides (Toussaint et al. 2008; Sikes et al. 2009).

Experimental studies showed that AMF positively affected the concentration of sugars, organic acids and ascorbic and folic acids in tomato, enhanced strawberry fruit anthocyanin content (Castellanos-Morales et al. 2010), increased antioxidant activity and quercetin concentrations in onion bulbs, and favoured the accumulation of secondary metabolites (carotenoids, phenolics), vitamins (ascorbate, tocopherol) and anthocyanins in lettuce leaves (Baslam et al. 2011; 2013). Other works reported a higher nutraceutical value in mycorrhizal globe artichoke and tomato, currently considered as functional foods and largely cultivated for human consumption (Ceccarelli et al. 2010; Giovannetti et al. 2012), suggesting that AMF inoculation may represent a suitable biotechnological tool to be implemented in agrifood chains aimed at producing safe and healthy food. Moreover, several mycorrhizal medicinal and aromatic plants showed higher accumulation of compounds with therapeutic value, compared with controls, *e.g*. *Foeniculum vulgare* seeds contained higher concentrations of essential oils (+62.5 %), *Echinacea purpurea* produced higher

levels of caffeic acid derivatives, alkylamides and terpenes (up to 30 times) (Gualandi 2010), *Hypericum perforatum* produced higher shoot levels of the anthraquinone derivatives hypericin and pseudohypericin (Zubek et al. 2012).

Some authors reported that selected plant growth promoting (PGP) bacteria were able to modulate the concentration of nutraceutical compounds in different inoculated plant species (Ordookhani and Zare 2011; Lingua et al. 2013; Bona et al. 2014). In a recent study (Battini et al. 2016) we reported the isolation of bacteria strictly associated with the spores of the AMF species *Rhizophagus intraradices*, showing multiple functional PGP traits, *i.e*. P solubilization from phytate, siderophore and indol acetic acid production, mineral P solubilization, and nitrogen fixation, which make them suitable candidates as biofertilizers and bioenhancers. So far, only a few studies investigated the mechanisms by which AMF or PGP bacteria modulate plant secondary metabolism and changes in the production of phytochemicals with health-promoting activity (Harrison and Dixon 1993; Blilou et al. 2000; Walter et al. 2000).

Ocimum basilicum (sweet basil), a source of natural antioxidants and flavonoids traditionally used for its pharmaceutical properties (Bais et al. 2002), accumulated enhanced concentrations of essential oils in shoots and leaves, when inoculated with different AMF species (Copetta et al. 2006; Rasouli-Sadaghiani et al. 2010). Other authors reported that increased levels of phenolic compounds, such as caffeic acid (CA) and rosmarinic acid (an ester of caffeic acid and 3,4-dihydroxyphenyllactic acid; RA), were triggered by specific AMF species (Toussaint et al. 2007), suggesting AMF inoculation as a natural way of producing basil plants with increased concentrations of RA, a bioactive compound showing antioxidant, antibacterial, antiviral and anti-inflammatory properties (Petersen and Simmonds 2003). Nevertheless, no studies investigated whether genes encoding key enzymes of the biochemical pathways leading to the production of RA are differentially expressed in *O. basilicum* inoculated with AMF or PGP bacteria.

Here, we inoculated *O. basilicum* plants with either the AMF species *R. intraradices* or a

combination of two PGP bacteria isolated from its sporosphere, *Sinorhizobium meliloti* TSA41 and *Streptomyces* sp. W43N, with the aim of assessing the expression of genes encoding for key enzymes involved in RA biosynthesis. As RA biosynthesis encompasses two biochemical pathways, the tyrosine-derived branch pathway and the phenylpropanoid branch (Fig. 1) (Petersen and Simmonds 2003), we investigated the expression levels of *tyrosine amino-transferase* (*TAT*) and *hydroxyphenylpyruvate reductase* (*HPPR*) in the tyrosine-derived pathway, plus *phenylalanine ammonia-lyase* (*PAL*) in the phenylpropanoid pathway by using quantitative real-time reverse transcription-PCR (qRT-PCR). In addition, we analysed the expression of *p-coumaroyl shikimate 3'-hydroxylase* (*CS3'H*), a gene encoding 3'-hydroxylation of 4-coumaroyl-4'-hydroxyphenyllactate, involved in one of the final steps of RA biosynthetic pathway and required to complete its biosynthesis (Petersen 2013).

Materials and methods

Plant, microorganisms and experimental conditions

Seeds of *O. basilicum* L. cv. "Tigullio" (a commercial cultivar of sweet basil purchased from a seed company, Franchi Sementi Spa, Bergamo, Italy) were germinated in plastic pots filled with a turf substrate (Hochmoor Hortus, TERFLOR Capriolo BS, Italy), which was not sterilised as a preliminary experiment showed the absence of naturally occurring AMF. In mycorrhizal treatments, each plant was inoculated with 20 % (*v/v*) of crude inoculum (mycorrhizal roots and soil containing spores and extraradical mycelium) of the AM fungal species *R. intraradices* (N.C. Schenck & G.S. Sm.) C. Walker & Schußler (formerly known as *Glomus intraradices*) (isolate IMA6), obtained from *Trifolium alexandrinum* and *Medicago sativa* pot cultures maintained in the collection of the Microbiology Labs of the Department of Agricultural, Food and Environment, University of Pisa,

Italy (International Microbial Archives, IMA). It is important to mention that the AMF inoculum encompassed low, natural levels of bacterial communities living tightly associated with their spores (5-23 CFU spore^{-1}) (Battini et al. 2016).

In bacterial treatments, each plant was inoculated with two bacterial strains, previously isolated from *R. intraradices* IMA6 spores (Battini et al. 2016), *S. meliloti* TSA41 and *Streptomyces* sp. W43N, selected for their PGP properties, such as the production of indole acetic acid (IAA) and siderophores, phosphatase-solubilizing and phytase-mineralizing activities. Each bacterial strain was cultured on Tryptic Soy Broth (TSB, Difco, NJ, USA) at 28 °C for 24 h. Bacterial cells were centrifuged (7800 rpm 10 min) and suspended in sterile physiological saline solution (0.9 % NaCl), washed twice and finally suspended in sterile physiological saline solution. For each strain, bacterial density of the suspension was assessed using a Thoma cell chamber and adjusted to inoculate plants at sowing with 10^6 CFU g^{-1}.

Control and bacteria-treated plants received a mock inoculum produced by sterilizing the appropriate amount of mycorrhizal inoculum. All pots received 2 mL of a filtrate, obtained by sieving the mycorrhizal inoculum through a 50 μm pore diameter sieve and a Whatman paper no. 1 (Whatman International Ltd, Maidstone, Kent, UK), to ensure common microflora for all treatments.

The experiment consisted of three treatment groups: control (with low, natural levels of mycorrhizosphere bacteria, including the two inoculant strains), AMF-inoculated (similar to the controls but with the addition of AMF) and bacterial-inoculated (no AMF inoculation, with high levels of the two inoculant bacterial strains). A total of 5 replicate plants were set up for each treatment, giving a total of 15 pots. Plants were grown in a glasshouse, under ambient natural light and temperature conditions and supplied with tap water as needed. In addition, they received weekly fertilization of Hoagland's solution with half strength of P (10 mL per pot). After 42 days' growth, basil plants were harvested and processed.

Mycorrhizal colonization

At harvest each plant root system was extracted from soil and carefully washed with tap water, cut into 5 cm pieces, cleared with 10 % KOH in water bath at 80 °C for 15 min, neutralized in 2 % aqueous HCl, and stained with 0.05 % Trypan blue in lactic acid. Percentage of colonised root length was assessed on each root sample under a dissecting microscope (Wild, Leica, Milano, Italy) at ×25 or ×40 magnification, by the gridline intersect method (Giovannetti and Mosse 1980). Colonised roots were selected, mounted on microscope slides and observed under a Reichert-Jung (Wien, Austria) Polyvar light microscope to detect intraradical fungal structures.

Isolation of *18S ribosomal* and *ubiquitin* orthologous genes

In order to design an *18S ribosomal* gene for use as a housekeeping gene, PCR primers were constructed on the homologous regions of *18S rDNA* sequences selected after BLAST analysis, using as query partial *18S ribosomal RNA* gene of *Schizonepeta tenuifolia*, *Solenostemon scutellarioides* and *Rosmarinus officinalis* with GenBank acc. nos. JN802671, EU019244 and JX974579.1 respectively. After CLUSTALW multi-alignment, primers (F: 5'-CAACCATAAACGATGCCGACCAG-3'; R: 5'-CCACCAACTAAGAACGGCCATGC-3') were used to amplify by RT-PCR the orthologous sequences of *O. basilicum*. The product was sequenced and the *O. basilicum 18S rDNA* sequence analysis showed 100% homology with the three Lamiaceae species considered. Gene-specific primers were then designed on the obtained *O. basilicum* sequences - deposited in GenBank with the accession numbers LN999821 - for the subsequent qRT-PCR (Table 1). The primers used to design housekeeping gene *ubiquitin* (UbiquitinKF: 5'-ACCACGGAGACGGAGGACAAG-3'; UbiquitinKR: 5'-

ACCCTCACGGGGAAGACCATC-3'), were previously reported for *Salvia miltiorrhiza* (Song and Li 2015). The RT-PCR product obtained using the above primers was sequenced and the sequences corresponding to *ubiquitin* in *O. basilicum* were deposited in GenBank with the accession numbers LN999820. Gene-specific primers were then designed on the obtained *O. basilicum* sequences for the subsequent qRT-PCR (Table 1).

qRT-PCR primers design

The primers used for detecting *TAT*, *HPPR*, *PAL* and *CS3'H*, gene expression were designed from known nucleotide sequences of *O. basilicum* available in GenBank (Table 1). The primer pairs were designed from nucleotide sequences (150 bp maximum length, optimal Tm at 60 °C, GC % between 20 % and 80 %) using Primer3 software (Applied Biosystems).

For *p-coumaroyl shikimate 3'-hydroxylase* (*CS3'H*) we designed primers for the identification of isoforms after Multiple Sequence Alignment of *CS3'H isoform 1* (GenBank accession nos AY082611) and *CS3'H isoform 2* (GenBank accession nos AY082612) using CLUSTALW. Primers were constructed on heterologous region specific for discriminating the two isoforms: *CS3'H iso1* e *CS3'H iso2* (COU1 e COU2 respectively). Another pair of primers (COU) able to amplify both isoforms (*CS3'H iso1 + CS3'H iso2*) was constructed on the homologous regions (Table 1).

Quantitative analysis of gene transcription

After 42 days of plant cultivation, total RNA was extracted from 100 mg of fresh leaves using RNeasy Plant Mini Kit (Quiagen) following manufacturer's instructions. The concentration of each RNA sample was measured using a WPA biowave DNA spectrophotometer (Biochrom Ltd.,

Cambridge, England) and their integrity was evaluated by agarose gel electrophoresis. The RNA samples were treated with Amplification Grade DNase I (Sigma-Aldrich) and reverse-transcribed into cDNA (400 ng per sample) using iScript cDNA synthesis kit (BioRad, Hercules, CA, USA). The synthesized cDNA was used for quantitative real time polymerase chain reaction (qRT-PCR) using gene specific primer pairs (Table 1). In our study *18S rRNA*, *actin* (Rather et al. 2015) and *ubiquitin* were selected as housekeeping genes. Although all the endogenous control genes tested exhibited stable expression among the different treatments (inoculated *vs.* uninoculated plant), among the three reference genes evaluated, *18S rRNA* was chosen to normalize gene expression data for its high transcriptional stability.

qRT-PCR reactions (20 µL) were carried out with 10 ng of cDNA, 250 nM primers, and 1x Fast SYBR Green Master Mix (Applied Biosystems, Foster City,CA, USA) following the manufacturer's instructions. PCR were run in a StepOne real-time PCR System (Applied Biosystems, Foster City,CA, USA) by using the recommended thermal-cycling conditions (hold 95 °C, 20 s; 40 cycles 95 °C, 3 s; 60 °C, 30 s). Before performing the relative expression calculation, the performance of each amplification was checked to ensure maximum specificity and efficiency. Primer specificity and the absence of primer-dimer formation during qRT-PCR analysis was indicated in each sample by the presence of a single peak in the dissociation (melt) curve at the end of the amplification program. Amplification efficiency, linearity and working range were determined by linear regression analysis of serial dilutions of cDNA. The amplifications of the target genes and the endogenous controls were run using three biological replicates (selecting plants showing identical growth performance among the five replicates), each with three technical replicates and were analyzed on the same plate in separate tubes. The relative abundance of transcripts was calculated by using the $2^{-\Delta\Delta Ct}$ method (Livak and Schamittgen 2001). Before the quantification, a validation experiment was performed to ensure that the amplification efficiencies of the target and reference genes were approximately equal.

Statistical analyses

Gene expression data were analysed by one-way analysis of variance (ANOVA) with treatment differences among means tested at $p<0.05$ by Tukey post-hoc test. Before statistical analysis, the Shapiro–Wilk and Levene's tests were applied to check for normality and homoscedasticity. All statistical analyses were performed on SPSS 20.0 software (IBM Corp., Armon, NY Inc, USA).

Results

Mycorrhizal colonisation

At harvest, *R. intraradices* successfully established mycorrhizal symbioses with basil plants, showing percentages of colonised root length ranging from 14.3 to 37.9 %. No colonisation was observed in control and bacteria-inoculated plants.

Transcriptional expression of genes related to RA biosynthesis

To gain further insight into the effect of AMF and PGP bacteria on *O. basilicum* secondary metabolism, we evaluated the expression of genes encoding key enzymes involved in RA biosynthesis (*TAT, HPPR, PAL* and *CS3'H*) using qRT-PCR (Fig. 1). qRT-PCR analysis revealed that both AMF and PGP bacteria significantly up-regulated the expression level of *TAT*, the first gene within the tyrosine-branch pathway. Specifically, taking into account that uninoculated plants were used as the calibrator sample (control sample), *TAT* expression levels increased 5.7 and 1.7-fold in plants inoculated with PGP bacteria and *R. intraradices*, respectively (Fig. 2a, b). Transcript

levels of *HPPR*, which encode the enzyme acting in the tyrosine pathway, downstream of *TAT*, were up-regulated 2-fold in leaves produced by plants inoculated with bacteria, while in mycorrhizal plants were down-regulated (Fig. 2a, c). The expression level of *PAL*, the first gene in the phenylpropanoid branch pathway, was not affected by both inoculation treatments (Fig. 3a, b).

In this study, we also evaluated the expression pattern of *CS3'H*, a gene directly linked to the accumulation of RA. *CS3'H* increased its expression in bacterial treatment and decreased in mycorrhizal treatment (1.5-fold and 0.8-fold, respectively); however, the differences were not statistically significant (Fig. 4a, b). In order to discriminate between the *CS3'H* isoforms, *CS3'H iso1* and *CS3'H iso2*, and to monitor their isoform-specific expression, specific primers were designed against variable regions of the gene. To verify the specificity of each primer set, the expected amplification products were confirmed on an agarose gel and sequenced (data not shown). *CS3'H iso1* transcript levels were significantly upregulated (2.4-fold) and downregulated (0.7-fold) in leaves of basil plants inoculated with PGP bacteria and *R. intraradices*, respectively (Fig. 4a, c). In contrast to the expression pattern of *CS3'H iso1, CS3'H iso2* showed consistent expression levels in both treatments (Fig. 4a, d).

Discussion

To the best of our knowledge, this is the first study showing the effects of AMF and their associated bacteria on the expression levels of transcripts encoding for key enzymes involved in RA biosynthetic pathways. Here we provided evidence that inoculation with the AM fungus *R. intraradices* IMA6 or the PGP bacteria *Sinorhizobium meliloti* TSA41 and *Streptomyces* sp. W43N, differentially modulate the expression levels of *TAT*, *HPPR*, *CS3'H* and *CS3'H iso1* in *O. basilicum* leaves.

Overexpression of *TAT*, the first enzyme of tyrosine-derived metabolic pathway, was detected in leaves of *O. basilicum* inoculated with either the AMF *R. intraradices* or PGP bacteria. Previous works carried out on *S. miltiorrhiza* showed that overexpression of *TAT* was significantly correlated with RA biosynthesis in different developmental stages, such as seedlings, flowering and late growth stage (Song et al. 2015). Consistent results were also found in *Perilla frutescens*, where overexpression of *TAT* led to increased RA yield, directly linked to *TAT* up-regulation (Lu et al. 2013).

HPPR catalyses the second reaction of tyrosine-derived pathway, encoding the enzyme transaminating 4-hydroxyphenylpyruvate to 4-hydroxyphenyllactate. In our experiments, *HPPR* transcriptional activity was enhanced in basil plants inoculated with PGP bacteria, while it was downregulated in mycorrhizal plants. *HPPR* gene, along with *TAT*, significantly contributes to RA biosynthesis. Previous studies, using *S. miltiorrhiza* transgenic hairy root cultures, showed that RA production was enhanced by increasing, either separately or simultaneously, the expression of *TAT* and *HPPR* (Xiao et al. 2011).

PAL, a fundamental gene in the biosynthesis of secondary metabolites, is the entry point in the phenylpropanoid pathway encoding enzymes that catalyse the deamination of phenylalanine to trans-cinnamic acid (Zhang and Liu 2015). *PAL* transcriptional activation represents the early response to biotic and abiotic stresses, leading to the accumulation of phenolic compounds, including RA (Song et al. 2009; Zhang and Liu 2015) and it is rapid and transient, reaching the maximum levels within few hours/days after elicitation, followed by a return toward normal control levels (Song and Wang 2009; Cabanás et al. 2014; Sadat Ejtahed et al. 2015). In this work, the expression levels of *PAL* in mycorrhizal and PGP bacterial treatments were not significantly different from uninoculated controls, possibly depending on the experimental design (harvest time 42 days after inoculation). Accordingly, previous works showed that 21 days after inoculation the expression levels of *PAL* in *Petunia hybrida* roots were not affected by the AM fungus

Funneliformis mosseae when compared with uninoculated controls (Hayek et al. 2014). Similar results were also observed in *Rubus* sp. where inoculation with *Pseudomonas fluorescens* N21.4 did not upregulate *PAL* expression levels in different fruit ripening stages (Garcia-Seco et al. 2015).

The final step of the biosynthetic pathway leading to RA involves the introduction of hydroxyl groups in meta-position of phenylpropanoid-derived compounds. Such a reaction is catalysed by enzymes belonging to the CYP98As cytochrome P450 family in different RA producing plants, *i.e. Lithospermum erythrorhizon* (Boraginaceae) and *Coleus blumei* (Lamiaceae) (Eberle et al. 2009; Ehlting et al. 2006; Matsuno et al. 2002; Petersen et al. 2009). In *O. basilicum*, CYP98A13 isoforms (CYP98A13v1 and CYP98A13v2), encoded by *CS3'H* genes, were shown to produce RA from p-coumaroyl-4'-hydroxyphenyllactate (Gang et al. 2002). In this work, the expression levels of *CS3'H iso2* were not affected by the two inoculation treatments. By contrast, the isoform *CS3'H iso1* was significantly overexpressed ($p<0.001$) in plants inoculated with PGP bacteria, suggesting that such an isoform was the main responsible for the differences in *CS3'H* expression found between PGP and AMF inoculated treatments.

In conclusion, this work shows for the first time that the inoculation with conventional high doses of PGP bacteria *Sinorhizobium meliloti* TSA41 and *Streptomyces* sp. W43N were able to trigger the overexpression of *TAT*, *HPPR*, and *CS3'H iso1* genes, encoding for the key enzymes of RA biosynthetic pathways tyrosine amino-transferase, hydroxyphenylpyruvate reductase and p-coumaroyl shikimate 3'-hydroxylase in *O. basilicum* leaves. By contrast, inoculation with the AM fungus *R. intraradices* IMA6 triggered the upregulation of *TAT* and the downregulation of *HPPR* and *CS3'H iso1*. Such downregulation is consistent with previous data showing lower levels of RA in basil plants inoculated with *R. intraradices*, compared with increased levels triggered by inoculation with *Funneliformis caledonius* (formerly *Glomus caledonium*) and *F. mosseae* (Touissant et al. 2007). Our data suggest that, although mycorrhizosphere bacteria occurring in all three treatments might have had the ability to modulate the expression of the relevant genes, their

concentrations and activity were greatly lower than the inoculant strains. Accordingly, the inoculation with the two selected strains of PGP bacteria utilised here could represent an efficient biotechnological tool for the production of *O. basilicum* plants with increased levels of key enzymes for the biosynthesis of RA, a bioactive compound showing important functional properties as related to human health.

Acknowledgements

This work was funded by a University of Pisa grant (PRA, Progetti di Ricerca di Ateneo).

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Table 1. List of *Ocimum basilicum* specific primer sequences used in qRT-PCR assays.

Gene	Primer sequence	Gene sequence accession number
18S rDNA	F: 5'-GGCGGATGTTGCTTTTAGGA-3'	LN999821
	R: 5'-TTCAGCCTTGCGACCATACTC-3'	
ubiquitin	F: 5'-GGTGGACTCCTTCTGGATGTTG-3'	LN999820
	R: 5'-GGAAGCAGTTGGAGGATGGA-3'	
β-actin	F: 5'-CCGTGGAGAAGAGCTACGAG-3'	Rather et al. 2015
	R: 5'-TCACACTTCATGATGGAGTTGTAGG-3'	
PAL	F: 5'-ATCCTACATTGCTGGTGTCCTCTT-3'	AB436791
	R: 5'-GCCTGGCCCGCATCTAG-3'	
TAT	F: 5'-TGACCCAAAGTTTATTGAGC-3'	KJ004760
	R: 5'-TGAATGTAGCAGGACCTCCA-3'	
HPPR	F: 5'-GCGAGCACAGGCATTTGA-3'	KJ004761
	R: 5'-TGTTGGGTTTCTTGGATCTTGAG-3'	
CS3'H	F: 5'-GATCCGCCGCCAAGTTC-3'	AY082611/12
	R: 5'-CCCATAATCCGCCCAAATC-3'	
CS3'H iso1	F: 5'-CTCCTTCCCCTCCTCCTTC-3'	AY082611
	R: 5'-GCGAAGGCGGTAATAGAGGT-3'	
CS3'H iso2	F: 5'-TCCTCCTCCTTCTCCTCCTC-3'	AY082612
	R: 5'-GAAGCGAAGGCGGTAATAGA-3'	

FIGURE LEGEND

Fig. 1 Schematic view of rosmaric acid biosynthetic pathway. PAL, phenylalanine ammonialyase; C4H, cinnamate 4-hydroxylase; 4CL, 4-coumarate: coenzyme A ligase; TAT, tyrosine aminotransferase; HPPR, hydroxyphenylpyruvate reductase; RAS, rosmarinic acid synthase, CS3'H, coumaroyl shikimate 3'-hydroxylase (modified from Petersen, 2013)

Fig. 2 (A) Tyrosine-derived pathway of rosmaric acid biosynthesis, (B) expression levels of *tyrosine amino-transferase* (*TAT*) and (C) *hydroxyphenylpyruvate reductase* (*HPPR*) genes in leaves of *Ocimum basilicum* cv "Tigullio", as affected by inoculation with the arbuscular mycorrhizal fungus *Rhizophagus intraradices* IMA6 (AMF) and the bacteria *Sinorhizobium meliloti* TSA41 and *Streptomyces* sp. W43N (Bacteria). Data are presented as means ± standard error (SE) of the means (n=3). Bars topped by the same letter do not differ at $P \leq 0.05$ by Tukey's HSD test.

Fig. 3 (A) Phenylpropanoid pathway of rosmaric acid biosynthesis (first step) and (B) expression levels of *phenylalanine ammonia-lyase* (*PAL*) gene in leaves of *Ocimum basilicum* cv "Tigullio", as affected by inoculation with the arbuscular mycorrhizal fungus *Rhizophagus intraradices* IMA6 (AMF) and the bacteria *Sinorhizobium meliloti* TSA41 and *Streptomyces* sp. W43N (Bacteria). Data are presented as means ± SE (n=3). Bars topped by the same letter do not differ at $P \leq 0.05$ by Tukey's HSD test.

Fig. 4 (A) Final step of rosmaric acid biosynthesis, (B) expression levels of *p-coumaroyl shikimate 3'-hydroxylase* (*CS3'H*), (C) *p-coumaroyl shikimate 3'-hydroxylase isoform 1* (*CS3'H iso1*) and (D) *p-coumaroyl shikimate 3'-hydroxylase isoform 2* (*CS3'H iso2*) genes in leaves of *Ocimum basilicum* cv "Tigullio", as affected by inoculation with the arbuscular mycorrhizal fungus *Rhizophagus intraradices* IMA6 (AMF) and the bacteria *Sinorhizobium meliloti* TSA41 and *Streptomyces* sp. W43N (Bacteria). Data are presented as means ± SE (n=3). Bars topped by the same letter do not differ at $P \leq 0.05$ by Tukey's HSD test.

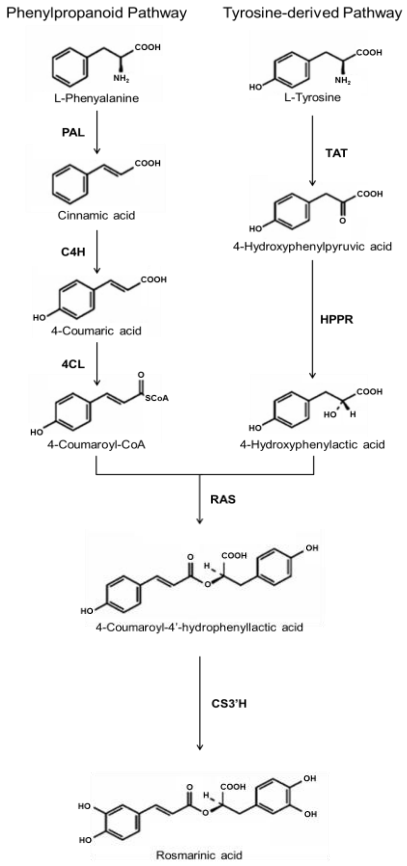
Phenylpropanoid Pathway
Tyrosine-derived Pathway
L-Phenyalanine
L-Tyrosine
PAL
TAT
Cinnamic acid
4-Hydroxyphenylpyruvic acid
C4H
HPPR
4-Coumaric acid
4CL
4-Coumaroyl-CoA
4-Hydroxyphenylactic acid
RAS
4-Coumaroyl-4'-hydrophenyllactic acid
CS3'H
Rosmarinic acid

Fig. 1

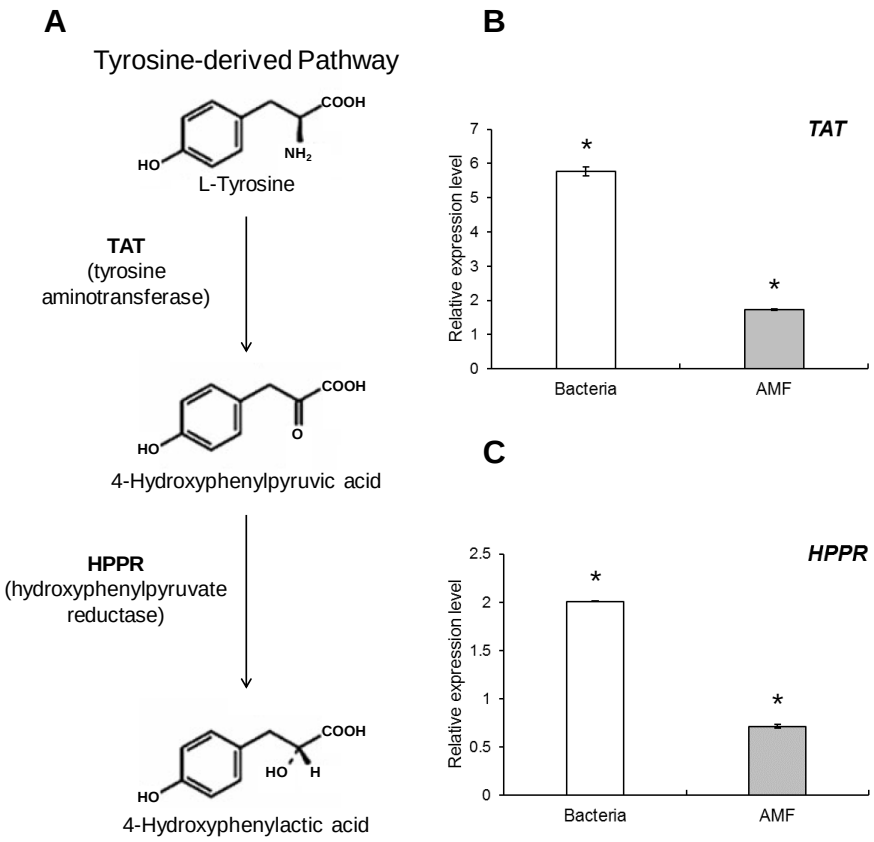
A
Tyrosine-derived Pathway
COOH
HO
NH2
L-Tyrosine
TAT
(tyrosine
aminotransferase)
COOH
HO
O
4-Hydroxyphenylpyruvic acid
HPPR
(hydroxyphenylpyruvate
reductase)
COOH
HO
HO
H
4-Hydroxyphenylactic acid
B
TAT
Relative expression level
0
1
2
3
4
5
6
7
*
*
Bacteria
AMF
C
HPPR
Relative expression level
0
0.5
1
1.5
2
2.5
*
*
Bacteria
AMF

Fig. 2

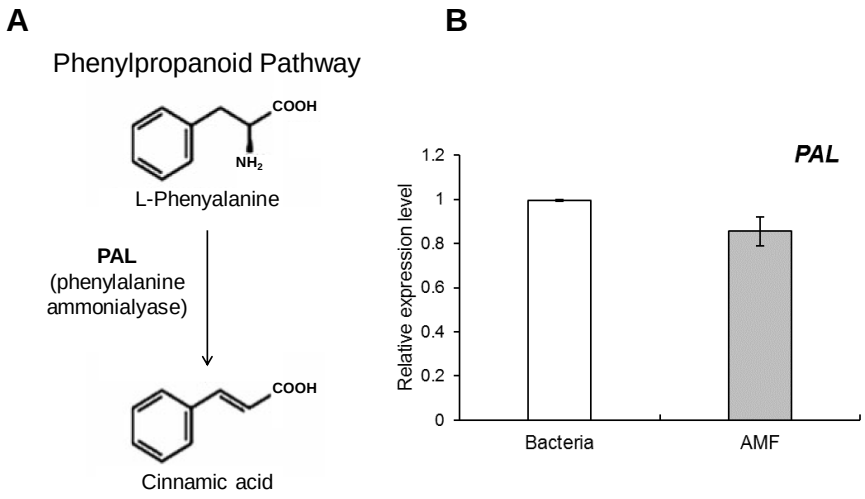
A
Phenylpropanoid Pathway
COOH
NH2
L-Phenyalanine
PAL
(phenylalanine
ammonialyase)
COOH
Cinnamic acid
B
PAL
Relative expression level
1.2
1
0.8
0.6
0.4
0.2
0
Bacteria
AMF

Fig. 3

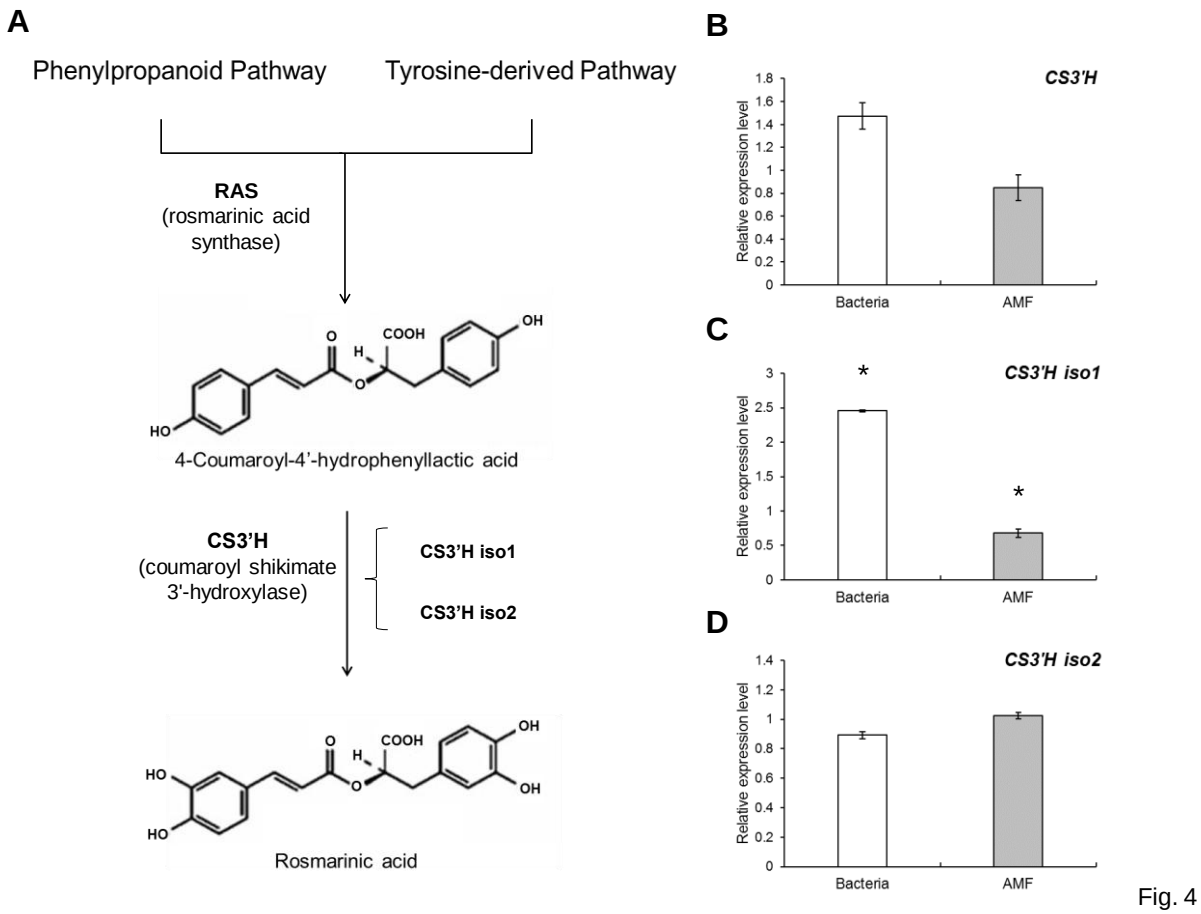
A
Phenylpropanoid Pathway
Tyrosine-derived Pathway
RAS
(rosmarinic acid synthase)
4-Coumaroyl-4'-hydrophenyllactic acid
CS3'H
(coumaroyl shikimate 3'-hydroxylase)
CS3'H iso1
CS3'H iso2
Rosmarinic acid
B
CS3'H
Relative expression level
Bacteria
AMF
C
CS3'H iso1
D
CS3'H iso2

Fig. 4