Journal of Plant Physiology 190 (2016) 79–94

Contents lists available at ScienceDirect

Journal of Plant Physiology

journal homepage: www.elsevier.com/locate/jplph

Molecular biology

Suppression Subtractive Hybridization analysis provides new insights into the tomato (*Solanum lycopersicum* L.) response to the plant probiotic microorganism *Trichoderma longibrachiatum* MK1

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ARTICLE INFO

Article history:
Received 28 July 2015
Received in revised form 3 November 2015
Accepted 6 November 2015
Available online 17 November 2015

Keywords:
Induced systemic resistance
Plant growth promotion
Differential cDNA library
Transcriptome analysis
Rhizosphere microbiome
Plant pathogens

ABSTRACT

Trichoderma species include widespread rhizosphere-colonising fungi that may establish an opportunistic interaction with the plant, resulting in growth promotion and/or increased tolerance to biotic and abiotic stresses. For this reason, *Trichoderma*-based formulations are largely used in agriculture to improve yield while reducing the application of agro-chemicals. By using the Suppression Subtractive Hybridization method, we identified molecular mechanisms activated during the *in vitro* interaction between tomato (*Solanum lycopersicum* L.) and the selected strain MK1 of *Trichoderma longibrachiatum*, and which may participate in the stimulation of plant growth and systemic resistance. Screening and sequence analysis of the subtractive library resulted in forty unique transcripts. Their annotation in functional categories revealed enrichment in cell defence/stress and primary metabolism categories, while secondary metabolism and transport were less represented. Increased transcription of genes involved in defence, cell wall reinforcement and signalling of reactive oxygen species suggests that improved plant pathogen resistance induced by *T. longibrachiatum* MK1 in tomato may occur through stimulation of the above mechanisms. The array of activated defence-related genes indicates that different signalling pathways, beside the jasmonate/ethylene-dependent one, collaborate to fine-tune the plant response. Our results also suggest that the growth stimulation effect of MK1 on tomato may involve a set of genes controlling protein synthesis and turnover as well as energy metabolism and photosynthesis. Transcriptional profiling of several defence-related genes at different time points of the tomato–*Trichoderma* interaction, and after subsequent inoculation with the pathogen *Botrytis cinerea*, provided novel information on genes that may specifically modulate the tomato response to *T. longibrachiatum*, *B. cinerea* or both.

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1. Introduction

Soil-borne beneficial microbes, like plant growth promoting rhizobacteria (PGPR) or fungi (PGPF), rhizobia, and mycorrhizal fungi, are well-known plant stimulators and can protect plants from abiotic and biotic stresses (Pozo and Aguilar, 2007; Bonfante and Genre, 2010; Berendsen et al., 2012; Caporale et al., 2014; Ruocco et al., 2015; Vos et al., 2015).

The direct and indirect biocontrol activity of rhizosphere-competent fungi of the genus *Trichoderma* is widely recognized (Harman et al., 2004; Shoresh et al., 2010). Several strains are also

Abbreviations: BCA, biocontrol agent; ET, ethylene; DPI, days post inoculation; ISR, induced systemic resistance; LHC, light-harvesting complex; MAMPS, Microbe-Associated Molecular Patterns; PGP, plant growth promotion; PGPF, plant growth promoting fungi; PGPR, plant growth promoting rhizobacteria; SAR, Systemic Acquired Resistance; SGN, SOL Genomics Network; SSH, Suppression Subtractive Hybridization.

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http://dx.doi.org/10.1016/j.jplph.2015.11.005
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able to stimulate plant growth (Lorito and Woo, 2015), improve nutrient uptake (Zhao et al., 2014), and contribute to plant hormonal balance and volatile production (Harman et al., 2004; Shoresh et al., 2005; Battaglia et al., 2013; Ruocco et al., 2015). It has also been demonstrated that these PGPF can protect plants from abiotic stresses and affect their direct and indirect resistance to insect pests (Bae et al., 2009; Mastouri et al., 2010, 2012; Battaglia et al., 2013; Brotman et al., 2013; Caporale et al., 2014). Thanks to their proved efficacy, a number of *Trichoderma* strains have been selected for application in agriculture, with a few hundreds of formulations registered worldwide, while several strains have been deeply studied at the laboratory level for their peculiar biological and genetic features (Lorito and Woo, 2015).

The molecular mechanisms that regulate direct *Trichoderma* biocontrol activity have been ascertained (Atanasova et al., 2013), those enabling *Trichoderma* strains to promote indirect defence against pathogens and pests and, even more so, plant growth have not been fully uncovered. Several reports indicate that the ability of *Trichoderma* spp. to activate induced systemic resistance (ISR) against pathogen infections is mediated by jasmonate (JA)- and ethylene (ET)-dependent mechanisms and requires transient expression of defence genes (Shoresh et al., 2005; Korolev et al., 2008). Moreover, long-lasting up-regulation of salicylic acid (SA)-responsive genes was demonstrated in tomato interacting with *Trichoderma harzianum* T22, whose modulation, together with JA-induced gene expression, contributed to increased resistance to the pathogen *Botrytis cinerea* (Tucci et al., 2011). Recently, increasing evidence is accumulating that both JA/ET and SA signalling may be triggered by *Trichoderma* in crop and model plants (Segarra et al., 2007; Tucci et al., 2011; Mathys et al., 2012; Perazzolli et al., 2012; Martinez-Medina et al., 2013). Only a few studies have addressed the molecular mechanisms underlying the promotion of plant growth by *Trichoderma* species. Auxin signalling was demonstrated to be important for biomass production induced by *Trichoderma virens* (Contreras-Cornejo et al., 2009) and increased transcription of IAA-related genes was observed in *Arabidopsis thaliana* roots after *T. harzianum* inoculation (Brotman et al., 2013). Moreover, proteomic approaches indicated increased photosynthesis and carbohydrate metabolism in *Trichoderma*-treated plants (Segarra et al., 2007; Shoresh and Harman, 2008), which were suggested to be related to enhanced growth response.

It is generally assumed that establishment of the plant–*Trichoderma* interaction triggers an extensive transcriptional reprogramming of genes involved in defence, growth and secondary metabolism, though the alteration of gene expression levels is often quantitatively small (Alfano et al., 2007; Shoresh and Harman, 2008; Moran-Diez et al., 2012). In search for genetic components of this beneficial response that undergo small transcriptional changes, we used PCR-Select cDNA Suppression Subtractive Hybridization (SSH), which has been proven useful for the identification of rare differentially expressed transcripts, to study transcriptome remodelling of tomato plantlets interacting with the MK1 strain of *Trichoderma longibrachiatum*. This *Trichoderma* strain was selected since it has been well characterised *in vitro* and has a demonstrated ability to increase plant growth and enhance pathogen resistance (Battaglia et al., 2013; Ruocco et al., 2015). Moreover, it is a rich source of bioactive molecules (Ruocco et al., 2015), some of which are the subject of a few patent applications.

Our results identified several plant genes activated by *T. longibrachiatum* strain MK1, which may mediate, at least in part, the ability of this PGPF to stimulate plant growth as well as ISR against pathogen infections. These genes were fully annotated and available transcriptomic data of tomato and *Arabidopsis* were exploited to obtain indications on their possible role and regulation by different signals. Further transcriptional characterisation showed that some defence-related transcripts are induced by the PGPF but not by inoculation with the pathogen *B. cinerea* and suggested that alleviation of disease symptoms in *Trichoderma*-treated plants may be achieved by boosting the expression of some components of the plant defence machinery while maintaining others at a low transcription level.

The repository of differentially expressed genes produced in this study can therefore be useful to acquire further knowledge on the mechanisms activated by *Trichoderma* strains to stimulate ISR, while also contributing to unravel the less known plant growth promotion (PGP) activity, thus supporting the development of more effective biostimulator formulations. Moreover, this gene catalogue represents a useful tool for designing new breeding strategies for the selection of crop varieties with improved ability to benefit from the interaction with *Trichoderma*.

2. Materials and methods

2.1. Fungal strain and plant material

T. longibrachiatum PGPF strain MK1 (referred to as MK1 along the paper), isolated from tomato roots, was obtained from the collection of the Department of Agricultural Sciences of the University of Naples Federico II, Italy and grown on potato dextrose agar. Colonies were allowed to sporulate at 25 °C in the dark for 7 days, then collected by washing the plates with sterile distilled water and brought to a concentration of 10^9 mL^{-1}.

Solanum lycopersicum cv. 'Crovarese' seeds were kindly provided by La Semiorto Sementi, Sarno (SA, Italy). The seeds, sterilized in 2% sodium hypochlorite for 20 min and washed in sterile distilled water, were germinated *in vitro* on Murashige and Skoog (MS) solid medium (Duchefa Biochemie, Haarlem, Netherlands) with 3% (w/v) sucrose at 24 °C and 16 h light/8 h dark photoperiod with an irradiance of 80 μmol m^{-2} s^{-1} until second true leaf appearance.

In vitro-grown plantlets were then transferred to petri dishes (12×12 cm) on half strength MS medium with Mk1 pre-germinated spores and grown as above indicated. Inoculum was diluted at a concentration of 1×10^5 spores mL^{-1} and applied at a rate of approximately 0.02 mL per plant. Mock inoculated plantlets were treated using the same conditions, but with sterile water. Control and treated shoots were collected at 1, 2 and 3 days post inoculation (DPI) and immediately frozen and kept at −80 °C until total RNA extraction. To reduce the impact of biological variation, at least six plantlets at 2 DPI were pooled for the construction of the subtractive library. Moreover, at least three biological replicates of 3–4 plantlets each were collected at each time point for the qRT-PCR analyses.

For *B. cinerea* infection, sterilized tomato seeds were incubated in a 10^6 mL^{-1} fresh MK1 spore suspension (treated) or in water (control) according to Tucci et al. (2011). Germinated seeds were transferred to pots in soil and grown in the greenhouse of the CNR-IBBR in Portici for two months.

2.2. Botrytis cinerea *infection*

B. cinerea strain 309, isolated from tobacco, was obtained from the culture collection available at the Department of Agricultural Sciences, University of Naples Federico II, Italy. Untreated control and MK1-treated 2-month-old tomato plants were infected with *B. cinerea* by inoculating the third true leaf with 10 μL of a 10^6 mL^{-1} spore suspension of the pathogen as reported in Tucci et al. (2011). Three plants per treatment were used for the inoculation. Immediately before infection, the fourth leaf of control and MK1-treated plants of each replicate were collected as the uninfected control. At 48 h after inoculation, the fifth or sixth leaf from each replicate

plant were collected, frozen in liquid nitrogen, and stored at −80 °C until RNA extraction.

2.3. Construction of the SSH library

Total RNA was isolated from frozen tomato shoots of at least six plantlets of both MK1-treated and control plants at 2 DPI using the RNeasy Plant Mini Kit (Quiagen, Valencia, CA, USA) following the manufacturer's instructions. Poly A^+ RNA was separated through the Dynabeads mRNA purification kit (Life Technologies, Carlsbad, CA, USA). The PCR-Select cDNA Subtraction kit (BD Biosciences Clontech, Palo Alto, CA) was then used to generate tester and driver double-stranded cDNAs from 2 μg of poly A^+RNA according to the manufacturer's protocol. The cDNAs of MK1-treated and control shoots were the source of tester and driver populations for the construction of the forward SSH library, respectively.

2.4. Cloning and differential screening using cDNA array

Products (3 μL) of the secondary PCR from the forward subtraction were directly inserted into the pCR® 2.1 plasmid vector (Life Technologies, Carlsbad, CA, USA) for T/A cloning. The ligated products were transformed into One-Shot TOP10 chemically competent *Escherichia coli* cells (Life Technologies, Carlsbad, CA, USA). Approximately 300 white putative recombinant colonies were picked and grown overnight with shaking at 37 °C in 100 μL kanamycin (50 μg mL^{-1})-containing Luria-Bertani medium. cDNA inserts were then amplified by colony PCR using a GeneAmp PCR System 9600 (Life Technologies, Carlsbad, CA, USA) and Nested Primer 1 and Nested Primer 2R, provided by the PCR-select differential screening kit (BD Biosciences Clontech, Palo Alto, CA), as the forward and reverse primers, respectively, to check for the presence and size of individual inserts. The final reaction volume of 25 μL contained 1× reaction buffer, 1.5 mM $MgCl_2$, dNTPs mix (0.2 mM each), 0.5 μM of each primer, 1U recombinant Taq DNA polymerase (Life Technologies, Carlsbad, CA, USA) and 1 μL of each bacterial culture. The PCR was run using the following cycling conditions: 3 min at 94 °C, 30 cycles at 20 s at 95 °C, 68 °C 3 min and a final extension step of 10 min at 72 °C. All PCR products were electrophoresed on 2% agarose gel, stained with ethidium bromide and then visualized under UV light.

Reverse RNA gel blotting analysis was used to verify the differential expression of the library clones. For the differential screening, 265 insert-containing clones were arrayed onto Hybond-N+ nylon membranes (GE Healthcare Life Sciences, Buckinghamshire, UK) as described in the Clontech PCR-select differential screening kit protocol. All clones were hybridized with four [α-^{32}P] dCTP labelled probes generated from unsubtracted tester and driver, and forward and reverse subtracted cDNAs in standard aqueous solution at 72 °C overnight. Membranes were washed at 68 °C to reduce the number of undesired background cDNA clones, representing non-differentially expressed transcripts, and exposed to a phosphor screen and scanned with a Typhoon Variable Mode Imager 9200 (GE Healthcare Life Sciences, Buckinghamshire, UK). Hybridization results were interpreted by comparing the differences in signal intensity with each of the four probes in the membrane cDNA arrays.

2.5. DNA sequencing and similarity-based functional annotation

Plasmid DNA was isolated from 72 differentially screened clones using the Qiaprep spin miniprep kit (Qiagen, Valencia, CA, USA). Cycle sequencing reactions were prepared using the Big Dye Terminator Version 3.1 kit (Life Technologies, Carlsbad, CA, USA) and the M13 reverse primer 5′-CAGGAAACAGCTATGAC-3′ according to the manufacturer's protocol. DNA sequencing was performed on an ABI 3700 DNA Sequencer (Life Technologies, Carlsbad, CA, USA).

Base calling on each electropherogram was performed by Phred (Ewing et al., 1998) with a quality cut-off of 0.05. Vector contaminations were identified using RepeatMasker [http://www.repeatmasker.org] and the sequences of both the vector pCR2.1 T/A and the PCR-Select adaptors as filtering reference data. All the ESTs were assembled into contigs using CAP3 (Huang and Madan, 1999) with an overlapping window of 25 nucleotides and a minimum percentage identity of 98. Functional annotation was determined by BLASTx comparisons (E-value <10^{-5}) *versus* the UniprotKB/TrEMBL database, the tomato protein complement (available at the Tomato Genomics Network, www.sgn.cornell.edu) and the *A. thaliana* (TAIR10, www.arabidopsis.org) protein database. Finally, transcripts were aligned along the reference tomato genome (version 2.40) using GenomeThreader (Gremme et al., 2005) with identity greater or equal to 90% and coverage of the transcript sequence of at least 90%. Tomato gene annotations by ITAG were automatically transferred to the transcripts under investigation.

BLASTN based analyses *versus* the dbEST partition of the NCBI were exploited to detect ESTs associated to the transcript indices and the tomato library they resulted from. Parameters used for this analysis considered an EST associated to a transcript index when aligned for greater or equal to 70% of its length (EST coverage) and with identity value in the alignment greater or equal to 95%.

The "Meta-analyzer" tool of the Genevestigator software, version V3 (www.genevestigator.ethz.ch) was used to identify the stimuli triggering transcription of *A. thaliana* orthologs to the tomato SSH transcripts.

The tomato MapMan ontologies (http://www.gomapman.org/export/current/mapman/sly_SL2.40_ITAG2.3_2015-01-09_mapping.txt.tgz) were retrieved from the GOMapMan web resource (Ramsak et al., 2014) and imported in the MapMan tool version 3.6.0 (Thimm et al., 2004). Then, the tomato genes matching the SSH transcripts were mapped to bins for data visualization and pathway analysis.

2.6. RNA isolation, cDNA synthesis and real time PCR analysis

Total RNA from at least three biological replicates was isolated from shoots of control and MK1-treated plantlets harvested at 1, 2 and 3 DPI, as well as from *B. cinerea* uninfected and infected leaves of control and 60 DPI MK1-treated plants, using the Purelink Micro-to-Midi Total RNA Purification System (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. To prepare cDNA template for qRT-PCR, total RNAs were treated with RNase-free DNase I (Life Technologies, Carlsbad, CA, USA) and cDNA was synthesized from 1 μg of total RNA using SuperScript II reverse transcriptase (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instruction. For each RNA sample, a reaction without RT was performed as a control for contamination by genomic DNA. Three technical replicates were analysed for each of three biological replicate cDNA samples. PCR reactions were prepared in a total volume of 20 μL with 10 μL of the 2X Power SYBR Green PCR Master Mix (Life Technologies, Carlsbad, CA, USA), 0.2 pmol of target gene primers or 0.4 pmol of *actin* primers, and 4 μL of 1:4 diluted cDNA template. qRT-PCR was performed using a 7900HT Fast Real-Time PCR System (Life Technologies, Carlsbad, CA, USA). PCR primers were designed, whenever possible, on the relative SSH clone sequence, or on the best matching Solyc sequence, using the Primer 3 program (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). The pathogenesis related *PR1b1* (accession number Y08804) and *PR-P2* (accession number X58548) genes were analysed as markers of the plant response to the PGPF. The *actin* gene (accession number BT013524) was used

Table 1
List of primers used for qRT-PCR assays.

Primers	Sequence	Tm (°C)	PCR efficiency
F-Contig 2	5′-ACGTGGAAGCCCCGAGTCTA-3′	61	107
R-Contig 2	5′-CACGGGGACATTGTTTCCAA-3′	57	
F-Contig 3	5′-CGGTGAAGCTGGTTTGGGAAA-3′	59	101
R-Contig 3	5′-TAAATGAACCCTGTGATGGTGGA-3′	59	
F-Contig 4	5′-TCTATGGTGATGTGAGCCGC-3′	59	110
R-Contig 4	5′-AGCATTCCCTTCATTGGCCT-3′	57	
F-15	5′-GTGCCGGAGAGGCTGGGACT-3′	65	102
R-15	5′-TTTTTCGTGTTTCCTCCACGGGTA-3′	61	
F-100	5′-CCGTGGAATGTCCGATCACA-3′	59	105
R-100	5′-CCACCACCAACATCAACAATGG-3′	60	
F-198	5′-TGGTGGTGCACTTCAAAAGG-3′	57	108
R-198	5′-TTGTTGCTGAATGTGGCAAAG-3′	56	
F-244	5′-TGGCCCTTCACAGCCCTTTA-3′	59	100
R-244	5′-GCACCCAAAGCAGTTAGGGTTTC-3′	62	
F-276	5′-ACCACCACTGCACGGCTGAG-3′	63	90
R-276	5′-TTCTTGGAGGGCTTTGAAGTTGG-3′	61	
F-292	5′-GATGGTGCTGGTAGAGGTTGGTG-3′	64	90
R-292	5′-GCGTATTCAGCTAAGGTGTTTGGTG-3′	63	
F-298	5′-CATTTGGACCTCGCCTCTCT-3′	59	104
R-298	5′-CAGCAGCATTAGCAACCAACA-3′	58	
F-PR1b1	5′-GCACTAAACCTAAAGAAAAATGGG-3′	58	100
R-PR1b1	5′-AAGTTGGCATCCCAAGACATA-3′	56	
F-PR-P2	5′-GCTTGTCAGCATCCCAGGTA-3′	57	92
R-PR-P2	5′-CCCAGGTAGCGCAGTAAACGC-3′	63	
F-Actin	5′-CACCACTGCTGAACGGGAA-3′	59	107
R-Actin	5′-GGAGCTGCTCCTGGCAGTTT-3′	61	

as an endogenous reference gene to normalize gene expression. Gene-specific primers are listed in Table 1. qRT-PCR experiments were carried out in triplicate. PCR was conducted with the following programme: an initial step of 10 min at 95 °C, followed by 40 cycles of two steps at 95 °C for 15 s and 60 °C for 1 min and melt curve analysis was performed at the end of each run to confirm that there was no signal from non-specific binding products. No template controls were included in each run to test for possible contamination of assay reagents. Reaction products were also resolved in agarose gel to verify amplicon size. PCR efficiencies calculated by the standard curve method were 90–110% for each primer pairs with a correlation coefficient (R^2) of 0.99. The relative expression of each gene, in the different conditions, was calculated by using the untreated control at the same time point as calibrator, whose expression was set equal to 1. The $\Delta\Delta$CT method was used for relative gene expression analysis (Pfaffl, 2001). Statistically significant differences were determined through the Student's *t*-test.

3. Results and discussion

3.1. Differential screening and sequence analysis of SSH clones

Molecular mechanisms underlying the effects of beneficial rhizosphere-competent microorganisms on plants were investigated by transcriptomic analysis of the mutualistic interaction between *S. lycopersicum* cv. 'Crovarese' and *T. longibrachiatum* strain MK1, used as a model system. Recent microarray data indicate that *Trichoderma*-induced systemic plant responses may not be very extensive (Alfano et al., 2007; Moran-Diez et al., 2012), while the root may show larger transcriptomic changes (Brotman et al., 2013), since it is the site of *Trichoderma* colonisation. Therefore, for the first time for the plant response to *Trichoderma*, we used a normalised subtractive library approach for the identification of tomato genes up-regulated in the shoots during the early response to MK1, since this method allows the enrichment of rare transcripts that other transcriptomic techniques may fail to detect (Cao et al., 2004). The *in vitro* axenic system used in this study ensured the isolation of sequences specifically modulated in tomato by this biocontrol agent (BCA), limiting interference from other pathogenic or non-pathogenic microorganisms.

Quantitative Real Time PCR of *PR1b1* and *PR-P2* of tomato shoots demonstrated that the transcript levels of these two *Trichoderma*-responsive marker genes increase already one day after MK1 root inoculation (DPI), and remains high at 2 and 3 DPI (data not shown). This is consistent with the demonstrated ability of some *Trichoderma* strains to extensively colonize tomato root surface after 24 h and grow inside the roots, mainly intercellularly, by 48 h (Yedidia et al., 1999; Lace et al., 2015). Therefore, 2 DPI was chosen as a relevant time point to investigate the tomato response to MK1.

A forward SSH library, enriched with up-regulated transcripts after 48 h of tomato root treatment with MK1, was obtained using cDNAs from *Trichoderma*-treated and untreated (control) shoots (tester and driver, respectively). Differential screening by cDNA dot blots of the 265 insert-containing library clones showed that the most frequent hybridization signal patterns were: (i) absent with both un-subtracted and subtracted probes, as for clone E11; (ii) present with all the probes, as for clone B2; (iii) high signal intensity with the forward-subtracted probe and absent or very weak hybridization signal with the reverse-subtracted and unsubtracted driver probes, as for clones D3, H2; (iv) present only with the forward-subtracted tester probe, as for clone G9 (Fig. 1). Moreover, we found several clones that did not hybridise to either the forward- or the reverse-subtracted cDNA probes, confirming that the hybridization background remained within acceptable levels. Hybridization results allowed selection of 72 differential clones. This quite low subtraction efficiency (27.2%) may reflect small differences in the abundance of mRNA species between control and MK1-treated shoots, as was expected from previous data (Alfano et al., 2007; Moran-Diez et al., 2012). This peculiar response is possibly due to the plant failure in perceiving the fungus as a potential pathogen and instead responding by priming defence responses against subsequent invaders.

Sequence pre-processing reduced the original dataset to 51 high quality sequences with an average length of 302 nucleotides (nts) and a maximum length of 673 nts, 88% of which were longer than 100 nts. Five sequences equal or shorter than 30 nts were discarded. The assembly process resulted in 40 up-regulated tran-

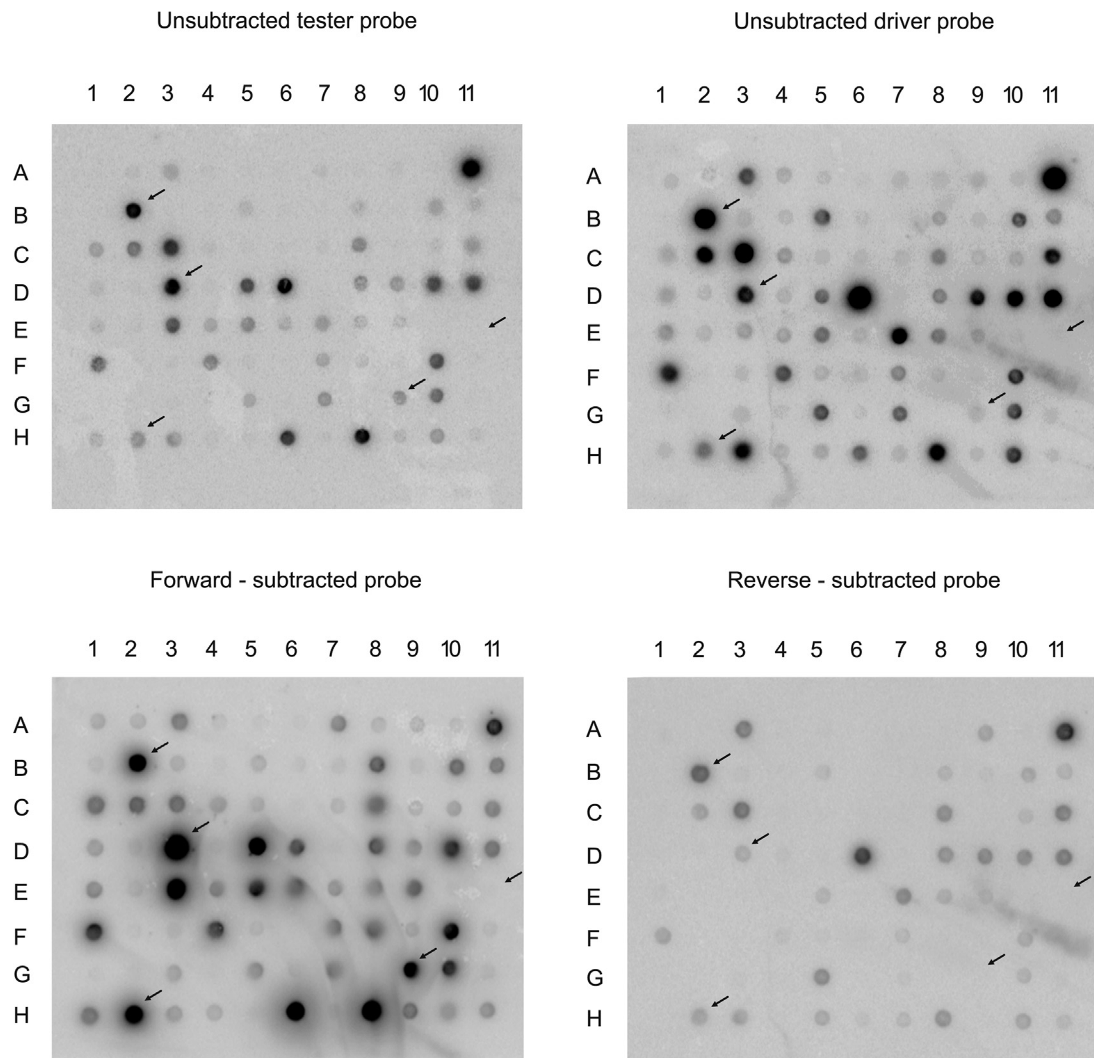

Fig. 1. Differential screening of PCR-positive clones of a *T. longibrachiatum* Mk1-induced cDNA library. Dot blots of the forward subtracted cDNA library clones were hybridized with α^{32}P dCTP labeled unsubtracted (tester and driver, upper panel) or subtracted (forward and reverse, lower panel) probes, respectively. Eighty-eight representative clones are shown out of the 265 insert-containing ones and the most frequent hybridization signal patterns are indicated by arrows.

scripts (35 singletons and 5 contigs), whose nucleotide sequences were deposited in the dbEST division of the GenBank repository under the accession numbers from JZ845517 to JZ845554. Transcripts with similarities to mitochondrial/plastidial or ribosomal sequences were not submitted to GenBank. The size of the obtained dataset is similar to those identified by microarray analysis of tomato–*Trichoderma hamatum* and *Arabidopsis*–*T. harzianum* interactions, with 45 (36 up and 9 down) and 66 (33 up and 33 down) differentially expressed plant systemic genes, respectively (Alfano et al., 2007; Moran-Diez et al., 2012), Instead, leaves of *T. hamatum*-treated *Arabidopsis* revealed a much larger number (1377 up and 698 down) of affected genes (Mathys et al., 2012), possibly because of the different experimental system and/or of the statistics used to identify differentially expressed genes. It is worth highlighting that the low number of up-regulated transcripts detected in our work may also be due to a weaker transcriptomic responses in the aerial parts of the plant in comparison to the roots, which directly interact with *Trichoderma*. In fact, increased expression of 249 genes and decreased expression of 29 genes was demonstrated in *A. thaliana* roots responding to *Trichoderma asperelloides* colonisation (Brotman et al., 2013).

3.2. *Sequence annotation*

Results of the BLASTx similarity search of the 40 transcripts *versus* the UniProtKB/TrEMBL (The UniProt Consortium, 2014) and TAIR10 (www.arabidopsis.org) databases demonstrated that the tomato up-regulated transcripts after 48 h interaction with MK1 share the highest similarities with plant sequences, with most of the best matches being found within Solanaceous species (Table 2). Table 2 also lists the best matching *S. lycopersicum* genes and their associated functional annotation as well as the genome coordinates resulting from the alignment of the 40 transcripts against the tomato reference genome. This latter analysis allowed all but two transcripts to be mapped, with a random distribution on the 12 tomato chromosomes. Transcript 138, which had no match in both UniprotKB/TrEMBL and the TAIR10 databases, mapped very close to a chitinase gene (Solyc04g072000.2.1) indicating that it may represent the 3′ UTR of this gene. Clone 145 and contig 2 overlapped the same tomato gene (Solyc12g094620.1.1), thus suggesting they could be the N- and the C-termini of a catalase enzyme. Similarly, both clone 83 and contig 1 mapped to Solyc09g010630.2.1, which codes for a heat shock protein HSP70.

Table 2
Results of the similarity search in the UniprotKB/TrEMBL database of the transcripts up-regulated in tomato 2 DPI with *T. longibrachiatum* MK1, classified in different functional categories. The best matches in the TAIR database are also shown, together with the coordinates of the transcripts onto the reference tomato genome. Transcripts lacking a GenBank accession number were not submitted to dbEST since they are similar to mitochondrial/plastidial or ribosomal sequences.

			Best match vs TrEMBL			Best match vs TAIR			Best match vs tomato genes		
Clone n.	Length(bp)	GenBank Acc.n.	ID	Function/organism	*E*-value	TAIR locus	Function	*E*-value	Solyc id	Function	Chromosome position (SL2.40ch/start-stop)
Cell defence and stress											
Contig 1	490	JZ845517	B3GPH1_ CAMSI	Heat shock protein 70/*Camellia sinensis*	1.00E-71	AT3G12580.1	HSP70 (heat shock protein 70)	2.00E-72	Solyc09g010630.2.1	Heat shock protein 70	ch09/3967681–3968135
83	222	JZ845551	Q943K7_ ORYSJ	Putative uncharacterized protein/*Oryza sativa* subsp. *japonica*	5.00E-34	AT5G02500.2	HSC70-1 (heat shock cognate protein 70-1)	1.00E-35	Solyc09g010630.2.1	Heat shock protein 70	ch09/3967341–3967562
Contig 2	590	JZ845518	Q2PYW5_ SOLTU	Catalase/*Solanum tuberosum*	1.00E-113	AT1G20630.1	CAT1 (Catalase 1)	3.00E-93	Solyc12g094620.1.1	Catalase	ch12/63143091–63143777
145	288	JZ845527	A8QID6_ CAPAN	Catalase/*Capsicum annuum*	3.00E-27	AT1G20620.4	CAT3 (Catalase 3)	1.00E-21	Solyc12g094620.1.1	Catalase	ch12/63144518–63144980
Contig 3	283	JZ845519	Q9FUN5_ CAPAN	Beta-1,3-glucanase-like protein/*Capsicum annuum*	8.00E-40	AT4G16260.1	Catalytic/ cationbinding/ hydrolase	1.00E-25	Solyc01g059980.2.1	Beta-glucanase	ch01/62202939–62203195
12	213	JZ845525	B2LW68_ SOLLC	PR1 protein/*Solanum lycopersicum*	1.00E-32	AT2G14580.1	ATPRB1	6.00E-17	Solyc01g106620.2.1	Pathogenesis-relatedprotein 1a	ch01/86187880–86188092
89	301	JZ845552	Q96477_ SOLLC	LRR protein/*Solanum lycopersicum*	2.00E-30	AT5G21090.1	Leucine-rich repeat protein/putative	3.00E-27	Solyc10g081190.1.1	LRR resistancepro-teinfragment	ch09/61642247–61643735
174	242	JZ845530	Q40487_ TOBAC	Cationic peroxidase isozyme 40 K/*Nicotiana tabacum*	9.00E-20	AT5G15180.1	Peroxidase/putative	3.00E-12	Solyc01g067860.2.1	Peroxidase 24	ch01/69231582–69231804
227	308	*	B2BAK7_ LILLO	Putative senescence-associated protein/*Lilium longiflorum*	1.00E-43	–	–	–	Solyc06g024230.1.1	Unknown Protein	ch00/5655985–5656271
240	115	JZ845538	M1D198_ SOLTU	Uncharacterizedprotein/ *Solanum tuberosum*	4.00E-10	AT1G16180.2	Serinc-domain containing serine and sphingolipid biosynthesis protein	1.00E-10	Solyc06g054420.2.1	Serine incorporator 1/TMS membrane protein_ tumourdif-ferentially	ch06/33690186–33690281
292	286	JZ845545	Q84U70_ SOLTU	Osmotin-like protein (Fragment)/*Solanum tuberosum*	6.00E-52	AT4G11650.1	ATOSM34 (osmotin 34)	4.00E-35	Solyc08g080670.1.1	Osmotin-likeprotein	ch08/61057399–61057663
Primary metabolism											
Contig 4	561	JZ845520	Q9LM03_ SOLTU	Methioninesynthase/ *Solanum tuberosum*	1.00E-104	AT3G03780.3	Atms2, 5-methyltetrahydropteroyl-triglutamate-homocysteine *S*-methyltransferase/ methionine synthase	1.00E-101	Solyc10g081510.1.1	5-methyltetrahydropteroyl-triglutamate-homocysteine methyltransferase	ch10/61893273–61893938
Contig 5	418	JZ845521	O78327_ CAPAN	Transketolase 1/*Capsicum annuum*	1.00E-66	AT3G60750.1	Transketolase/putative	2.00E-63	Solyc05g050970.2.1	Transketolase 1	ch05/60331673–60332072
15	253	JZ845529	O04936_ SOLLC	Malicenzyme/*Solanum lycopersicum*	1.00E-40	AT1G79750.1	ATNADP-ME4 (NADP-malicenzyme 4); malate dehydrogenase	3.00E-35	Solyc05g050120.2.1	Malicenzyme	ch05/59253026–59254005
50	222	JZ845549	B0YQX2_ GOSAR	Plastid fructose 1,6 bisphosphate aldolase/*Gossypium arboreum*	2.00E-33	AT4G38970.2	Fructose-bisphosphatealdolase/ putative	1.00E-35	Solyc02g084440.2.1	Fructose-bisphosphatealdolase	ch02/42112290–42112684
225	427	JZ845536	A7PIZ4_ VITVI	Oxoglutaratedehydrogenase/*Vitis vinifera*	1.00E-56	AT5G65750.1	2-oxoglutarate dehydrogenase E1 component/putative/ oxoglutaratedecarboxylase/ putative	1.00E-56	Solyc05g054640.2.1	2-oxoglutarate dehydrogenase E1 component	ch05/63658421–63658890
232	419	JZ845537	Q9SPF9_ MESCR	Ubiquitincarrierprotein/ *Mesembryanthemum crystallinum*	2.00E-61	AT4G27960.2	UBC9 (ubiquitinconjugatingenzyme 9)	8.00E-63	Solyc05g050230.2.1	Ubiquitin-conjugatingenzyme E2 10	ch05/59425881–59429043
244	280	JZ845540	Q2XTD0_ SOLTU	Adenosylhomocysteinase/ *Solanum tuberosum*	1.00E-33	AT4G13940.4	Adenosylhomo-cysteinase	5.00E-35	Solyc09g092380.2.1	Adenosylhomo-cysteinase	ch09/66843900–66844158
249	202	JZ845541	Q2VY16_ SOLLC	CONSTANS interacting protein 3/ *Solanum lycopersicum*	8.00E-30	AT5G64350.1	FKBP12 (FK506-binding protein)/ peptidyl-prolyl cis-trans isomerase	1.00E-26	Solyc01g105710.2.1	Peptidyl-prolyl *cis-trans* isomerase	ch01/85540025–85541286
285	187	JZ845544	K4BK61_ SOLLC	Uncharacterizedprotein/ *Solanum lycopersicum*	2.00E-05	AT3G19480.1	D-3-phosphoglycerate dehydrogenase/putative	9.00E-07	Solyc03g112070	Uncharacterized protein	–
295	140	JZ845546	Q9ZWH9_ NICPA	Elongation factor 1-alpha/*Nicotiana paniculata*	4.00E-15	AT5G60390.3	Elongationfactor 1-alpha	2.00E-17	Solyc06g009960.1.1	Elongationfactor 1-alpha	ch06/4359581–4359701
Secondary metabolism											
98	286	JZ845553	Q9M4X2_ SOLLC	Putative cytochrome P450/*Solanum lycopersicum*	6.00E-41	AT3G14630.1	CYP72A9; electron carrier/heme binding/iron ion bind-ing/monooxygenase/oxygen binding	2.00E-17	Solyc07g043460.2.1	Cytochrome P450, E-class, group I	–

100	463	JZ845522	Q42958_TOBAC	Catechol O-methyltransferase/*Nicotiana tabacum*	4.00E-78	AT5G54160.1	ATOMT1 (*O*-methyltransferase 1)	4.00E-73	Solyc03g080180.2.1	*O*-methyltransferase	ch03/45616218–45616833
125	467	JZ845523	A1XEM2_TOBAC	CYP72A58/*Nicotiana tabacum*	7.00E-55	AT3G14660.1	CYP72A13; electron carrier/heme binding/iron ion binding/monooxygenase/oxygen binding	2.00E-46	Solyc07g062500.2.1	Cytochrome P450	ch07/62438003–62438450
198	293	JZ845533	B5LAW0_CAPAN	Phenylalanineammonia-lyase/*Capsicum annuum*	2.00E-46	AT3G10340.1	PAL4 (Phenylalanineammonia-lyase 4)	4.00E-45	Solyc09g007920.2.1	Phenylalanine-ammonia-lyase	ch09/1437130–1437406
263	313	JZ845542	Q40132_SOLLC	2-oxoglutarate-dependent dioxygenasehomolog (Fragment)/*Solanum lycopersicum*	3.00E-52	AT4G10500.1	Oxidoreductase/2OG-Fe(II) oxygenase family protein	5.00E-20	Solyc07g043420.2.1	2-oxoglutarate-dependent dioxygenase	ch07/54504273–54504656
Transport											
81	267	JZ845550	Q940G0_ARATH	Endomembraneprotein 70 putative/*Arabidopsis thaliana*	7.00E-17	AT1G10950.1	Endomembraneprotein 70/putative	4.00E-19	Solyc01g103930.2.1	Transmembrane 9 superfamilyproteinmember 3	ch01/84168568–84168966
156	635	JZ845528	Q9SPD5_SOLLC	Plasma membrane H^+-ATPase/*Solanum lycopersicum*	1.00E-105	AT5G62670.1	Arabidopsis H(+)-ATPase 11)	1.00E-105	Solyc03g113400.2.1	H-ATPase	ch03/40061992–40063631
188	375	JZ845531	A9PHT6_POPTR	Proteaseinhibitor/Populustrichocarpa	5.00E-21	AT2G45180.1	Lipid transfer protein (LTP) family protein	4.00E-20	Solyc06g065970.1.1	Corticalcell-delineatingprotein	ch06/37743245–37743570
223	273	JZ845535	B9HQM5_POPTR	ABC transporter family protein/*Populus trichocarpa*	1.00E-38	AT5G60790.1	ATGCN1; transporter	6.00E-40	Solyc11g069090.1.1	ATP-binding cassette protein (AHRD V1 ***-C0NDN3_AJECG)	ch11/50760933–50761767
278	366	*	Q8M9S4_9ASTE	ATP synthase epsilon chain (Fragment)/*Desfontainia spinosa*	9.00E-50	ATCG00470.1	ATPase epsilon subunit	2.00E-47	Solyc01g007320.2.1	ATP synthase subunit beta chloroplastic	ch01/1849406–1849747
Energy metabolism											
2	215	JZ845548	Q41423_SOLTU	Chlorophyll a/b binding protein/*Solanum tuberosum*	4.00E-16	AT2G34430.1	LHB1B1; chlorophyllbinding	3.00E-13	Solyc02g071000.1.1	Chlorophyll a/b binding protein	ch02/35097606–35097804
243	186	JZ845539	B0ZSC8_9ROSI	Chloroplast ribulose-1,5-bisphosphate carboxylase/oxygenase small subunit/*Jatropha curcas*	1.00E-12	AT5G38410.3	Ribulose bisphosphate carboxylase small chain 3B	2.00E-11	Solyc02g085950.2.1	Ribulose bisphosphate carboxylase small chain	ch02/43293319–43293484
276	673	JZ845543	K4D246_SOLLC	Uncharacterizedprotein/*Solanum lycopersicum*	1.00E-95	AT3G56940.1	CRD1 (copper response defect 1); DNA binding/magnesium-protoporphyrin IX monomethyl ester (oxidative) cyclase	6.00E-97	Solyc10g077040.1.1	Magnesium-protoporphyrin IX monomethylester	ch10/59279031–59280420
298	451	JZ845547	Q8LSZ3_TOBAC	NADPH:protochlorophyllide oxidoreductase/*Nicotiana tabacum*	2.00E-62	AT4G27440.2	PORB (protochlorophyllideoxidoreductase B)	8.00E-44	Solyc10g006900.2.1	Protochlorophyllide reductase	ch10/1335160–1336611
Signal transduction											
9	412	JZ845554	A7PS51_VITVI	Kinaseprotein/*Vitis vinifera*	3.00E-07	AT5G27620.1	CYCH1 (cyclin h1); cyclin-dependent protein kinase/protein binding/protein kinase	2.00E-05	Solyc04g072880.2.1	RNA polymerase II holoenzyme cyclin-like subunit	ch04/57430428–57431041
197	454	JZ845532	A5H7H5_9SOLA	Mitogen-activated protein kinase Naf3 (Fragment)/*Nicotiana attenuata*	7.00E-74	AT1G59580.2	ATMPK2 (*Arabidopsis thaliana* mitogen-activated protein kinase homolog 2); MAP kinase	9.00E-72	Solyc04g080730.2.1	Mitogen-activated protein kinase 9	ch04/62422222–62426785
Unknown											
202	489	JZ845534	K4CU91_SOLLC	Uncharacterizedprotein/*Solanum lycopersicum*	2.00E-10	–	–	–	Solyc09g064630.2.1	TPR domain protein Tetratricopeptide-like helical	ch09/57552194–57552682
No identity											
128	181	JZ845524	–	–	–	–	–	–	Solyc01g088660.2.1	Protein of unknown function DUF581	ch01/75186830–75186988
138	225	JZ845526	–	–	–	–	–	–	–	2600 bp downstream to the gene Solyc04g072000.2.1	ch04/56656742–56657151

Availability of the fully annotated tomato genome sequence (The Tomato Genome Consortium, 2012) allowed the resolution of partial transcripts obtained by sequencing and bioinformatic processing, overall confirming the annotation obtained through comparisons with the TAIR database. Conversely, assigned functions of similar sequences in the UniprotKB/TrEMBL and TAIR databases helped in the annotation of clones 227 and 285, classified as unknown or uncharacterised in SGN, but matching a *Lilium longiflorum* putative senescence protein and an *A. thaliana* putative D-3-phosphoglycerate dehydrogenase, respectively.

The most numerous functional categories were cell defence/stress, including 11 transcripts (27.5%), and primary metabolism, represented by 10 transcripts (25.0%). Further 5 transcripts were assigned to secondary metabolism (12.5%) and 5 to transport (12.5%) categories (Table 2). Four transcripts were catalogued in the photosynthesis and 2 in the signal transduction class. Three sequences (7.5%) showed identity with unknown genes or had no identity in the protein databases (Table 2).

The MK1-induced tomato transcripts were also annotated according to the MapMan ontology (http://ww.gomapman.org/; Supplemental Table S1). This analysis mapped several transcripts to the categories (bins) associated to the biotic and abiotic stress as well as the development, along with bins of the cell wall, lipid and photosynthesis metabolism and of phenylpropanoid and flavonol biosynthesis (Fig. 2A and B).

3.3. Identification of gene functions

Upon perception of beneficial *Trichoderma* strains, plants activate a first line of defence, including transcription of defence genes, directed to limiting potential root invasion. When the BCAs is recognised as non pathogenic, generating an incompatible interaction, defence gene expression returns in part to previous levels, unless being induced more rapidly and more intensely upon pathogen challenge, through a mechanism known as priming. As a contribution to the understanding of the molecular mechanisms activated by *Trichoderma* spp. to induce plant growth and ISR, which have not been completely clarified, we will discuss the *Trichoderma*-responsive tomato genes identified in the present work in view of their potential involvement in either ISR and/or PGP.

As highlighted above, plant colonisation by *Trichoderma* triggers transcription of defence genes, which are recruited to limiting invasion by the PGPF and/or to participating in ISR. Consistently, the most numerous functional category in our *Trichoderma*-induced tomato gene catalogue was found to be cell defence and/or stress (Table 2).

Plant interaction with MK1 induced accumulation of catalase transcripts (contig 2 and singlet 145), indicative of production of reactive oxygen species (ROS) during early stages of the tomato–MK1 interaction (Fig. 2A). Further, up-regulation of a peroxidase (singlet 174), matching a cationic peroxidase with affinity for 5-aminosalicylic acid and H_2O_2 and associated to cellular phase-dependent alteration of cell walls (Narita et al., 1995), may suggest a response to oxidative stress, although peroxidases are also involved in lignin biosynthesis, suberification and cross-linking of wall components and hence reinforcement of cell walls. ROS production is induced by a wide array of stimuli and may be responsible of severe injury to the plant. However, plants have also evolved detoxifying mechanisms that, in most instances, are effective in controlling damage and in turning ROS in signalling molecules activating defence responses (Mittler et al., 2014). It has been reported that *Trichoderma* improves ROS scavenging in colonised plants (Mastouri et al., 2012). The importance of this activity for the beneficial properties of MK1 is further supported by up-regulation of a $NADP^+$-malic enzyme (singlet 15), since these enzymes are a crucial source of NADPH during oxidative stress, in addition to being relevant for lignin and flavonoid metabolism. This finding corroborates the hypothesis that ROS detoxification contributes to the ability of MK1 to trigger ISR and improve tolerance to abiotic stresses. Accordingly, two enzymes related to malate metabolism were up-regulated in *Trichoderma asperellum* treated cucumber plants (Segarra et al., 2007). Noticeably, HYTLO1, a hydrophobin secreted by *T. longibrachiatum* MK1 as part of its Microbe-Associated Molecular Patterns (MAMPS), was found to induce transient ROS accumulation and superoxide dismutase activation in tomato leaves, as well as increased pathogen resistance (Ruocco et al., 2015), suggesting that induction of ROS as signalling molecules may also be relevant for *T. longibrachiatum* MK1-mediated ISR. Results reported for other *Trichoderma* species (Yedidia et al., 2000; Segarra et al., 2007; Shoresh and Harman, 2008; Mastouri et al., 2012; Perazzolli et al., 2012; Brotman et al., 2013) and for treatments with the *Trichoderma* hydrophobin SM1 (Djonovic et al., 2006) indicate that beneficial microorganisms exploit different ROS signalling mechanisms for improving plant tolerance to biotic and abiotic stresses.

As already mentioned, up-regulation of the peroxidase-encoding singlet 174 suggests a role of cell wall strengthening in the ISR induced by MK1. This view is reinforced by the presence, in the SSH library, of singlet 98, classified in the secondary metabolism category, whose best match is a tomato wound-inducible P450 monooxygenase (Bartoszewski et al., 2000), although it could not be aligned to the tomato reference genome. It is annotated as a cytochrome P450 monooxygenase CYP72A9 in the TAIR database, and *A. thaliana* cytochrome P450 co-expression data in the CYPedia website (http://www-ibmp.u-strasbg.fr/~CYPedia/index.html; Ehlting et al., 2006) indicate that enzymes in this family are mainly involved in the cell wall carbohydrate metabolism. Moreover, induction of phenylalanine ammonia-lyase (PAL), the key regulatory enzyme of the phenylpropanoid pathway, encoded by singlet 198, as well as of the P450 monooxygenase CYP72A13 encoded by singlet 125, which participates in the biosynthesis of phenylpropanoids (http://www-ibmp.u-strasbg.fr/~CYPedia/index.html), indicate increased biosynthesis of phenolic compounds that can be made available for lignin biosynthesis and reinforcement of cell walls. MK1-induced cell wall strengthening is further supported by increased transcription of singlet 100, showing the highest similarity with a class I *O*-methyl transferase-encoding gene of *Nicotiana tabacum*, which is induced in leaves hypersensitively reacting to TMV infection (Jaeck et al., 1996). This gene is thought to be involved in the production of syringyl units of lignin, generally considered more resistant to enzymatic degradation (Pinçon et al., 2001). Microarray analysis also suggested increased transcription of lignin biosynthesis- and cell wall assembly-associated genes in *A. thaliana* and *S. lycopersicum* interacting with *T. hamatum* (Alfano et al., 2007; Mathys et al., 2012), while proteomic studies demonstrated over-expression of proteins related to accumulation of structural carbohydrates in *T. harzianum*-treated maize seedlings (Shoresh and Harman, 2008).

According to *A. thaliana* co-expression data, CYP72A13 enzymes are also involved in the biosynthesis of isoprenoids and hence in the production of plant hormones. Similarly, systemic up-regulation of PAL transcripts (singlet 198) in response to colonisation by *T. longibrachiatum* MK1 could lead to accumulation of SA and of several defence compounds, including phytoalexins, as part of the increased resistance responses induced by the BCA. This mechanism of ISR appear to be shared by other, but not all, plant–*Trichoderma* interactions. Indeed, over-expression of PAL proteins or transcripts was reported in maize and cucumber shoots and in *A. thaliana* roots during interaction with *Trichoderma* (Shoresh et al., 2005; Shoresh and Harman, 2008; Brotman et al., 2013), but not systemically in *T. hamatum* T382-treated *A. thaliana* (Mathys et al., 2012).

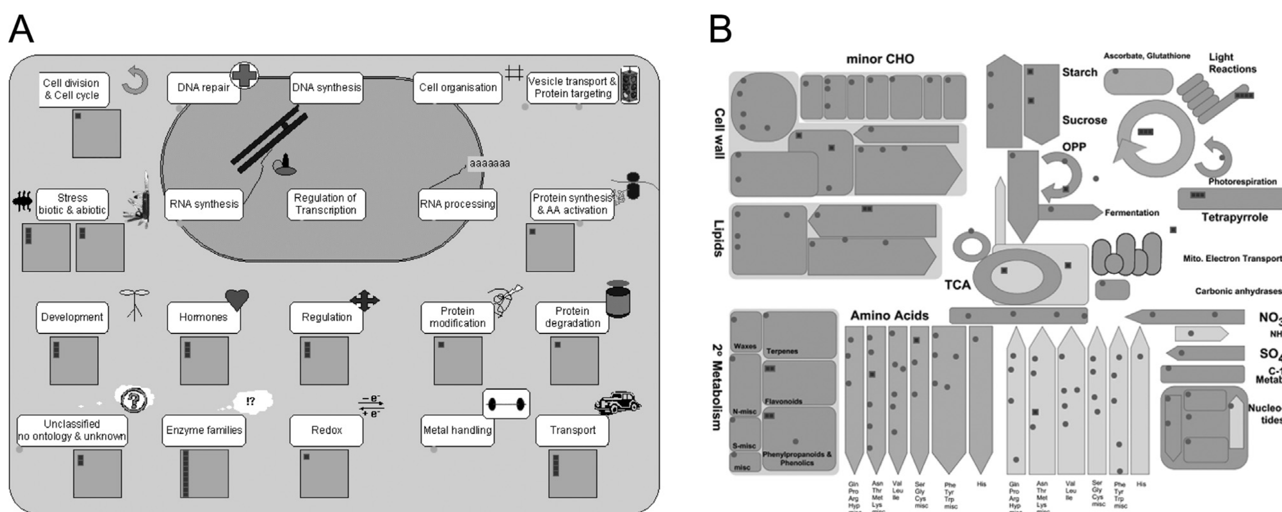

Fig. 2. MapMan overview showing all differentially expressed SSH transcripts involved in cellular functions (A) and metabolism (B). Individual tomato genes matching the SSH transcripts are represented by small squares.

The 2-oxoglutarate-dependent dioxygenase homolog encoded by singlet 263 may also participate in plant defences systemically activated by MK1, since these enzymes catalyse the biosynthesis of several secondary metabolites, including alkaloids and glucosynolates, as well as of ET and gibberellic acid (GA).

Among MK1 up-regulated defence transcripts, pathogenesis-related (PR) genes are represented by a β-1,3 glucanase (contig 3), a PR1 (singlet 12), a osmotin-like gene (singlet 292), and a possible chitinase (singlet 138), as listed in Table 2. Earlier results demonstrated augmented β-1,3 and β-1,4 glucanase and chitinase activities in cucumber plants treated with *T. harzianum* T-203 at the site of hyphae penetration (Yedidia et al., 2000), were they may be recruited to limit *Trichoderma* invasion to the first layers of cortical cells, and increased expression of the corresponding genes in systemic leaves (Shoresh et al., 2005), where they may participate in ISR. Similarly, induction of the same PR5 (Alfano et al., 2007) and of another isoform of PR1 (*PR1b1*, Tucci et al., 2011) was demonstrated in tomato interacting with other *Trichoderma* species (*i.e. T. hamatum* and *T. harzianum*). Interestingly *Trichoderma* MAMPs, including chitin, are known to contribute to ISR (Lorito et al., 2010), and increased expression of several chitinases as well as of chitin responding genes was reported in *A. thaliana* both in roots interacting with *Trichoderma* spp. and in systemic leaves (Mathys et al., 2012; Brotman et al., 2013). Therefore, triggering of PR gene transcription and chitin-induced responses appear to be active mechanisms participating to the ability of *Trichoderma* spp. to increase plant pathogen resistance through ISR.

It is worth noting that our tomato SSH gene catalogue includes singlet 89, a member of the leucine-rich repeat proteins involved in molecular recognition and/or interaction between plants and beneficial or pathogenic microbes (Tornero et al., 1996; Marra et al., 2006; Shoresh and Harman, 2008). This gene may therefore be part of a signalling cascade induced by root interaction with *Trichoderma*, resulting in activation of systemic immune responses in tomato shoots.

A heat shock protein (HSP)-encoding transcript, identified by contig 1 and singlet 83, was also induced by the MK1. Since HSPs function mainly as molecular chaperones in the control of protein folding, accumulation, localization and degradation (Priya et al., 2013), up-regulation of contig 1 and singlet 83 suggests that increased pathogen and stress resistance by the PGPF may involve mechanisms directed to preventing irreversible protein aggregation and to refolding or degrading damaged proteins. Moreover, up-regulation of HSP70 transcription could also be indicative of increased protein synthesis and turnover, which may be necessary to support PGP by *Trichoderma* (see below). Consistent with our findings, accumulation of HSP70 isoforms matching contig 1 and singlet 83 was also demonstrated by proteomic studies on maize and cucumber treated with *Trichoderma* (Segarra et al., 2007; Shoresh and Harman, 2008), while other isoforms were down-regulated (Shoresh and Harman, 2008).

Other SSH transcripts, not classified in the cell defence/stress category, may be involved, though not exclusively, in defence responses. These include singlet 232, coding for a ubiquitin conjugating enzyme, since ubiquitylation is involved not only in protein localisation and turnover (see below), but also plays a major role in the regulation of the plant response to external stimuli. Moreover, up-regulation of singlet 295, annotated as an elongation factor, may also reflect induction of a general plant adaptive response to changing environment by MK1, since several elongation factors showed increased expression after osmotic stress (Costa et al., 2010) or mechanical wounding (Morelli et al., 1994).

It is worth noting that, in the absence of any potential pathogen, the SSH library of genes, systemically induced by MK1, is enriched both of JA/ET and of SA-responsive defence genes. These results demonstrate that, in contrast to earlier results obtained with other strains (Shoresh et al., 2005; Korolev et al., 2008), MK1 activates not only JA/ET but also SA signalling, thus sharing common features with both ISR and SAR. Accordingly, persistent up-regulation of SA-mediated defence genes was shown in tomato after two-month interaction with *T. harzianum* T22, which, together with primed JA-dependent gene expression, resulted in increased pathogen resistance (Tucci et al., 2011). In fact, our results corroborate recent data demonstrating that both JA/ET as well as SA signalling are activated during the plant response to *Trichoderma* species (Segarra et al., 2007; Shoresh and Harman, 2008; Martinez-Medina et al., 2013), resulting in ISR (Mathys et al., 2012). This suggests that, opposite to previous indications, the involvement of both signalling pathways may be a common feature of most plant–*Trichoderma* interactions.

Induced transcription of contig 4, encoding a methionine synthase, could be indicative of increased ethylene production, since almost 80% of the cellular methionine is converted to *S*-adenosyl-L-methionine (SAM) (Ravanel et al., 1998), the substrate of the first committed step of the ET biosynthetic pathway. The identification of an adenosylhomocysteinase-coding gene (singlet 244) could also support MK1-mediated regulation of the SAM pool, since this enzyme catalyses conversion of *S*-adenosylhomocysteine to homo-

cysteine and adenosine and participates in the methylmethionine cycle. Thereby, it can influence the availability of activated methyl groups in the form of SAM for the biosynthesis of ET as well as of several other molecules, including DNA, lignin, and flavonoids, which can participate in defence responses. The main role of the systemic accumulation of methionine synthase in maize treated with *T. harzianum* was therefore suggested to be ET biosynthesis (Shoresh and Harman, 2008), although the ET pathway did not appear to be a major player in the *A. thaliana* response to *T. hamatum* (Mathys et al., 2012).

All the above results are strongly suggestive of an activation of the plant defence machinery by MK1 as part of the *Trichoderma*-mediated systemic induction of plant defence mechanisms against subsequent invaders, although it cannot be excluded that these defences may also be recruited to limit MK1 root tissue colonisation.

As for the growth-promoting activity of *T. longibrachiatum* MK1, several genes of the PGPF-interacting tomato SSH library could also be implicated in increased plant biomass. Among those, the above discussed up-regulation of methionine synthase could also be indicative of increased protein synthesis. This was not considered the case in *T. harzianum*-treated maize, where it was not accompanied by accumulation of other proteins involved in this process (Shoresh and Harman, 2008) nor in *T. asperellum*-treated cucumber, where protein synthesis and folding were down-regulated (Segarra et al., 2007). On the contrary, in *T. longibrachiatum* MK1-responding tomato plants methionine synthase up-regulation is paralleled by increased transcription of other genes related to protein synthesis and turnover (Table 2 and Fig. 2A and B). These include the D-3-phosphoglycerate dehydrogenase encoded by singlet 285, involved in the biosynthesis of amino acids like serine, glycine and cysteine, and singlet 232, sharing similarity with ubiquitin conjugating enzymes, thus possibly being involved in protein turnover (Fig. 2A and B). Reprogrammed and increased protein synthesis is also suggested by the up-regulation of an elongation factor (singlet 295), since these genes are over-expressed in regions of high protein synthesis (Ursin et al., 1991). Likewise, this elongation factor was induced in *T. hamatum*-treated tomato plants (Alfano et al., 2007).

Several other SSH transcripts in the primary metabolism category may also participate in MK1-induced PGP. Transketolase, encoded by contig 5, is a key enzyme involved in sugar metabolism, functioning both in the Calvin cycle for carbon fixation and in the pentose phosphate pathway, thus playing a critical role in the biosynthesis of sugars, amino acids and nucleic acids, as well as in carbohydrate degradation. Interestingly, this enzyme was also found to be over-expressed in *T. asperellum*-interacting cucumber (Segarra et al., 2007). Fructose-bisphosphate aldolase (singlet 50) catalyses reversible reactions in glycolysis, gluconeogenesis and the Calvin cycle, and was also found up-regulated by microarray analysis in *T. harzianum*-treated *Arabidopsis* plants (Moran-Diez et al., 2012). Singlets 15, annotated as a $NADP^+$-malic enzyme, and 225, coding for a oxoglutarate dehydrogenase, catalysing the conversion of 2-oxoglutarate to succinyl-CoA and CO_2 in the citric acid cycle, are crucial for respiration and production of chemical energy through ATP generation. Few studies have addressed the molecular basis of plant growth promotion by PGPFs. However, accumulation of several proteins involved in carbohydrate metabolism and in the citric acid cycle in maize in response to *T. harzianum* was proposed to be related to accelerated seedling growth (Shoresh and Harman, 2008). Similarly, increased transcription of the above genes may contribute to improved biomass production induced by MK1, as highlighted by the corresponding bins in the MapMan ontology (Fig. 2B). However, it must be highlighted that the plastid aldolase sharing highest identity with the tomato singlet 50 in the UniProtKB/TrEMBL database was isolated from drought stressed *Gossypium arboreum* and could therefore be also implicated in stress responses. Similarly, singlet 225 also contributes to the NADH pool, together with the D-3-phosphoglycerate dehydrogenase, encoded by singlet 285, thus influencing the cell redox state. Their up-regulation during tomato-MK1 interaction could therefore further support an improved ability to tolerate oxidative stress.

MK1-induced plant genes annotated in the energy metabolism category may as well be essential for the increased plant growth stimulated by the PGPF (Table 2 and Fig. 2B). Among them, two genes (singlets 298 and 276) are involved in porphyrin and chlorophyll metabolism. Singlet 298 codes for a NADPH:protochlorophyllide oxidoreductase (POR B), which catalyses the light-dependent reduction of protochlorophyllide to chlorophyllide, particularly in green plants (Holtorf et al., 1995), while singlet 276 is involved in the formation of the isocyclic ring of chlorophyll molecules. Singlet 2 encodes a chlorophyll A–B binding protein, a member of the light-harvesting complex together with chlorophylls A and B. Singlet 243 codes for RuBisCO (ribulose-1,5-bisphosphate carboxylase/oxygenase), involved in fixing carbon dioxide in the first step of the Calvin cycle. Retrieving of these genes among the up-regulated transcripts in MK1-treated plants confirms previous findings in cucumber and maize (Segarra et al., 2007; Shoresh and Harman, 2008), and further corroborates the hypothesis that the establishment of the tomato–MK1 interaction triggers increased photosynthetic light energy capture, which can in turn support energy needs for increased growth and improved resistance against pathogens.

Overall, the above results on the tomato transcriptomic response to MK1 colonisation indicate that the growth promotion properties of the PGPF (Ruocco et al., 2015) could be mediated by reprogramming of the cellular protein set, increased sugar synthesis and activation of photosynthesis and energy metabolism.

Along with the genes discussed so far, the SSH library included transport and transporters related transcripts (Table 2 and Fig. 2A), which may have a role in defence signalling and in the transport of molecules necessary for the establishment of microorganism interaction. Among them, a gene coding for an ABC transporter (singlet 223) was found to be up-regulated in tomato by MK1. This gene could be relevant for the tomato response to the PGPF, since several plant ABC transporters are involved in secreting toxic secondary metabolites with protecting roles against pathogen attack, as well as signalling molecules fundamental for the establishment of microorganism interactions (Ruocco et al., 2009, 2011). A possible role of specific ABC transporters in plant–*Trichoderma* interactions is supported by the finding that other ABC transporter genes are up- or down-regulated in tomato following treatment with *T. hamatum* (Alfano et al., 2007). MK1 root treatment also activated the expression of a lipid transfer protein (LTP) encoding gene (singlet 188), similarly to what was observed for *LTP b1* in rice roots during colonization by the arbuscular mycorrhizal fungus *Glomus mosseae* (Blilou et al., 2000), but in contrast with strongly reduced expression of the *LTP4* gene in *Arabidopsis* roots treated with *T. asperelloides* T203 (Brotman et al., 2012). Further, up-regulated genes in this category included transcripts involved in the primary (H^+) and secondary transport (ions and organic compounds). Singlets 156 and 278 are members of the proton-pumping ATPase (H^+-ATPase) system that generates the proton motive force across plant plasma membrane necessary to activate most of the ion and metabolite transport. As such, in MK1-colonised tomato they may be essential for the uptake of metabolites contributing to plant–microbe interaction.

Finally, MK1 treatment activated the expression of two genes involved in signal transduction processes. Singlets 9 and 197 encode for a RNA polymerase II holoenzyme cyclin-like subunit and a MAPK9, respectively, indicating that they may be related to the initial events of recognition of the beneficial microorganism and of

induction of resistance, similarly to what proposed for *T. harzianum* T39-induced resistance in susceptible grapevines (Perazzolli et al., 2012).

3.4. Interrogation of expression databases

To further investigate the *S. lycopersicum* sequences up-regulated during interaction with *T. longibrachiatum* MK1, a BLASTn search was carried out against the dbEST database. This allowed detecting transcripts from our collection that were associated to ESTs from publically available stress-related tomato libraries. The distribution of ESTs associated to each of our transcripts at the filtering conditions implemented for this analysis (70% coverage, 95% identity) is reported in Table 3. A total of 373 ESTs from abiotic and biotic stress-related libraries were found to match with our transcripts. It is evident that the majority of matches comes from biotic stress-related libraries, which contain the larger number of ESTs, while transcript 227 is the only one recursively associated to ESTs from abiotic stresses. Interestingly, many differential transcripts from our library did not found extensive matches in EST repositories, revealing that they are indeed rare transcripts with small differences in the expression levels, as we also confirmed by real time analysis in some cases (see below).

Further, we also interrogated microarray data of *A. thaliana* orthologs through Genevestigator (www.genevestigator.com). The stimuli that mainly induce expression of *Arabidopsis* orthologs are reported in Fig. 3. This analysis showed that infection by the biotrophic pathogen *Pseudomonas syringae* or the necrotrophic pathogen *B. cinerea* as well as treatment with the phytohormone salicylic acid (SA) trigger transcription of most *Arabidopsis* orthologs (40%). Interestingly, besides SA, other phytohormones, *i.e.* ET, GA and ABA, induce expression of 15–24% of the *A. thaliana* orthologous transcripts, suggesting possible communication between different response pathways. These results further corroborate recent lines of evidence indicating that priming of defence responses induced by *Trichoderma* spp. is not dependent solely on JA/ET mediated gene expression (Tucci et al., 2011; Mathys et al., 2012; Martinez-Medina et al., 2013), but requires cross-talking between different signalling pathways. This network may contribute to fine tune the enhancement of plant growth and resistance responses induced by beneficial *Trichoderma* strains at a low energy cost.

3.5. Gene expression by quantitative RealTime PCR

Validation of the SSH results through qRT-PCR of 4 randomly selected cDNAs confirmed all sequences to be up-regulated, though only slightly (1.3–2.6 fold), after 48 h of interaction with MK1 and demonstrated that the subtraction process of the SSH technique used in this study was successful in selecting over-represented transcripts, including low-abundance differentially expressed cDNAs (Fig. 4). These results indicate that the onset of the symbiosis with *Trichoderma* does not induce dramatic transcriptional changes in the plant in terms of expression levels, suggesting that the beneficial effects of the PGPF are exerted in an energy-effective way and confirming similar findings for other beneficial *Trichoderma*–plant interactions (Alfano et al., 2007; Moran-Diez et al., 2012), while the number of affected genes can be quite high.

Since annotation of SSH clones as well as similarity search against tomato ESTs and *Arabidopsis* microarray data clearly suggested the induction of a systemic defence response during the first stages of tomato interaction with MK1, we characterised the transcription profile of some defence-related SSH transcripts, along with the marker gene *PR1b1*, through a time-course qRT-PCR analysis of *in vitro* plantlets at 1, 2 and 3 DPI and of *in vivo*-grown plants after 2 months from the MK1 treatment (Fig. 5). Transcription of catalase and glucanase (contig 2 and 3) and *PR1b1* increased during the first hours of the tomato–MK1 interaction and remained up-regulated for up to 2 months (Fig. 5), possibly contributing to the enhancement of disease resistance induced by MK1. Interestingly, expression of methionine synthase, methyltransferase and phenylalanine ammonia-lyase (*PAL*), namely contig 4, and singlets 100 and 198, which were classified in metabolism categories but are

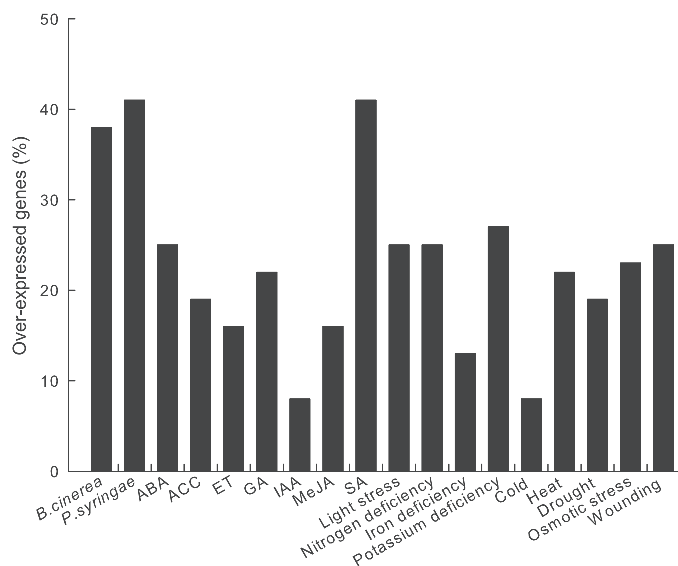

Fig. 3. Abiotic and biotic stimuli driving expression of *A. thaliana* genes orthologous to the tomato SSH transcripts. Bars indicate the percentage of *Arabidopsis* orthologs responding to a specific stimulus over the total number of *Arabidopsis* genes orthologous to the up-regulated transcripts in tomato-MK1 interaction. ABA, abscisic acid; ACC, 1-Aminocyclopropane-1-carboxylic acid (ACC); ET, ethylene; GA, gibberellic acid; IAA, indoleacetic acid; MeJA, methyl-jasmonate; SA, salicylic acid.

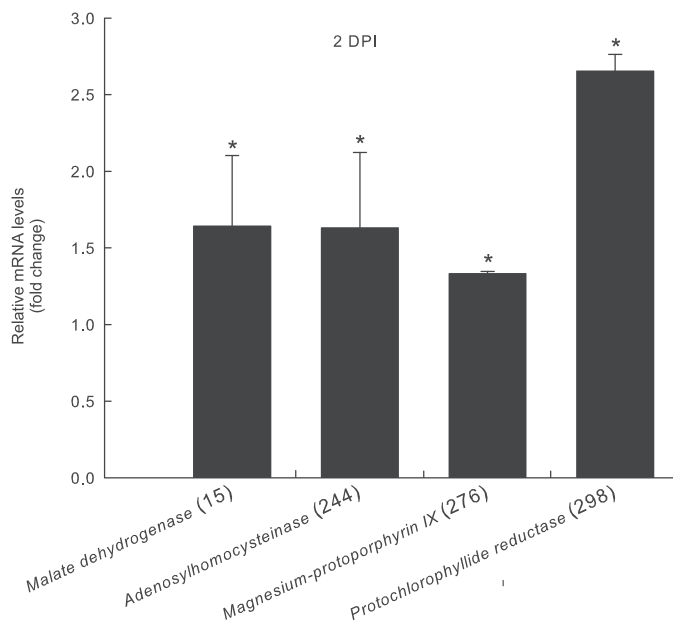

Fig. 4. Validation of the subtraction efficiency through qRT-PCR of randomly selected SSH clones. The expression levels of *Malate dehydrogenase*, *Adenosylhomocysteinase*, *Magnesium-protoporphyrin IX* and *Protochlorophyllide reductase* genes are reported as means of their fold increase relative to that of the control plants (=1) ± SD (*n*. of biological replicates = 4). SSH clone identifiers are indicated in parentheses. The tomato *actin* gene was used as an internal control to normalize the expression level. Asterisks indicate statistically significant differences between MK1-treated and control plants (Student's *t*-test, $P < 0.05$).

Table 3
Distribution of the tomato ESTs associated to the SSH transcripts identified in this study. The number of ESTs matching the SSH transcripts is indicated for each tomato stress-associated library (A: Abiotic, B: Biotic stresses). ID indicates the library identifier in dbEST.

EST libraries				Transcripts																																									
Type of stress	ID[a]	Description	Size	Cell defence/stress											Primary metabolism										Secondary metabolism					Transport					Energy metabolism				Signal trans-duction		Un-known	No hits		N. of hits	N. of tran-scripts
				C1	83	C2	145	C3	12	89	174	227	240	292	C4	C5	15	50	225	232	244	249	285	295	98	100	125	198	263	81	156	188	223	278	298	276	2	243	9	197	202	128	138		
A[b]	15371	Subtractive library root under excess copper	53								1	1																																2	2
A	24564	Drought Stressed leaves	66									6																																6	1
A	27417	Heat stressed leaf	75									13																																13	1
A	19204	ZS-5 Reverse SSH library salt stress	123					1				10			2																													13	3
A	19202	LA2711 Reverse SSH library salt stress	150					2				10																	1															13	3
A	19203	ZS-5 Forward SSH library	152									2																																2	1
A	18162	Subtractive library heat-shock down-regulated genes	154		1																												1											2	2
A	19201	LA2711 Forward SSH library salt stress	171									2																																2	1
A	152268/015365	Seedlings treated with Cadmium	789			1						11																									1							13	3
A	15349	Subtractive library roots under phosphate starvation	823							1																																		1	1
A	2537	Root deficiency, Al, Zn, P, K, F	1007																					7				2												1				10	3
B	22811	Plant treated with RNMV infected rice leaf sap	12																										1															1	1
B	23304	Fruit SSH library	50											1																														1	1
B	25492	Leaf inoculated with *Phytophthora infestans*	113																	1																								1	1
B	18166	Forward subtractive library inoculated with *Ralstonia solanacearum*	333				1																			1																		2	2
B	18165	Reverse subtractive library inoculated with *Ralstonia solanacearum*	385	1			4								4					1																								10	4
B	24222	PAMP-elicited in leaf	591																																	1		3						4	2
B	8633	Tomato crown gall	5204			3	3	2	2	2										1	6	2		5	1	1		1		1														30	13
B	2476	Tomato mixed elicitor	10016	1		1	3		6	3				2				12		3				3	3			1	4			1			2	4	10	146						205	17
B	27146	Micro-Tom root	27146			7		1	1					2				9		4	2	1		6				1								3					5			42	12
N. of hits				2	1	12	11	6	9	6	1	55	0	5	6	0	0	21	0	10	8	3	0	21	4	2	0	5	6	1	0	1	1	0	2	8	11	149	0	1	5	0	0		
N. of libraries				2	1	4	4	4	3	3	1	8	0	3	2	0	0	2	0	5	2	2	0	4	2	2	0	4	3	1	0	1	1	0	1	3	2	2	0	1	1	0	0		

[a] Library ID in dbEST.
[b] A, abiotic stress-associated library; B, biotic stress-associated library.

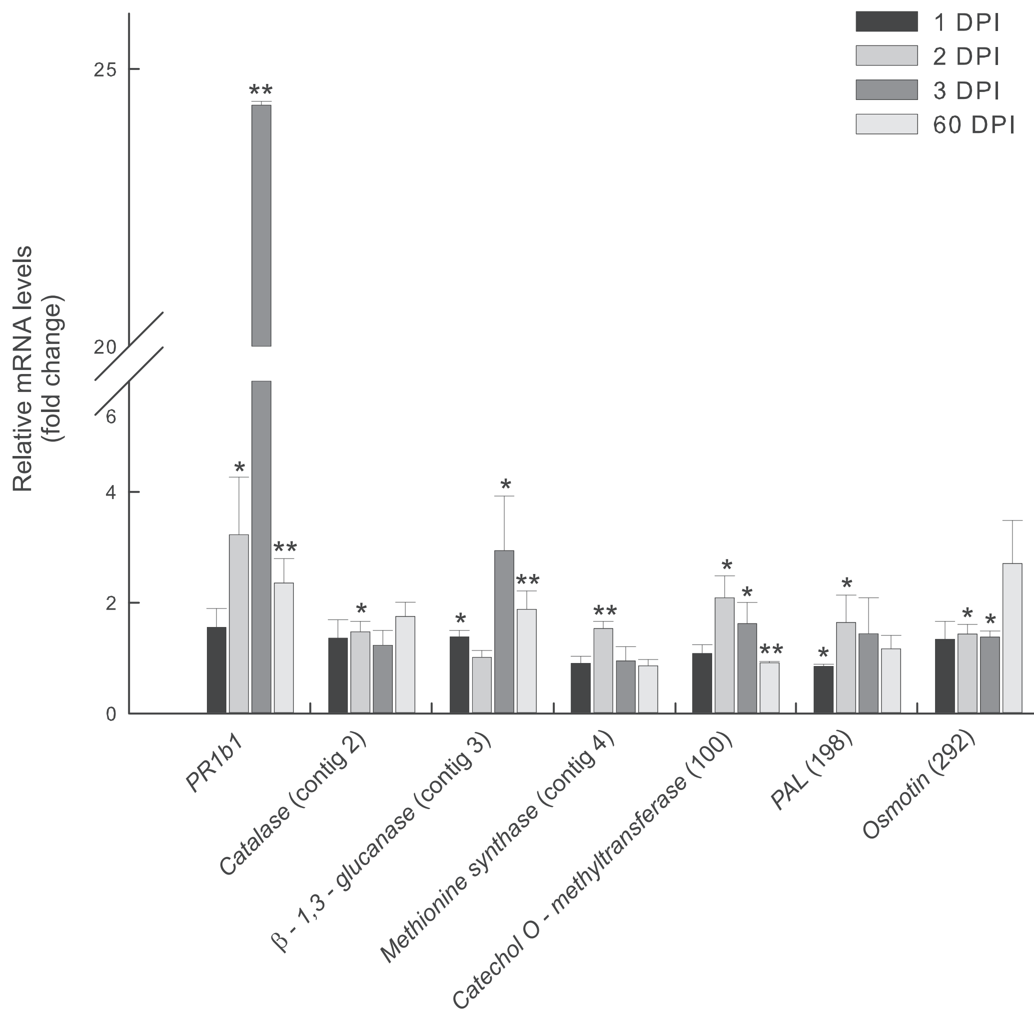

Fig. 5. Induction of systemic defence responses in tomato plants after 1, 2, 3, and 60 days of interaction (DPI) with *T. longibrachiatum* MK1. The expression levels of defence-related genes, determined through qRT-PCR, are reported as means of the fold increase relative to that of the control plants (=1) at each time point ± SD (*n.* of biological replicates = 3). SSH clone identifiers are indicated in parentheses. The tomato *actin* gene was used as an internal control to normalize the expression level. Asterisks indicate statistically significant differences between MK1-treated and control plants for each gene at each time point (Student's *t*-test, $^{*}P < 0.05$; $^{**}P < 0.01$).

known to be also involved in defence responses, increased at early stages of the interaction, but not after 2 months, thus suggesting they may specifically participate in the early defence responses to limit plant tissue colonization by MK1 (Fig. 5).

The role of the same defence-related SSH genes was further investigated in response to biotic stress by testing their expression during infection by the pathogen *B. cinerea* of MK1-treated and control plants. After 48 h of *B. cinerea* inoculation, transcription of most defence genes (contigs 2 and 3 and singlet 292) as well as of *PAL* (clone 198) significantly increased in both control (C + B) and MK1-treated (T + B) plants as compared to their expression in the untreated uninfected control (C), which was set equal to 1 (Fig. 6). The expression levels of glucanase and osmotin-like genes (contig 3 and singlet 292) were higher in MK1-treated than in control plants after *B. cinerea* inoculation, indicating that they may participate in the increased resistance to the pathogen observed in tomato plants interacting with the PGPF (Fig. 6). Moreover, catalase, *PAL* and *PR1b1* showed a slightly reduced expression in MK1-treated than in control plants after inoculation with *B. cinerea*, confirming that the PGPF ability to reduce pathogen symptoms is achieved at a diminished intensity of some plant responses, as already reported (Tucci et al., 2011). This effect is noticeable for catalase, since detoxification of ROS induced by the pathogen is very important for resistance to *B. cinerea* (Elad 1992; Govrin and Levine, 2000; Temme and Tudzynski, 2009). On the contrary, down-regulation of methionine synthase and methyltransferase (contig 4 and singlet 100) by *B. cinerea* infection both in control (C + B) and in *Trichoderma*-treated (T + B) plants confirmed that their induction during the first hours after MK1 treatment could be part of the specific early response to the PGPF.

4. Conclusions

The subtractive library approach presented in this paper resulted in the compilation of an inventory of 40 tomato genes responding to colonisation by the rizhosphere fungus *T. longibrachiatum* MK1, enriched with transcripts displaying even small differences in gene expression levels. This technique, together with the use of a crop model species, such as tomato, and of the growth-promoting, ISR-inducing and bioactive molecules-producing MK1 strain, hold promise that this gene catalogue will represent a valid contribution to the investigation of plant systemic responses to the interactions with beneficial components of the soil microbiome, which are often accompanied by small perturbations in the gene

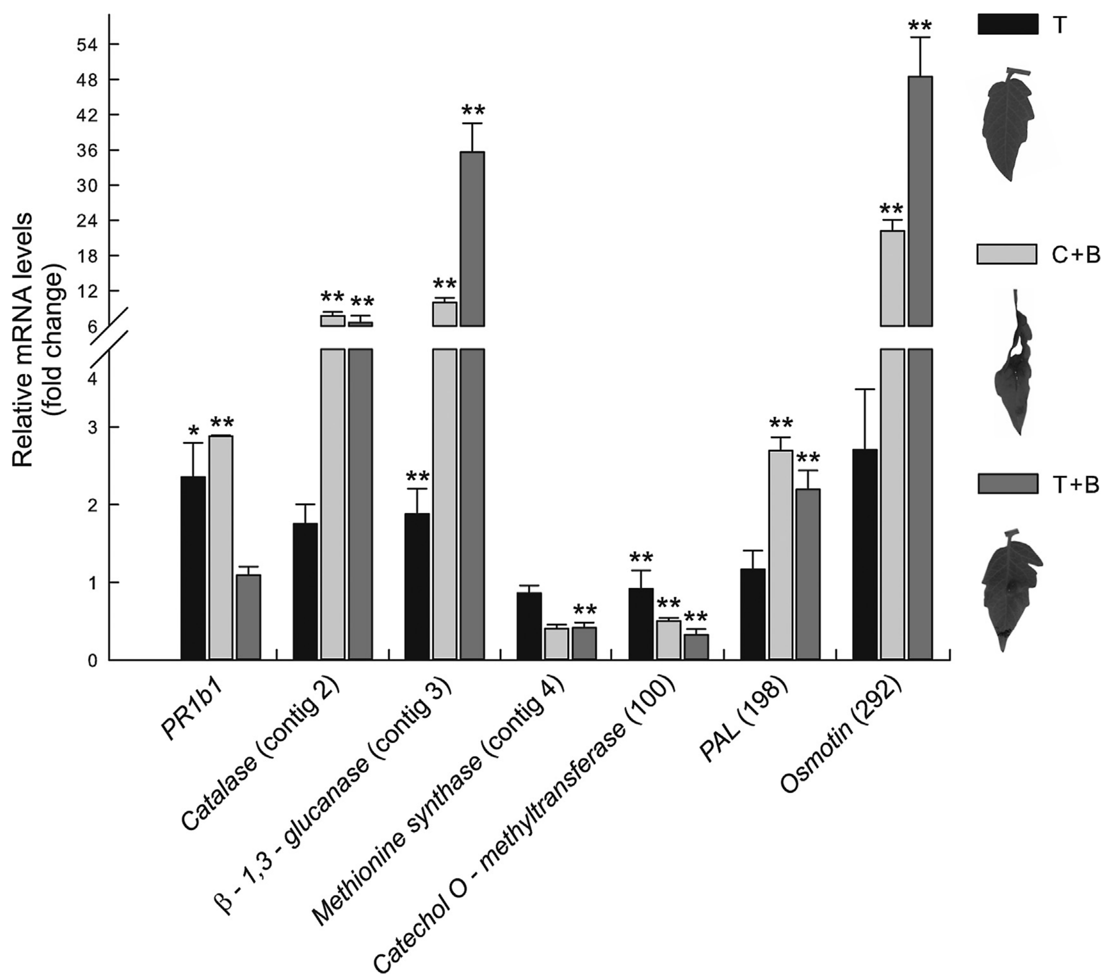

Fig. 6. Induction of systemic defence responses in MK1-treated tomato plants after infection with *B. cinerea*. Transcription of defence-related genes was determined through qRT-PCR in plants interacting with MK1 for 60 DPI, not infected (T) or infected (T + B) for 48 h with *B. cinerea*, along with plants not treated with MK1 (C+B). Gene expression levels are reported as means of the fold increase relative to that of MK1-untreated, pathogen-uninfected control plants (=1) ± SD (*n*. of biological replicates = 3). SSH clone identifiers are indicated in parentheses. The tomato *actin* gene was used as an internal control to normalize the expression level. Asterisks indicate statistically significant differences between the different treatments and control plants (Student's *t*-test, $^{*}P < 0.05$; $^{**}P < 0.01$). Representative phenotypes of not infected and *B. cinerea*-infected leaves are also shown.

transcription rate. To date, investigation of the plant response to *Trichoderma* species by RNA-sequencing approaches is only available for grapevine (Perazzolli et al., 2012). Further RNA-sequencing data may prove useful in confirming our findings with larger datasets.

The genes up-regulated in tomato by MK1 and identified in this study could be divided in two main groups. The first includes genes that are involved in cell wall reinforcement, ROS scavenging, as well as in the synthesis of defence compounds and hormones. The second group includes genes relevant for protein synthesis, localisation and turnover, along with genes influencing carbohydrate metabolism and photosynthesis. These mechanisms appear therefore crucial for the ability of beneficial *Trichoderma* strains to activate ISR and/or plant growth. Our findings also indicate that some responses may be specific to the interaction tomato–*T. longibrachiatum* MK1.

Comparison of early *vs* late responses to the PGPF through transcriptional profiling allowed listing a number of genes that may include those necessary for controlling tissue colonization by MK1, or pathogen invasion or both. It is tempting to speculate that the observed return to the level of the untreated control of the transcription rate of some genes, two months after the first contact with *T. longibrachiatum* MK1, is due to the full establishment of the mutualistic interaction with the PGPF, also considering that the same genes were not activated by pathogen infection. These genes could be specifically involved in limiting a potentially aggressive behaviour of some root-colonizing *Trichoderma* (Lace et al., 2015).

Transcript annotation as well as interrogation of available ESTs and microarray data demonstrated that different signalling pathways interact to activate the ISR and growth-promoting response induced in tomato by MK1. These results add further support to the recently accumulating evidence that the JA/ET pathway is not the only one operating in plant–PGPF interactions.

Our results may help improving the exploitation of beneficial fungi, and identifying gene markers for selecting plant genotype–rhizosphere microorganism combinations particularly effective both in terms of increased growth and improved pathogen resistance.

Acknowledgements

This work was partially supported by research grants from the Italian Ministries of Education, University and Research (projects GenoPOM, D.D. 14/03/2005 prot. 602, and CL.A.N. Agri-Food CTN01_00230_248064). The Authors acknowledge that M.L. Chiusano was responsible for the coordination of bioinformatics in this work and thank Mr. G. Guarino, R. Nocerino and A. Scafarto of the CNR-IBBR for excellent technical assistance.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jplph.2015.11.005.

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