Plant Mol Biol (2014) 84:345–357
DOI 10.1007/s11103-013-0142-0

Expression of Terpenoids 1, a glandular trichome-specific transcription factor from tomato that activates the terpene synthase 5 promoter

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Received: 23 July 2013 / Accepted: 7 October 2013 / Published online: 20 October 2013

Abstract Terpene biosynthesis in tomato glandular trichomes has been well studied, with most if not all terpene synthases (TPSs) being identified. However, transcription factors (TFs) that regulate TPSs have not yet been discovered from tomato. In order to unravel the transcriptional regulation of the *Solanum lycopersicum* linalool synthase (*SlMTS1*, recently renamed *SlTPS5*) gene in glandular trichomes, we functionally dissected its promoter. A 207 bp fragment containing the minimal promoter and the 5′UTR appeared to be sufficient for trichome-specific expression in transgenic plants. Yeast-one-hybrid screens with this fragment identified a glandular trichome-specific transcription factor, designated Expression of Terpenoids 1 (*SlEOT1*). SlEOT1 is a member of a conserved family of TFs that includes the *Arabidopsis Stylish 1* (*AtSTY1*) and *Short Internode* (*AtSHI*) genes. The EOT1 protein localized to the nucleus and specifically transactivated the *SlTPS5* promoter in *Nicotiana benthamiana* leaves.

Keywords Glandular trichomes · Promoter · Terpene biosynthesis · Tomato · Transcription factor

Electronic supplementary material The online version of this article (doi:10.1007/s11103-013-0142-0) contains supplementary material, which is available to authorized users.

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Introduction

Plants produce a plethora of isoprenoids (or terpenoids), which constitute a large, structurally diverse class of chemical compounds. Terpenoid molecules occur both in primary and in secondary metabolism. As products of primary metabolism they can be found in membranes (phytosterol), are building blocks of photosynthetic pigments (carotenoids and phytol side chain of chlorophyll) or hormones (strigolactones, gibberellins, brassinosteroids, abscisic acid). However, the majority of plant isoprenoids are known as secondary (also called specialized) metabolites (Lange et al. 2000; Flores-Perez et al. 2010). These specialized metabolites are non-volatile or volatile chemicals that can be sequestered in the plant or secreted in the environment serving various roles (Langenheim 1994). Non-volatile compounds can function as part of the plant's direct defenses either by inhibiting or suppressing growth of pathogens (Ahuja et al. 2012), or by exhibiting toxic or deterrent effects against herbivores (Balkema-Boomstra et al. 2003; Heiling et al. 2010; Bleeker et al. 2012). Some volatile terpenoids function as attractants of pollinators, seed dispersers and other beneficial animals (Pichersky et al. 1995) or can play a role in plant–plant communication: when released from damaged plants they can induce defense responses in neighboring plants (Arimura et al. 2000). Furthermore, many of the volatile terpenoids are emitted by herbivore-infested plants and serve as attractants of natural enemies (predators and parasitoids), thus providing indirect defense against herbivores. These compounds are either released when feeding ruptures the structures in which they are stored (i.e. glandular trichomes) or when synthesized de novo at different time points after the onset of herbivore feeding (Pichersky and Gershenzon 2002). Induced terpene synthesis after wounding or herbivore attack has been reported from

numerous plant species, including tomato (van Schie et al. 2007), maize (Schnee et al. 2002, 2006), poplar (Arimura et al. 2004a), lotus (Arimura et al. 2004b), cucumber (Mercke et al. 2004) and medicago (Gomez et al. 2005; Navia-Gine et al. 2009). As indicated in the examples above, regulation of induced terpene biosynthesis occurs mostly at the transcriptional level of the terpene synthases (TPSs), precursor biosynthesis genes (Sanchez-Hernandez et al. 2006; Paetzold et al. 2010) and/or downstream modifying enzymes (Son et al. 1998; Luo et al. 2001).

Relatively few transcription factors (TFs) that regulate terpenoid biosynthesis have been identified to date (De Geyter et al. 2012). Stress-induced biosynthesis of terpenoids has been well-studied in *Catharanthus roseus*, where a methyl-jasmonate (MeJA)-inducible transcription factor of the MYC family (*CrMYC2*) was found to regulate at least two TFs of the AP2 family (*ORCA2* and *ORCA3*) that in turn regulate expression of *Strictosidine Synthase* (*STR*) involved in terpene indole alkaloid biosynthesis (Zhang et al. 2011). The MeJA-inducible *Arabidopsis* MYC2 transcription factor was recently shown to regulate the sesquiterpene synthases AtTPS21 and AtTPS11 (Hong et al. 2012). Two JA-responsive AP2 family transcription factors from *Artemisia annua* (AaERF1 and 2) were shown to regulate the Amorpha-4,11-diene synthase (ADS), a sesquiterpene synthase involved in the biosynthesis of artemisinin (Yu et al. 2012). More recently another AP2/ERF TF from *A. annua* (*AaORA*) was identified. This trichome-specific TF was shown to positively regulate several genes in the artemisinin biosynthetic pathway, including *AaERF1* (Lu et al. 2013). Other examples related to terpene biosynthesis describe the identification of WRKY proteins of the zinc finger family from various plant species (*Gossypium arboreum*, Xu et al. 2004; *Nicotiana attenuata*, Skibbe et al. 2008; *Artemisia annua*, Ma et al. 2009; *C. roseus*, Suttipanta et al. 2011).

In tomato, the first monoterpene synthase identified (*SlMTS1*; van Schie et al. 2007; renamed *SlTPS5*; Falara et al. 2011) was a JA-inducible linalool synthase, expressed specifically in trichomes. However nothing is known about the tissue-specific regulation of this gene. A total of 44 terpene synthase (TPS) genes have been identified in the draft genome sequence of *Solanum lycopersicum* cv. Heinz (The Tomato Genome Consortium 2012), out of which 29 appear to have uncompromised open reading frames (containing no mutations or deletions). For 14 of these genes transcripts have been identified (also) in trichomes (Falara et al. 2011; Matsuba et al. 2013).

In order to identify transcription factor(s) involved in the regulation of *SlTPS5* (and possibly other *SlTPS* genes) we functionally dissected its promoter and screened for interactors of the promoter fragment conferring trichome specificity. We report the characterization of a novel zinc finger transcription factor from *S. lycopersicum*, EOT1. It contains a RING finger-like conserved N-terminal domain and a less conserved C-terminal IGGH domain found in proteins of many plant species. SlEOT1 is a trichome-specific transcription factor and is a member of a small gene family. In a transient transactivation assay in leaves of *Nicotiana benthamiana*, SlEOT1 specifically targets the *SlTPS5* promoter. Its potential role in plant defenses will be discussed.

Materials and methods

Construct design and stable transformations

1,254 bp of the genomic sequence (from cv. Moneymaker) upstream of the start codon of *SlTPS5* (AY840091) were cloned between restriction sites *Sac*I (5′) and *Xba*I (at the 3′ end of the sequence just prior to the ATG) replacing the 35S promoter in vector pJVII, a pMON999-based vector (Monsanto, St. Louis, MO) with a modified multiple cloning site, upstream of the ATG start codon of *uidA* (GUS) fused to yellow fluorescent protein (sYFP1) and followed by the *Nos* terminator (t*Nos*). Five 5′ deletion constructs were generated by designing forward primers at positions −1,046, −806, −612, −408 and −207 distal to the ATG and a reverse primer at position −18 (Fig. S1). These five PCR products were cloned in vector pJVII using the same restriction sites as for the full-length promoter and after verifying by sequencing, the expression cassettes were transferred to the binary vector pBINplus (van Engelen et al. 1995) by digesting with restriction enzymes *Sac*I and *Sma*I and ligating at the same restriction sites in the multiple cloning site (MCS) of pBINplus. These six constructs and an empty pBINplus vector were introduced to *Agrobacterium tumefaciens* strain EHA105 and used to create transgenic plants using explants derived from cotyledons of sterile seedlings of *Solanum lycopersicum* cultivar Moneymaker, as previously described (Cortina and Culianez-Macia 2004).

The coding sequence of *SlEOT1* was cloned in the pKG1662 vector (KeyGene, Wageningen, NL; for a map of the vector see patent nr US2011/0113512A1) between restriction sites *Nco*I (at the ATG) and *Sac*I (after the stop codon) followed by the *Nos* terminator (t*Nos)* and driven by the CaMV 35S promoter. 1,961, 1,236 and 1,398 bp genomic sequence upstream of the start codon of *SlTPS3*, *SlTPS7* and *SlTPS8* respectively, were cloned in pKG1662 between restriction sites *Hin*dIII (5′) and *Nco*I (at the 3′ end of the sequence just prior to the ATG) replacing the 35S promoter upstream of *uidA* (GUS) followed by t*Nos*. Similarly, 1,557 and 1,270 bp of the genomic sequence upstream from the start codon

of *SlTPS9* and *SlEOT1*, respectively, were cloned in vector pJVII. The first was cloned between restriction sites *Sac*I (5′) and *Nhe*I (at the 3′ end of the sequence just prior to the ATG) and the second between *Hind*III (5′) and *Xba*I (at the 3′ end of the sequence just prior to the ATG) replacing the 35S promoter, upstream of the ATG start codon of GUS fused to yellow fluorescent protein (sYFP1) followed by the t*Nos*. All constructs were verified by sequencing and then the expression cassettes were transferred to the MCS of the binary vector pBINplus (van Engelen et al., 1995) between *Hind*III and *Sma*I restriction sites for SlTPS3p, SlTPS7p, SlTPS8p and SlEOT1p and *Sac*I and *Sma*I restriction sites for SlTPS9p. The final constructs were transformed to *A. tumefaciens* GV3101 (pMP90).

A mutation was introduced in the YUC-like element (bases −110 to −104 from the start codon; Fig. S1) of the 207 bp *SlTPS5* promoter fragment (ACTCTAC->ACAAAAC). This fragment containing a 5′ *Hind*III and a 3′ *Nco*I restriction site was synthesized by Eurofins MWG Operon (Germany) and cloned into vector pCR2.1-TOPO (Invitrogen, Paisley, UK). After verifying by sequencing, it was digested between these sites and used to replace the full-length *SlTPS5* promoter in the pKG1662_SlTPS5p:GUS construct (see above). The expression cassette was then transferred to the MCS of the binary vector pBINplus (van Engelen et al. 1995) between *Hind*III and *Sma*I restriction sites. The final construct was transformed to *A. tumefaciens* GV3101 (pMP90).

Analysis of transgenic plants

One transgenic line was obtained with the empty pBINplus vector, four independent transgenic lines with the full-length (1,254 bp) *SlTPS5* promoter construct, three with the 1,046 bp *SlTPS5* promoter construct, five with the 806 and 612 bp *SlTPS5* promoter constructs, eight with the 408 bp *SlTPS5* promoter construct and nine with the 207 bp *SlTPS5* promoter construct. Presence of the transgenes was verified by PCR (target gene *GUS*) on genomic DNA isolated from leaves of the different T0 lines. For quantification of GUS expression five plants of each independent T1 line were grown in soil for 4 weeks. Expression of the transgenes was verified by YFP observation under a fluorescence stereomicroscope and one plant was chosen from each line. Trichomes were collected at the bottom of a 50 ml tube by vortexing stem pieces frozen in liquid nitrogen. Crude extracts were prepared according to Jefferson et al. (1987) and the enzymatic GUS activity was determined spectrophotometrically using 4-methylumbelliferyl-β-D-glucuronide (MUG) as substrate. Values were expressed relative to protein content, measured using Bradford reagent.

Hormone treatment, RNA isolation and cDNA synthesis

Tomato plants were grown in soil in a greenhouse with day/night temperatures of 23/18 °C and a 16/8 h light/dark regime for 4 weeks. Jasmonic acid (JA; Duchefa, NL) was applied to plants by spraying 1 mM solution made with tap water containing 0.05 % SilwetL-77 (GE Silicones, VA, USA). Control plants were sprayed with tap water containing 0.05 % SilwetL-77. Trichomes were collected at various time points as described above. Other tissue-types analyzed were also frozen in liquid N_2 and then the material was ground and total RNA was isolated using Trizol (Invitrogen, Paisley, UK). DNA was removed with DNAse (Ambion, Huntingdon, UK) according to the manufacturer and cDNA was synthesized from 1.5 μg RNA using M-MuLV H^- Reverse Transcriptase (Fermentas, St. Leon-Rot, Germany).

Quantitative real time PCR

For Q-RT-PCR cDNA equivalent to 100 ng total RNA was used as template in 20 μl volume and reactions were performed in the ABI 7500 Real-Time PCR System (Applied Biosystems) using the Platinum SYBR Green qPCR SuperMix-UDG kit (Invitrogen, Paisley, UK) with the following cycling program: 2 min 50 °C, 7 min 95 °C, 45 cycles of 15 s at 95 °C and 1 min at 60 °C, followed by a melting curve analysis. Primer pairs were tested for amplification kinetics and linearity with a standard cDNA dilution curve and new primers were designed if necessary. Expression levels were normalized using *ACTIN* (SGN-U579547) mRNA levels. Effects of JA in gene expression were analyzed in three biological replicates by ANOVA using PASW Statistics 17.0 (http://www.spss.com). The homogeneity of variance was tested by Levene's test and values were log transformed before the analysis if necessary.

Yeast-one-hybrid screens

In order to be used as target binding sequence in yeast-one-hybrid screens, the 207 bp *SlTPS5* promoter fragment was cloned between restriction sites *EcoR*I (5′) and *Xba*I (at the 3′ end of the sequence just prior to the ATG) in the MCS of the yeast vector pHISi (Clontech, Mountain View, CA, USA) upstream of the minimal promoter of *HIS3*. The *HIS3* gene of pHISi was used as selectable marker for integration into the nonfunctional *his3* locus of *Saccharomyces cerevisae* strain PJ69-4A. The vector was linearized at the *Xho*I restriction site before integration. Leaky *HIS3* expression enabled enough colony growth, making it possible to use this as a selectable marker. Background growth was controlled during library screening by the addition of 5 mM 3-amino-1,2,4-triazole (3-AT) in the selective medium

(synthetic dextrose, SD). For the construction of the cDNA library used, trichomes were shaken off frozen stem pieces of tomato *S. lycopersicum* cv. Moneymaker, RNA was isolated using the RNeasy Plant kit from Qiagen (Valencia, CA, USA). The cDNA library was constructed according to the instructions of the HybriZAP −2.1 XR library and HybriZAP −2.1 XR cDNA synthesis kit from Stratagene (Cedar Creek, Texas, USA). The size of the primary cDNA library was 3.0×10^6 pfu. The primary library was subsequently amplified and mass excised as described by the manufacturer. Screening of the library was performed using 20 μg library plasmid according to the Clontech Matchmaker One-Hybrid System user manual. Transformation efficiency was on average 10^5 colony forming units (cfu) per μg DNA. Positive colonies were transferred on medium containing higher 3-AT concentration (10 and 15 mM) and plasmid was isolated and sequenced from colonies that could grow on these stringent selection media.

Gateway cloning and confocal microscopy

Gateway technology (Invitrogen, Paisley, UK) was used for the construction of the CaMV 35S-driven SlEOT1—fluorescent protein fusion. The SlEOT1 ORF excluding the stop codon was PCR-amplified introducing the attB recombination sites and subsequently recombined in pDONR207 (BP reaction; Invitrogen, Paisley, UK) creating SlEOT1-pENTR, which was verified by sequencing. For the C-terminal fusion with mVenus (Nagai et al. 2002), SlEOT1-pENTR was recombined in a destination vector (LR reaction; Invitrogen, Paisley, UK) along with pGEM Box1 CaMV 35S and pGEM Box3 mVenus-t*Nos*. The construct was transformed to *A. tumefaciens* GV3101 (pMP90). Agroinfiltration of 5-week-old *N. benthamiana* leaves was performed as described by Verweij et al. (2008). A separate *A. tumefaciens* GV3101 (pMP90) culture harboring mCherry with a N-terminal nuclear localization signal (NLS; Grebenok et al. 1997) followed by t*Nos* (Galvan-Ampudia, Xu and Testerink; unpublished) was co-infiltrated in a 1:1 (v/v) ratio. Cells were observed 48 h later with a Zeiss LSM 510 inverted microscope. mVenus fluorescence was monitored with a 520–555 nm band pass emission filter (514 nm excitation) and the mCherry fluorescence was monitored with a 585–615 nm band pass emission filter (561 nm excitation). Images were taken with an open pinhole and processed by ImageJ (http://rsb.info.nih.gov/ij/).

Transient transactivation assay in *Nicotiana benthamiana* leaves

Agrobacterium tumefaciens GV3101 (pMP90) cultures were grown overnight from a single colony and diluted in infiltration buffer (50 mM MES pH 5.8, 0.5 % glucose, 2 mM NaH_2PO_4, 100 μM acetosyringone; Sigma-Aldrich) to OD_{600} of 0.3. Leaves from 5 weeks old *N. benthamiana* plants were then infiltrated with mixtures carrying various promoter:GUS or promoter:GUSsYFP1 reporter constructs and the 35S:EOT1 effector construct in a 1:1 ratio. In order to normalize for transformation efficiency and protein extraction efficiency, a construct containing *Photinus pyralis* (firefly) *luciferase* (*LUC*) driven by the CaMV 35S promoter was co-infiltrated with the reporter/effector mix in a 2:5 ratio (LUC: reporter/effector mix). Two leaves of three plants were infiltrated for each combination. Two days later leaf disks from the infiltrated areas were collected, frozen in liquid nitrogen and crude extracts were prepared in extraction buffer containing 25 mM Tris phosphate pH 7.8, 2 mM DTT, 2 mM CDTA pH 7.8, 10 % glycerol and 1 % Triton X-100. The enzymatic GUS activity was determined spectrophotometrically with 4-methylumbelliferyl-β-D-glucuronide (MUG) as a substrate (according to Jefferson et al. 1987). The luciferase assay was performed using the same extraction buffer, according to van Leeuwen et al. (2000) and measured in a FluoroCount Microplate Fluorometer (Packerd BioScience Company) using a 560 nm emission filter. Enzymatic GUS activity was normalized for luciferase activity for each sample. Significant differences between samples were tested by ANOVA followed by Tuckey's B posthoc test using software PASW Statistics 17.0 (http://www.spss.com). The homogeneity of variance was tested by Levene's test and values were log transformed before the analysis if necessary. The experiments were repeated at least twice with similar results.

Results

Identification of an *SlTPS5* promoter fragment sufficient for glandular trichome-specific expression and a transcription factor that interacts with it

The *Solanum lycopersicum TPS5* has been previously described as a linalool synthase, expressed in trichomes (van Schie et al. 2007). A promoter fragment of 1,254 base pairs upstream of the start codon ATG (Bleeker et al. 2012), designated here as the full-length promoter, was used to drive β-glucuronidase (GUS) fused to yellow fluorescent protein (sYFP1). This construct and five 5′ deletion constructs (Fig. S1) were used to create transgenic tomato (cv. Moneymaker) lines. YFP expression for the full-length *SlTPS5* promoter was evaluated in T0 stems and leaves and T1 seedlings under a fluorescence stereomicroscope. Fluorescense was specific for the four "head" cells of the type VI glandular trichomes (Fig. 1). Trichome-specific

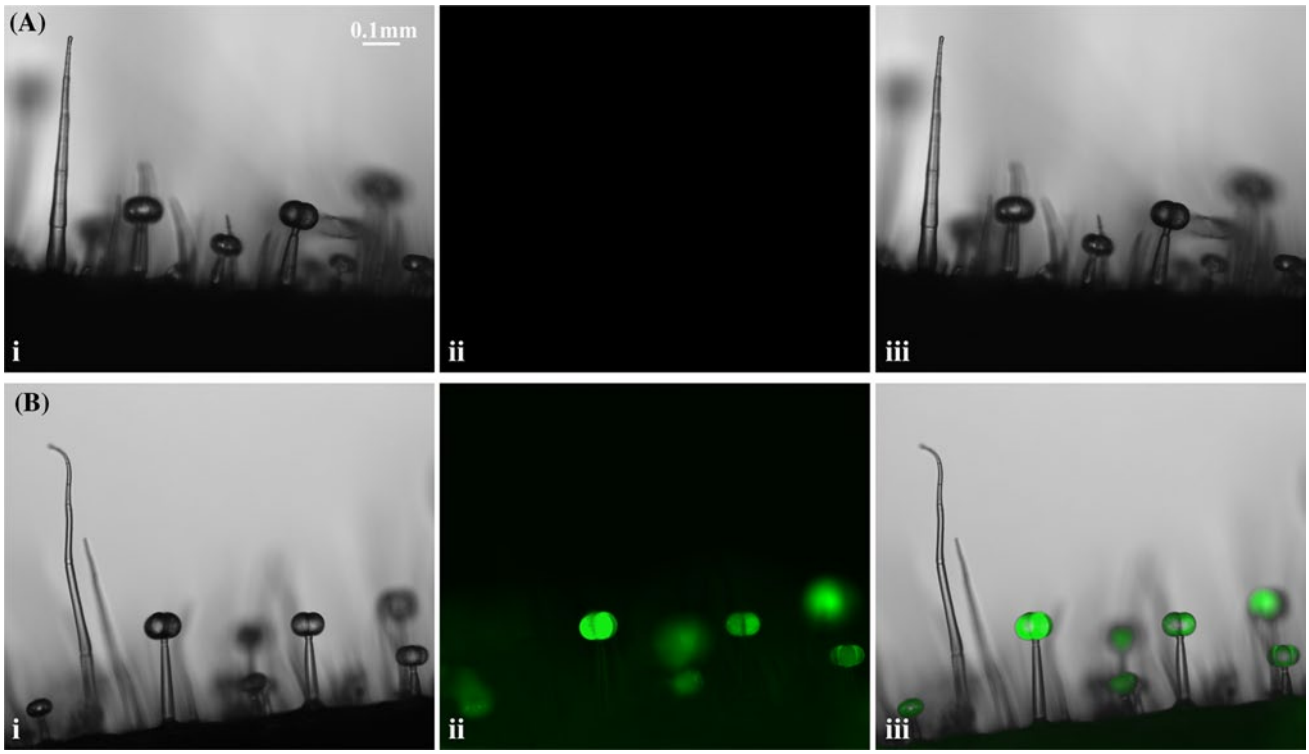

Fig. 1 The *SlTPS5* promoter drives specific expression in the "head" of type VI tomato glandular trichomes. **a** Trichomes of tomato Moneymaker plant and **b** trichomes of tomato Moneymaker plant transformed with the full-length *SlTPS5* promoter driving a GUS-sYFP1 reporter as seen under an EVOSfl (www.amgmicro.com) inverted microscope. (*i*) Normal light (brightfield), (*ii*) YFP filter (emission 525–545 nm) and (*iii*) Merged image

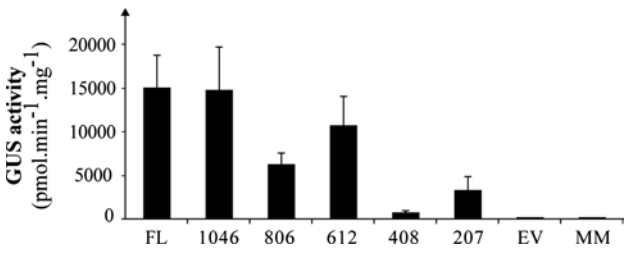

Fig. 2 Quantification of GUS activity in isolated stem trichomes of transgenic *SlTPS5* promoter deletion lines and control plants. Average relative GUS activity for each promoter GUS-sYFP1 reporter construct [full-length (FL), 1,046, 806, 612, 408, 207 bp), empty vector control (EV)] and untransformed Moneymaker plant (MM). The bars represent mean values for a different number of independent lines for each construct (for more details see Materials and methods section) and the error bars represent the standard error

expression of GUS was quantitatively evaluated by measuring GUS activity and was found to be lowest in plants with the 408 bp promoter:GUS construct. Expression directed by the 207 bp fragment (that included the 98 bp 5′UTR) remained specific for the type VI glandular trichome "heads", albeit less strong than that of the full-length promoter (Fig. 2).

This 207 bp trichome-specific *SlTPS5* fragment was used to screen a Y1H cDNA library prepared from isolated stem trichomes of tomato cv. Moneymaker plants in order to identify transcription factors that can interact with it. The screen was repeated three times yielding 71 clones (Table S1). One putative transcription factor appeared 34 times and this gene was designated as Expression of Terpenoids 1 (*SlEOT1*). The interaction was confirmed by using the full-length EOT1 clone (1,056 bp) in an independent yeast-one-hybrid assay with the same 207 bp trichome-specific *SlTPS5* fragment. The 326 bp minimal promoter fragment of *Petunia hybrida* cv. W115 (Mitchell) *ODORANT1* (*ODO1*; Van Moerkercke et al., 2011) was taken along as negative control (Fig. S2).

Furthermore it was tested if in yeast it could also interact with the full-length or shorter *SlTPS5* promoter fragments. To this end yeast cells with the full-length or various short promoter fragments integrated into their genome were transformed with pAD-GAL4-2.1_SlEOT1 and grown on selective medium. SlEOT1 could interact with the full-length promoter as well as the 207 bp *SlTPS5* promoter fragment, but none of the other promoter parts (Fig. S3). The mRNA, genomic and protein sequence of SlEOT1 (KC331910) are provided in Figure S4.

SlEOT1 is a zinc finger-like protein that has a conserved RING finger-type motif and a less conserved IGGH domain

Searches for homologous sequences of the *SlEOT1* ORF (BLASTN) in the potato CDS sequences (PGSC DM v3.4) available at the Solanum Genomics Network (SGN; http://solgenomics.net/) database returned a cDNA that after translation had 83.5 % identity with the SlEOT1 protein. Searches for homologous sequences of the *SlEOT1* ORF (BLASTN) in the *Petunia inflata* genomic scaffolds available from the Petunia Consortium (released through the Sol Genomics Network) returned a sequence from which the ORF was predicted and subsequently cloned full-length from *Petunia* x *hybrida* (*PhEOT1*, KC331913). The two sequences shared 56 % identity on protein level. Homology searches of the *SlEOT1* ORF after translation (BLASTX) against the National Center for Biotechnology Information (NCBI) non-redundant database returned sequences of putative transcription factors from various plant species such as *Ricinus communis*, *Vitis vinifera* and *Populus trichocarpa*. The best hits from *Arabidopsis thaliana* were a Short Internode (SHI)-related protein (Stylish 1; STY1, At3g51060) with which it had 42,4 % identity and SHI (At5g66350) with which it had 40.8 % identity. *STY1* and *SHI* of *Arabidopsis* belong to a gene family that consists of 10 members (Fridborg et al. 2001; Kuusk et al. 2002). Similarly in tomato, homology searches on nucleotide level against the SGN database revealed that *SlEOT1* belongs to a small family that consists of at least five more genes (Table 1). The EOT1/SHI family appears to be specific for plants, as homology searches in human, insect, fungus, nematode, microbe and protozoa genomes (http://www.ncbi.nlm.nih.gov/sutils/genom_table.cgi?organism) returned no hits. However, homologs have been found in the moss *Physcomitrella patens* (Eklund et al. 2010b). A homology tree with the SlEOT1 protein family and homologs from other plant species is shown in Fig. 3. Three tomato EOT proteins together with the other Solanaceae homologs formed a separate sub-cluster, part of a larger cluster that cannot be separated with confidence, as it was not supported by high bootstrap values. The tomato homologs in this sub-cluster consist of the trichome-specific KC331911 (Fig. S5), tentatively named SlEOT2 and the previously unidentified EST KC331912, tentatively named SlEOT3. What the above-mentioned proteins had in common was a zinc finger-like conserved domain with a less conserved C-terminal companion domain (NCBI: www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi; Fig. 4 and S6). The zinc finger-like domain was first described in the *Arabidopsis* Lateral Root Primordium 1 (LRP1) protein (Smith and Fedoroff 1995). *LRP1* and *STY1* belong to the *SHI* gene family (Fridborg et al. 2001; Kuusk et al. 2002). The sequence arrangement of the conserved N-terminal motif seen as $C\text{-}X_2\text{-}C\text{-}X_7\text{-}C\text{-}X\text{-}H\text{-}X_2\text{-}C\text{-}X_2\text{-}C\text{-}X_7\text{-}C\text{-}X_2\text{-}H$ was named C3HC3H RING domain (Fridborg et al. 2001) due to the similarity with the zinc binding RING finger motif identified in the human *RING1* gene ($C\text{-}X_2\text{-}C\text{-}X_{9\text{-}27}\text{-}C\text{-}X_{1\text{-}3}\text{-}H\text{-}X_2\text{-}C\text{-}X_2\text{-}C\text{-}X_{4\text{-}48}\text{-}C\text{-}X_2\text{-}C$ (C3HC4); Freemont 1993; Lovering et al. 1993). The second (C-terminal) domain of the SHI family proteins with high sequence identity (although less conserved than the N-terminal domain) was named IGGH domain due to these four highly conserved residues of the region (Fridborg et al. 2001) and is unique for this family proteins. Amino acid sequence similarities in the N- and C-terminal domains between the tomato family members and the *Arabidopsis* STY1, SHI and LRP1 are shown in Fig. 4.

SlEOT1 is specifically expressed in tomato trichomes and not induced by jasmonic acid

Four EOT homologs (SlEOT1, 2 and 3 and SGN-U569648) were expressed in tomato stem trichomes (Table 1). Transcripts were identified by RNAseq of stem trichome cDNA (Falara et al. 2011). In order to investigate the expression pattern of *SlEOT1*, Q-RT-PCR was performed on cDNA from *S. lycopersicum* cv. Moneymaker leaves, stems,

Table 1 *SlEOT1* family members in the tomato Heinz genome

SGN nr	NCBI nr	Chr	Trichome DB contig nr	Reads C	Reads JA	Fold JA-induction	ORF length (AA)	Origin cDNA
SGN-U566013		1	–	–	–	–	226	a
SGN-U587292	KC331910	2	4,489	5	7	1.4	351	SlEOT1
–	KC331912	2	8,844	39	40	1.02	345	b; SlEOT3
SGN-U563829	KC331911	3	12,542	26	53	2.04	321	SlEOT2
SGN-U569648		4	26,035	10	5	0.5	372	a, b
SGN-U5586059		11	–	–	–	–	350	a

When not available, putative full-length sequences were predicted by Genscan (http://genes.mit.edu/GENSCAN.html) using sequences obtained from SGN genomic sequence and *a*) available ESTs or *b*) contig sequences from a stem trichome database (DB; Falara et al. 2011). Reads Control (C) and Reads Jasmonic Acid (JA) refer to mRNAs obtained by sequencing trichomes isolated from plants sprayed with control or JA solution (Falara et al. 2011). *Chr* chromosome

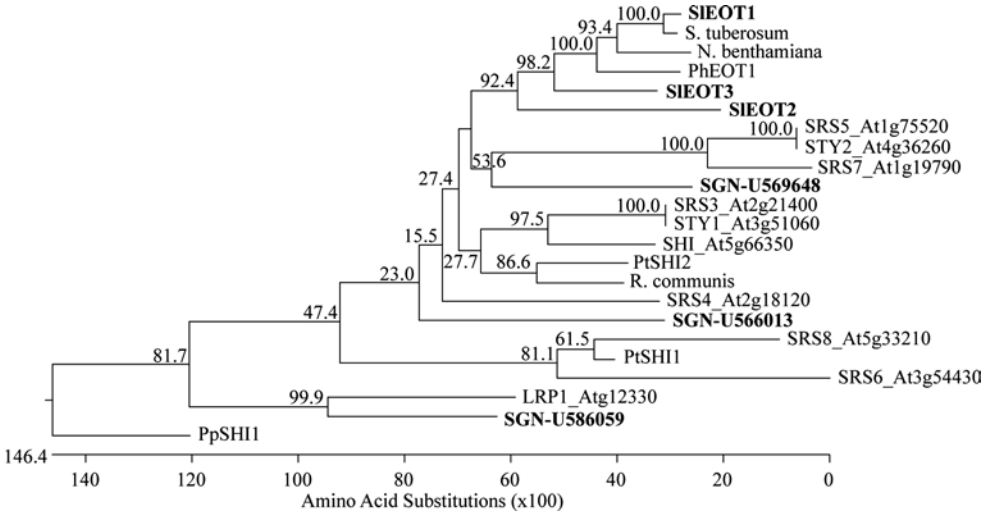

Fig. 3 Homology tree of SlEOT1 homologs from tomato and other plant species [*Arabidopsis thaliana*, *N. benthamiana* (NbS00014581g0024.1), *Petunia hybrida* (KC331913), *Physcomitrella patens*, *Populus trichocarpa*, *Ricinus communis* (XM_002533607) and *Solanum tuberosum* (PGSC0003DMT400040107)]. Amino acid sequences were aligned using ClustalW. The tree was constructed after bootstrap analysis (n = 1,000) using Lasergene DNAstar Megalign software (DNASTAR, Madison, USA). Tomato family members are shown in bold

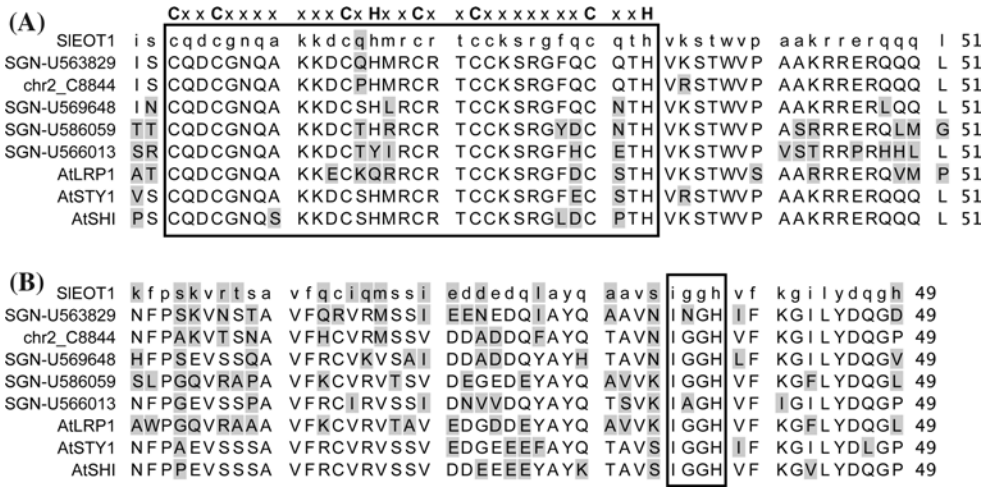

Fig. 4 Amino acid sequence alignment of the SlEOT1 tomato family members and *Arabidopsis STY1, SHI* and *LRP1* conserved domains. **a** N-terminal domain and **b** C-terminal domain. The alignment was generated in CLC Workbench (www.clcbio.com) using ClustalW. The C3HC3H RING and IGGH motifs are boxed

isolated stem trichomes and roots of 4-week-old plants, as well as flowers and fruit of mature plants. As shown in Fig. 5a, expression of *SlEOT1* was specific for trichomes. This was confirmed by creating transgenic tomato Moneymaker plants expressing a GUS-sYFP1 fusion under the control of the 1,270 bp *SlEOT1* promoter. In five independent transformants the expression of *SlEOT1* was similar to that of *SlTPS5* in the four "head" cells of the type VI glandular trichomes (Fig. 6). Since *SlEOT1* interacted with the promoter of *SlTPS5*, a JA-inducible gene (van Schie et al. 2007), we investigated whether expression of *EOT1* would also be regulated by application of JA. To this end, 4-week-old Moneymaker plants were sprayed either with control solution or with solution containing JA and at different time points stem pieces were collected and Q-RT-PCR was performed on cDNA from isolated stem trichomes. *SlEOT1* expression was not induced by JA (Fig. 5b), which was in agreement with the RNAseq data in Table 1, where the number of reads in JA-treated plants did not differ from control conditions for *SlEOT1*. Expression of *SlEOT2* did increase after JA treatment of the plants (Table 1).

SlEOT1 localizes to the nucleus

In order to determine the subcellular localization of SlEOT1, the mVenus (Nagai et al. 2002) reporter gene was fused in frame with the ORF of *SlEOT1* under the control of a double CaMV 35S promoter. The translational fusion was transiently expressed in *N. benthamiana* leaves using *A. tumefaciens* infiltration (Verweij et al. 2008). As illustrated in Fig. 7, confocal imaging showed co-localization of this fusion protein with the nuclear marker [nuclear localization signal (NLS)-mCherry; Galvan-Ampudia, Xu and Testerink; unpublished] in epidermal cells, as expected by its predicted function as a transcription factor. Co-localization was observed in independent experiments, also in mesophyll cells.

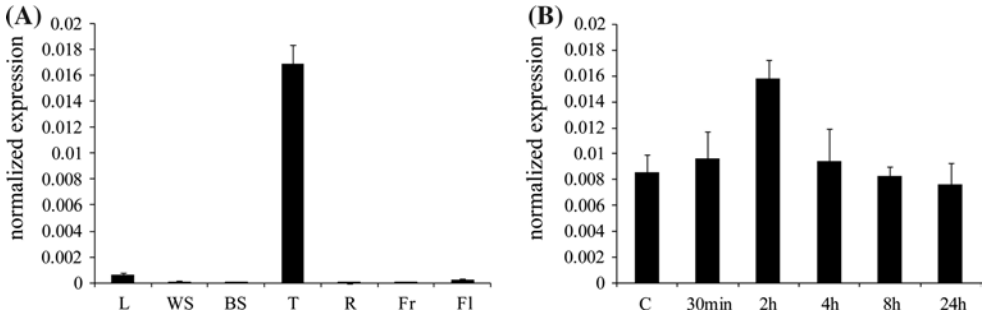

Fig. 5 Tissue specific expression of *SlEOT1* and response to JA treatment. Relative transcript levels for *SlEOT1* as determined by Q-RT-PCR in **a** *L* leaf, *WS* whole stem, *BS* bald stem, *T* stem trichomes, *R* root, *Fr* fruit, *Fl* flower and **b** *C* control and jasmonic acid-induced stem trichomes at different time points. JA treatment had no effect on *EOT1* expression (ANOVA, $F_{5,12} = 2.94$, $P = 0.058$). Mean values (+SE) of 3 biological replicas are shown, normalized for *Actin* expression

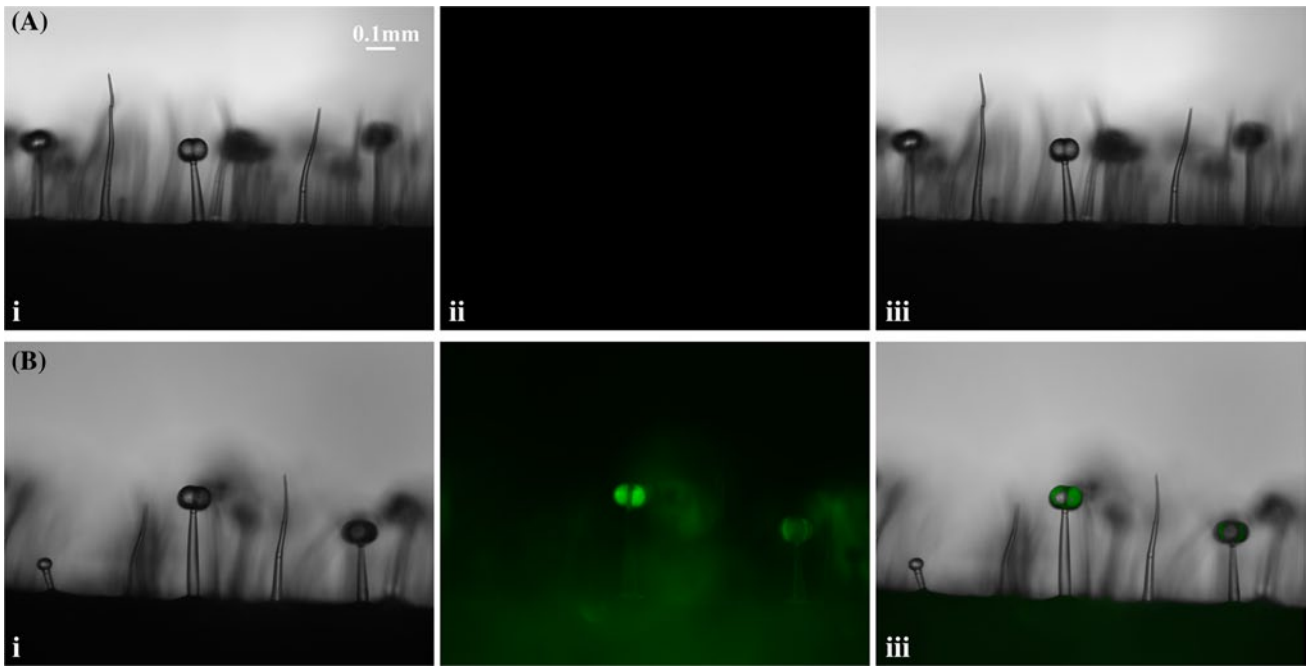

Fig. 6 The *SlEOT1* promoter is expressed in the "head" of type VI tomato glandular trichomes. **a** Trichomes of tomato Moneymaker plant and **b** trichomes of tomato Moneymaker plant transformed with the full-length *SlEOT1* promoter driving GUSs-YFP1 as seen under an EVOSfl (www.amgmicro.com) inverted microscope. (*i*) Normal light (brightfield), (*ii*) YFP filter (emission 525–545 nm) and (*iii*) Merged image

SlEOT1 specifically transactivates the *SlTPS5* promoter in *Nicotiana benthamiana* leaves

In order to investigate whether SlEOT1 can interact with the *SlTPS5* promoter *in planta*, a transient assay was used in *N. benthamiana* leaves, which has been previously used successfully to investigate transcription factor interactions with plant promoters (Van Moerkercke et al. 2011). In the reporter constructs, expression of β-glucuronidase (GUS) was driven by a trichome-specific promoter. Co-infiltration with the CaMV 35S:SlEOT1 effector construct, now expressed in leaves, should transactivate the promoters with which it could interact, leading to GUS expression in this heterologous system. Initially, in order to confirm that none of the promoter:GUS constructs used gave background expression in *N. benthamiana* leaves, the assay was performed excluding the CaMV 35S:SlEOT1 effector construct. GUS activity comparable to the negative control was detected for all mixtures infiltrated. Only co-infiltration of the full-length *SlTPS5* promoter reporter construct with a CaMV 35S:SlEOT1 effector construct resulted in enhanced GUS activity in *N. benthamiana* leaves (Fig. 8a). Because SlEOT1 was identified as a protein interacting with the 207 bp trichome-specific fragment of the *SlTPS5*

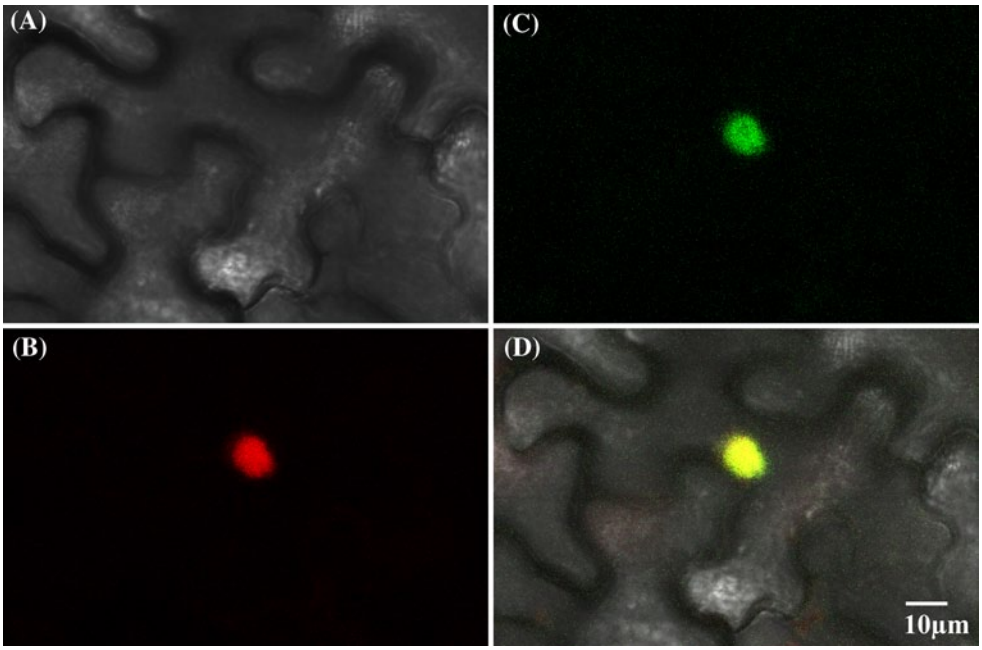

Fig. 7 Subcellular localization of SlEOT1. Transient expression of 2 × 35S:SlEOT1-mVenus in *N. benthamiana* leaves showing nuclear localization. **a** Epidermal cell bright field image, **b** NLS-mCherry image, **c** mVenus-SlEOT1 image and **d** merged image. Images were obtained with a Zeiss LSM 510 confocal laser scanning microscope

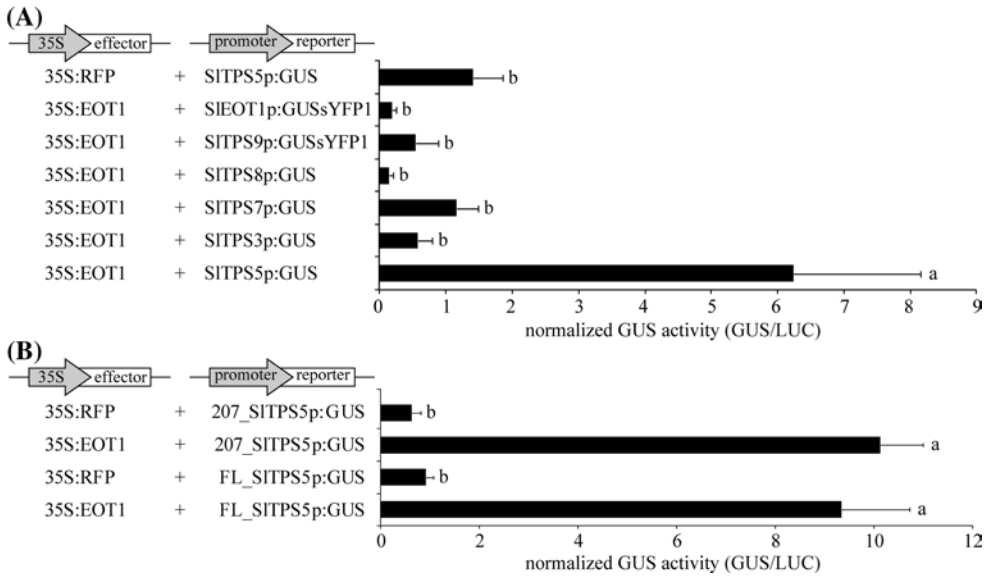

Fig. 8 Transactivation of promoters by SlEOT1 in *N. benthamiana* leaves. Normalized GUS activity after co-infiltration with *A. tumefaciens* harboring the 35S:SlEOT1 effector construct and **a** various promoter:GUS reporter constructs or **b** either the full-length (FL) or the 207 bp TPS5 promoter:GUS reporter construct. The 35S:RFP effector construct was used as negative control. The bars represent obtained mean values and the error bars the standard error (n = 3). *RFP*; red fluorescent protein. Letters indicate significant differences (ANOVA, $P < 0.05$ according to Tuckey's B posthoc test). Representative results from three experiments are shown

upstream sequence in yeast we also used this fragment in the transactivation assay. As shown in Fig. 8b, SlEOT1 transactivated the 207 bp *SlTPS5* promoter fragment *in planta*.

Subsequently, the assay was performed with promoter fragments from trichome-expressed TPS genes (*SlTPS3*, *SlTPS5*, *SlTPS9*), from a ubiquitously expressed TPS gene (*SlTPS7*) and from a root-expressed TPS gene (*SlTPS8*).

SlEOT1-enhanced SlTPS5 reporter activity, up to fivefold compared to the CaMV 35S:RFP-infiltrated plants, was detected but not for any of the other TPS promoters tested (Fig. 8a), indicating that from the TPS promoters tested, SlEOT1 can only regulate the *SlTPS5* promoter. Furthermore, the SlEOT1p:GUSsYFP1/CaMV 35S:SlEOT1 co-infiltration showed that SlEOT1 could not transactivate its own promoter (Fig. 8a).

Discussion

Reporter gene studies with consecutive deletion fragments of the *S. lycopersicum TPS5* promoter allowed us to delimit a 207 bp fragment that is sufficient for glandular trichome-specific expression. A yeast-one-hybrid screen with this fragment identified the tomato transcription factor Expression of Terpenoids 1 (*SlEOT1*). This gene is expressed only in glandular trichomes. In addition it can transactivate the *TPS5* promoter *in planta*. Several members of the small EOT gene family are expressed in glandular trichomes and may have a role in regulating expression of TPSs.

Deletion analysis of the trichome-specific *SlTPS5* promoter and identification of a novel tomato transcription factor through a yeast-one-hybrid screen

Relatively few trichome-specific promoters of genes involved in the terpenoid biosynthesis pathway have been described to date (Tissier 2012) and even fewer have been extensively characterized. In order to identify transcription factors that are involved in the regulation of terpene biosynthesis, the promoter of a trichome-specific monoterpene synthase from tomato (*SlMTS1*; van Schie et al. 2007, renamed *SlTPS5*; Falara et al. 2011) was isolated to function as bait in a yeast-one-hybrid screen. However, given that there are restrictions in the size of the DNA fragment that can be efficiently used as bait, since the majority of yeast promoters range from approximately 150–400 bp (Dobi and Winston 2007), the *SlTPS5* promoter (1,254 bp upstream of the ATG) was first dissected. We created transgenic tomato plants with 5′ deletion constructs driving a GUS-sYFP1 fusion, in order to identify the minimal promoter fragment necessary for trichome-specific expression. GUS-sYFP1 expression driven by a 207 bp fragment was specific for the secreting cells (“head”) of type VI trichomes (Fig. 1). Interestingly, all other promoter deletion fragments, except the 408 bp fragment, retained trichome-specific expression. The 408 bp promoter fragment did not drive expression in trichomes, which might indicate the presence of a repressor element between −408 and −208 bases and/or a transcriptional enhancer in the −409 to −612 bp fragment. Co-existence of both activating and repressing *cis*-elements in a promoter has been reported before (Ennajdaoui et al. 2010). A further investigation of these transgenic, promoter-deletion plants could reveal the fragment responsible for the JA-inducibility of *TPS5* and is the focus of future studies.

Several yeast-one-hybrid (Y1H) screens of a stem trichome cDNA library with the trichome-specific 207 bp *SlTPS5* fragment yielded one putative transcription factor (34 times; Table S1) that was designated Expression of Terpenoids 1 (SlEOT1; KC331910). Although two other putative TFs were found (Table S1), these only appeared once in individual experiments and were not further analyzed. The fact that SlEOT1 is low abundant in the trichome cDNA library used (see Table 1), but still was predominantly retrieved in the Y1H screens, warrants the conclusion that other EOT homologs present in this library (SlEOT2, 3 and SGN-U569648; Table 1) did not interact with the 207 bp *SlTPS5* promoter fragment.

SlEOT1 is a trichome-specific gene that interacts with the *SlTPS5* promoter both in yeast and *in planta*

SlEOT1 was shown to be expressed in the secreting cells of glandular trichomes (Fig. 6) like *SlTPS5*, but was not induced by jasmonic acid (JA; Fig. 5b). Therefore it is possible that *SlEOT1* is involved in the steady-state transcription of *SlTPS5*. Alternatively, it might be part of a transcription machinery involving (JA-inducible) co-regulator(s) and thus exert a combinatorial effect. SlEOT1 belongs to a small family (Fig. 3; Table 1), which contains proteins that carry an IGGH domain (Fig. 4), known from the *Arabidopsis* homologs to be responsible for intrafamily homo- and heterodimerization (Eklund et al. 2010a). According to stem trichome transcriptomic data, one of the tomato family members, *SlEOT2* (KC331911), is JA-inducible (Table 1). However, although this and other family members are also expressed in tomato trichomes (Table 1), the Y1H screens identified only SlEOT1. This does not however exclude the possibility that additional proteins (like the JA-inducible SlEOT2) interact under specific (stress) conditions with SlEOT1 *in planta*, affecting its activity, or binding to other parts of the promoter.

SlEOT1 was shown to function as a transcriptional activator *in planta* (Fig. 8). It contains three potential activation domains: two glutamine (Q)-rich regions and one acidic region cluster (Fig. S6), both of which are known to function as such (Giniger et al. 1985; Courey and Tjian 1988). Furthermore it was shown to localize in the nucleus (Fig. 7) and a putative NLS typically found in transcription factor proteins (Boulikas 1994) is present in its N-terminal conserved domain (Fig. S6). The interaction with the promoter is probably taking place through its conserved zinc finger

RING-like domain (Fig. S6), as was shown for *Arabidopsis* SHI family members (Eklund et al. 2010a).

A role for EOT1/SHI proteins in plant defense responses?

The family of SHI proteins from various plant species is undoubtedly involved in auxin responses and plant growth (Eklund et al. 2010a; Eklund et al. 2010b; Zawaski et al. 2011). In particular, the *Arabidopsis* AtSTY1 interacts with the promoter of at least one member of the YUCCA (YUC) family of flavin monooxygenases involved in tryptophan-dependent auxin biosynthesis (Sohlberg et al. 2006; Eklund et al. 2010a). Interestingly however, the *Arabidopsis* AP2/ERF transcription factor AtORA59 (Pre et al. 2008) is also a STY1 target, possibly through a YUC element found in the *ORA59* promoter (Eklund et al. 2010a). AtORA59 is involved in resistance against necrotrophic pathogens, integrating JA and ethylene signals to regulate expression of defense genes like Plant defensin 1.2 (*PDF1.2*) (Pre et al. 2008). AtORA59 was also shown to regulate three genes involved in tryptophan biosynthesis (Pre et al. 2008). Therefore it is possible that AtSTY1 regulates tryptophan availability and thus could promote either auxin biosynthesis or defense responses.

Intriguingly, a yeast-one-hybrid screen with a region from the *Catharanthus roseus ORCA3* promoter with a library of MeJA-treated *C. roseus* cells also resulted in the identification of a protein of the SHI family (Vom Endt et al. 2007). The *C. roseus* SHI ortholog (clone 2D81) shares highest sequence identity with AtSRS5 (http://www.arabidopsis.org/Blast/) and potentially binds to the YUC element found in the *ORCA3* promoter region (Eklund et al. 2010a). 2D81 could interact with this region also in vitro and its binding was not affected by mutations in jasmonate-responsive promoter elements (Vom Endt et al. 2007). Binding to a mutated YUC element was not tested. ORCA3 regulates JA-induced terpenoid indole alkaloid (TIA) biosynthesis by controlling expression of tryptophan decarboxylase (*TDC*) that converts tryptophan to tryptamin, as well as expression of strictocidine synthase (*STR*) and deoxy-xylulose phosphate synthase (*DXS*) (van der Fits and Memelink 2000). Therefore 2D81, by regulating ORCA3, could also be controlling tryptophan availability or TIA production. It is worth pointing out here, that in the promoter fragment of *SlTPS5* a YUC element can be found (bases −652 to −646; Fig. S1), however it is in a part of the promoter, with which SlEOT1 does not interact in yeast (Fragment C; Fig. S3) but could potentially serve as a binding site for another family member.

A similar element (YUC-like element; A**T**TCTA**T**) is found in the promoter region of the 207 bp minimal *SlTPS5*p fragment (bases −110 to −104; Fig. S1) and could be the binding site of SlEOT1. Attempts to confirm binding of SlEOT1 to this element or the whole 207 bp fragment in vitro by electrophoretic mobility shift assays (EMSA) were not successful, most likely due to inactive protein or the absence of an additional protein necessary for binding. *In planta* however SlEOT1 could transactivate the 207 bp *SlTPS5* promoter fragment with a mutated YUC-like element (Fig. S7), indicating that this is not its binding site or that the activation does not take place through direct binding.

Finally, another potential link of the EOT1/SHI family proteins with JA signaling comes from analysis of *AtSRS7*, an *AtSHI* homolog, which is mainly expressed in flower filaments and the shoot apex. In the *srs7-1* mutant expression of the Defective in Anther Dehiscence 1 (*DAD1*) gene was increased (Kim et al. 2010). DAD1 encodes the lipolytic enzyme that catalyzes the release of linolenic acid from cellular lipids in the JA biosynthesis pathway (Ishiguro et al. 2001). Furthermore, similarly to the JA-deficient mutants, in *srs7-1* plants anther dehiscence was inhibited (Kim et al. 2010), observations which suggest a link of *AtSRS7* with the JA pathway.

From the above-presented correlations of SHI family proteins with the jasmonate pathway and production of defense compounds it is already intriguing to speculate an additional role for (at least some of) these proteins in the plant defense system.

Acknowledgments We thank Mattijs Bliek (Vrije Universiteit, Amsterdam) for kindly providing the yeast strain PJ69-4A, Carlos Galvan-Ampudia, Ronald Breedijk and Maria Koini for help with confocal imaging constructs and microscopy, Kai Ament for constructing the trichome cDNA library, Juan M. Alba-Cano for help with the statistical analyses and Harold Lemeris, Ludeck Tikovsky and Thijs Hendrix for the excellent care of the tomato plants. This work was supported by KeyGene and the Technological Top Institute-Green Genetics Grant 1C002RP.

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