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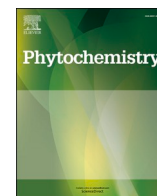
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Functional characterization of a small gene family coding for putrescine hydroxycinnamoyltransferases, involved in phenolamide accumulation, in tomato

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ABSTRACT

Phenolamides are specialized metabolites widely distributed in the plant kingdom. Their structure is composed by the association of hydroxycinnamic acid derivatives to mono-/poly-amine through an amination catalyzed by *N*-hydroxycinnamoyltransferases enzymes. Tomato plants accumulate putrescine-derived phenolamides in their vegetative parts. Recently, two first genes coding for putrescine-hydroxycinnamoyltransferase (PHT, *Solyc11g071470* and *Solyc11g071480*) were identified in tomato and demonstrated to control the leaf accumulation of caffeoylputrescine in response to leafminer infestation. In this study, two additional genes (*Solyc06g074710* and *Solyc11g066640*) were functionally characterized as new tomato PHT. The substrate specificity and the expression pattern *in planta* were determined for the four tomato PHT. Taken together the results give a comprehensive view of the control of the putrescine-derived phenolamide accumulation in tomato plant through the biochemical specificity and the spatial expression of this small family of PHT.

1. Introduction

Phenolamides are specialized metabolites widely distributed in plants. They result from the amination of phenolic acids and mono- or poly-amines (tyramine, dopamine, serotonin, putrescine, agmatine, spermidine, spermine), leading to a high structural diversity (for review, see Bassard et al., 2010; Macoy et al., 2015; Roumani et al., 2021). Phenolamides have been reported in flowers, seeds and pollen coat, but their biological role in these organs are not elucidated yet (Cabanne et al., 1981; Martin-Tanguy 1985, 1997; Vogt 2018). In Solanaceae, Brassicaceae and Poaceae, these metabolites are also found in vegetative organs. They accumulate in response to abiotic stresses (Matsuda et al., 2005; Demkura et al., 2010) and biotic stresses such as pathogen infection (Roepenack-Lahaye et al., 2003a,b; Zacarés et al., 2007; Muroi et al., 2009; Royer et al., 2016; Morimoto et al., 2018; Ube et al., 2019) or herbivory (Pearce et al., 1998; Tebayashi et al., 2007; Kaur et al., 2010; Onkokesung et al., 2012a; Marti et al., 2013; Alamgir et al., 2016). In these latter cases, phenolamide accumulation has been proven to contribute to the plant defense against pests through direct

antimicrobial activities (Zacarés et al., 2007; Yogendra et al., 2015; Dobritzsch et al., 2016; Yogendra and Kushalappa 2016; Ube et al., 2017; Morimoto et al., 2018), reinforcement of the plant cell wall (King and Calhoun 2005), perturbation of the insect oviposition (Tebayashi et al., 2007) and insect toxicity leading to an increase in mortality or at least impacting on the life cycle (Kaur et al., 2010a; Onkokesung et al., 2012a; Marti et al., 2013; Alamgir et al., 2016).

The biosynthesis of phenolamides involves *N*-hydroxycinnamoyltransferases that transfer the phenolic acid donors to mono- or poly-amine acceptors. To date, about fifty *N*-hydroxycinnamoyltransferases have been biochemically characterized (Wang et al., 2021; Roumani et al., 2021). They belong to two different enzyme classes. Hence, some of them are homologous to GNATs (Gen5-related *N*-acetyltransferases), but most of them belong to the BAHD acyltransferase superfamily, named after the four enzymes that were first characterized (BEAT, Benzyl alcohol *O*-acetyltransferase; AHCT, Anthocyanin *O*-HydroxyCinnamoyl Transferase; HCBT, *N*-HydroxyCinnamoyl/Benzoyl-Transferase; DAT, Deacetylvindoline 4-*O*-AcetylTransferase). GNATs are restricted to the biosynthesis of monoamine phenolamides (tyramine,

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dopamine, noradrenaline, octopamine). BAHD *N*-hydroxycinnamoyltransferases have a much broader activity and are able to catalyse the biosynthesis of a large diversity of phenolamides. Depending on their amine specificity and their phylogeny, we recently proposed classifying these BAHD *N*-hydroxycinnamoyltransferases into eight clusters (Roumani et al., 2021).

Tomato (*Solanum lycopersicum*) has been reported to constitutively produce several phenolamides. Indeed, as a common feature with all core eudicotyledons, tomato plant accumulates phenolamides in the flower parts and particularly, two- and three-substituted spermidine derivatives in pollen coats (Paupière et al., 2017). In addition, putrescine derivatives are found in healthy vegetative organs, notably stem (Larbat et al., 2014). The phenolamide chemodiversity is significantly increased when tomato plants are subjected to biotic stresses, leading to

the synthesis of dopamine, tyramine, noradrenaline, octopamine, and, more recently, tri-substituted spermine derivatives accumulating locally and/or systemically (Von Roepenack-Lahaye et al., 2003; Zacarés et al., 2007; Lopez-Gresa et al., 2016; Roumani et al., 2022). Interestingly, in comparison with this diversity, only a few *N*-hydroxycinnamoyltransferases have been reported in this plant. Among them is a 4-members family of tyramine-hydroxycinnamoyl transferases (THT) controlling the accumulation of tyramine-, noradrenaline-, octopamine- and dopamine-derived phenolamides in wounded tomato plants (Von Roepenack-Lahaye et al., 2003; Zacarés et al., 2007) and one catalyzing tri-acylation of spermidine in flowers (Perrin et al., 2021). Recently, two tomato genes encoding putrescine-hydroxycinnamoyltransferase (PHT; *Solyc11g071470/Solyc11g071480*) were reported. Their expression was shown to be dramatically induced in response to herbivory leading to an

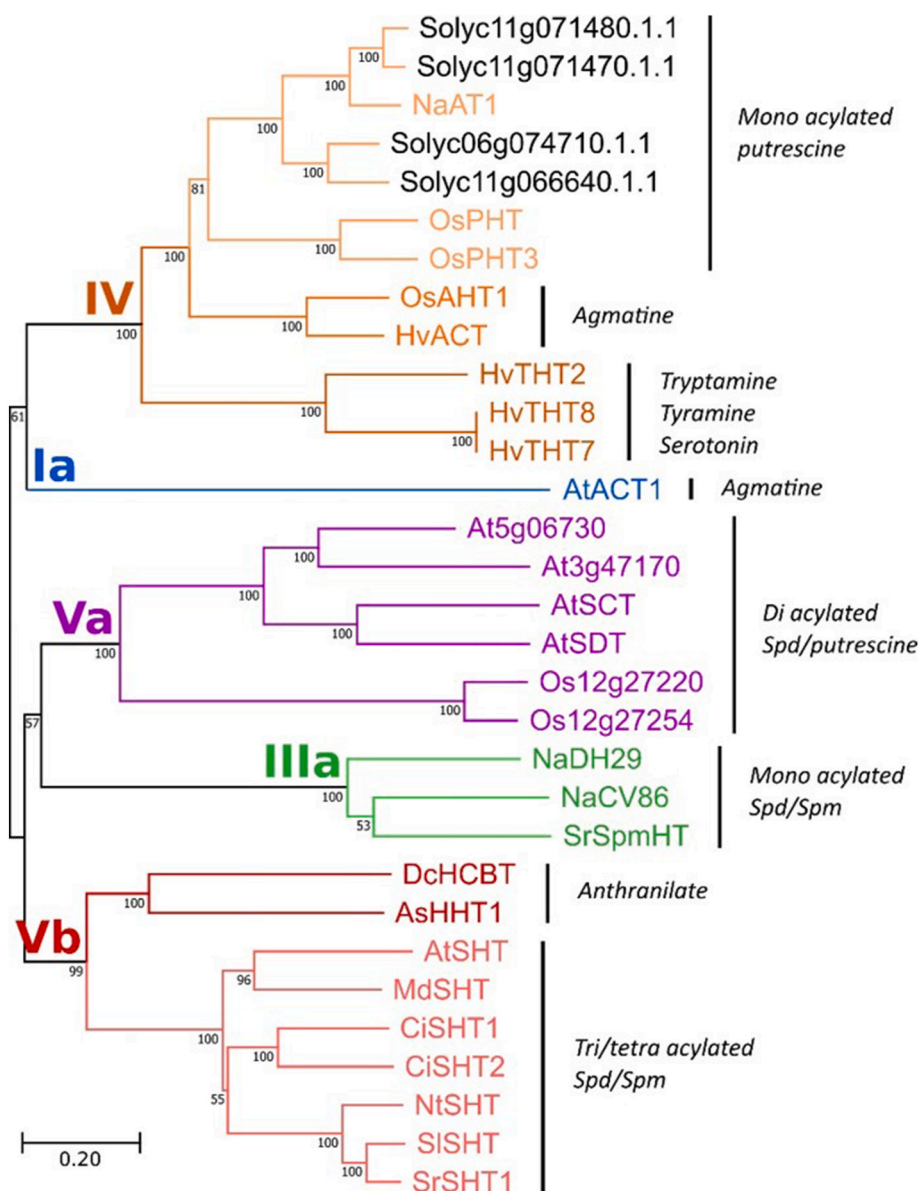


Fig. 1. Phylogenetic tree of BAHD *N*-hydroxycinnamoyltransferases. The tree was constructed by neighbor-joining distance analysis on protein sequences. The putative *N*-hydroxycinnamoyltransferases Solyc11g066640 and Solyc06g074710 from tomato were compared to a selection of BAHD *N*-hydroxycinnamoyltransferases from various species: *S. lycopersicum* soly11g071470, soly11g071480 and SiSHT (MN787044); *N. attenuata* NaAT1 (JN390826), NaDH29 (JN390824) and NaCV86 (JN390825); *O. sativa* OsPHT (BAT09226), OsPHT3 (BAT09225), OsAHT1 (BAS91492), Os12g27220 (ABA98379) and Os12g27254 (ABG22016); *H. vulgare* HvACT (AY228552), HvTHT7 (Hr1G019410.1), HvTHT8 (Hr1G077780.1) and HvTHT2 (Hr1G077790.1); *A. sativa* AsHHT1 (AB076980); *A. thaliana* AtSHT (NM127464), AtSDT (NM127915), AtSCT (NM128072), AtACT1 (BT011800), At5g06730 and At3g47170; *S. richardii* SrSHT (KP165411) and SrSpmHT (KR150683); *D. caryophyllus* DcHCBT (Z84383); *M. domestica* MdSHT (ALF00095); *C. intybus* CiSHT1 and 2 (MG457243, MG457244); *N. tabacum* NtSHT (MN787045).

increase of the accumulation of caffeoylputrescine in the damaged tissues (Roumani et al., 2022). Such genes coding for PHTs were already described in *Nicotiana attenuata* (Onkokesung et al., 2012), in *Arabidopsis* (Wang et al., 2021) and in rice where this activity was also supported by a small gene family displaying complementary expression profile (Dong et al., 2015; Tanabe et al., 2016).

The accumulation of putrescine-derived phenolamides has been shown to be spatially regulated in healthy tomato plants, but also highly inducible by pest attacks (Larbat et al., 2014; Royer et al., 2016; Paupière et al., 2017; Roumani et al., 2022). The aim of this study was, then, to characterize the determinants of this accumulation. Taking advantage of the identification of two first PHT in tomato (Roumani et al., 2022) this study describes the isolation of two additional genes encoding PHTs. The biochemical properties and the expression profiles of the four enzymes were investigated with regard to the phenolamide accumulation in *planta*.

2. Results

2.1. Seeking for homologous coding sequences of PHT *Solyc11g071470/Solyc11g071480* in tomato

Investigations of *Solanum lycopersicum* genomic data using BLASTp on predicted proteins revealed two homologous sequences for *Solyc11g071470* and *Solyc11g071480*. These two sequences are *Solyc06g074710* and *Solyc11g066640*, sharing around 65% protein sequence identity with both *Solyc11g071470* and *Solyc11g071480*. Their

corresponding genes are located respectively on a distant locus and a different chromosome compared to the pair *Solyc11g071470/Solyc11g071480* (Supplementary data 1-A). Phylogenetic analysis with representative BAHD *N*-hydroxycinnamoyl transferase members indicated that these 4 tomato sequences belong to the BAHD clade IV, clustering in a sub-group of enzymes involved in putrescine monoacylation, including the *N. attenuata* AT1 and the *O. sativa* PHT and PHT3 (Fig. 1). Considering the phylogenetic analysis of the protein sequences, the genomic organization of the genes, and the genetic distance between the promoter sequences of each gene (Supplementary data 1), it can be assumed that *Solyc11g071470/Solyc11g071480* are duplicated orthologs of *NaAT1*, whereas *Solyc06g074710/Solyc11g066640* are their paralogs.

2.2. Enzymatic characterization

The enzymatic activity of *Solyc06g074710* and *Solyc11g066640* was assessed *in vitro* on purified recombinant protein expressed in *E. coli*. As expected, each enzyme was proved functional and exhibited a putrescine hydroxycinnamoyl-CoA transferase activity. For this reason, and for ease of reading, we attributed the name *Solanum lycopersicum* putrescine hydroxycinnamoyl transferase (SIPHT) to each enzyme with the following nomenclature SIPHT1 (*Solyc11g071470*), SIPHT2 (*Solyc11g071480*), SIPHT3 (*Solyc06g074710*) and SIPHT4 (*Solyc11g066640*). In our *in vitro* conditions, SIPHT3 transferred efficiently caffeoyl-CoA, feruloyl-CoA and *p*-coumaroyl-CoA to both putrescine and agmatine (Fig. 2A and B; Supplementary data 2). In addition, this

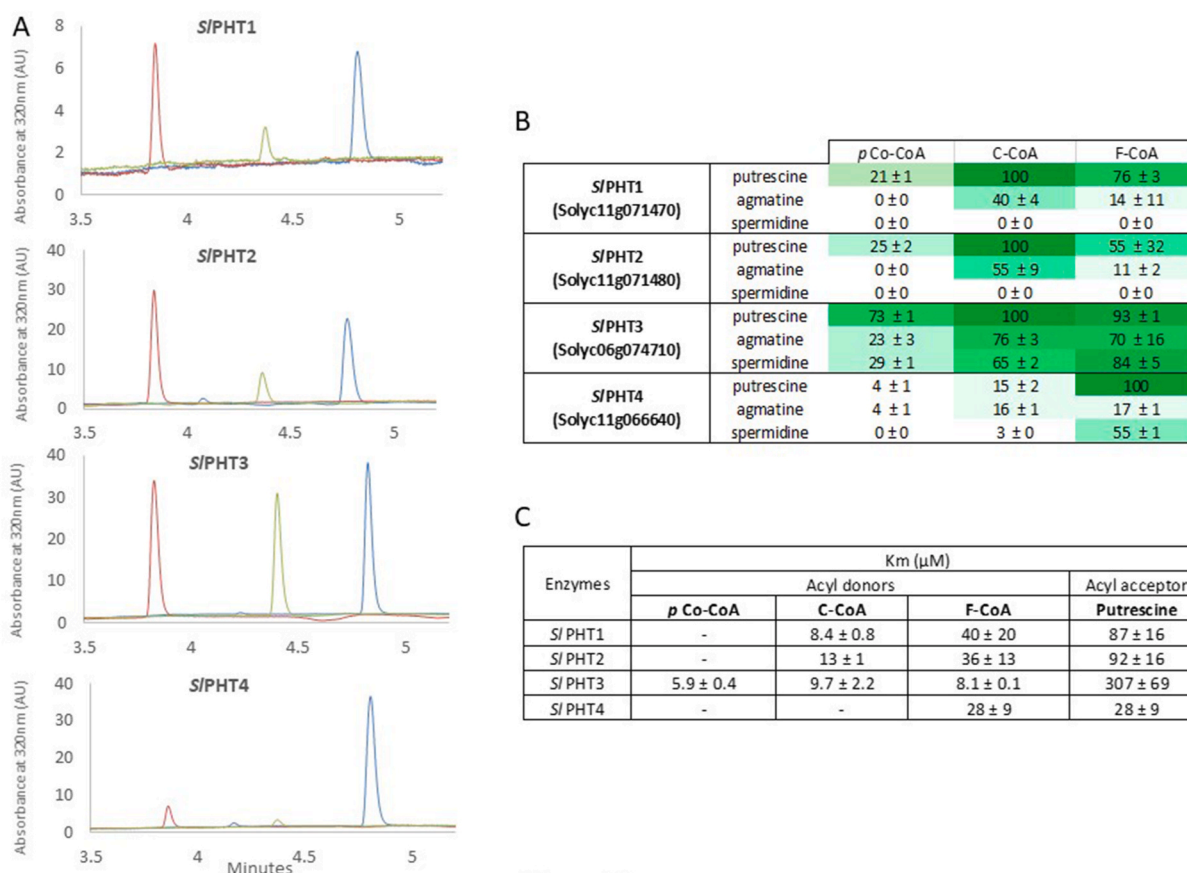


Fig. 2. Functional characterization of putrescine hydroxycinnamoyltransferases from tomato. A: Chromatograms showing the amount of *p*-coumaroyl putrescine (green), caffeoyl putrescine (red) and feruloylputrescine (blue) produced from independent reactions with the same reaction conditions and enzyme amount for the 4 tomato hydroxycinnamoyltransferases. B: Substrate specificity of the four enzymes. *p*-coumaroyl-CoA (pCo-CoA), caffeoyl-CoA (C-CoA) and feruloyl-CoA (Fer-CoA) were incubated with the respective acyl acceptors (putrescine, agmatine and spermidine). The amount of product formed per time unit was determined by measuring the area under peak at 320 nm and expressing as *p*-coumaric acid, caffeic acid and ferulic acid equivalents. Then, substrate preference was expressed relative to the best reaction. The color scale indicates the favorable reactions in green. C: Catalytic constants determined for putrescine and the three main acyl donors.

enzyme also transferred caffeoyl-CoA and *p*-coumaroyl-CoA to spermidine with a lower efficiency and led, at least, to isomers of mono-substituted spermidine (Fig. 2 B; Supplementary data 2). The second enzyme, SIPHT4, exhibited a narrow specificity to feruloyl-CoA which could be transferred on putrescine, agmatine and, to a lower extent, to spermidine (Fig. 2A and B; Supplementary data 2). These results demonstrate that both enzymes had different enzymatic specificities than SIPHT1 and SIPHT2 that exhibited a higher affinity for caffeoyl-CoA (Fig. 2A and B-C; Supplementary data 2). The four SIPHTs displayed optimal pH at 9 (SIPHT1-2) and 10 (SIPHT3-4). Interestingly, SIPHT1-2 exhibited a significant activity on a broader range of pH from 5 to 11 (Supplementary data 3). They all had a maximum activity between 30 and 40 °C, precisely 40 °C for SIPHT1-2, 30 °C for SIPHT3 and 35° for SIPHT4 (Supplementary data 4). In addition to the polyamines cited above, these enzymes were also assayed with spermine, tyramine, octopamine, noradrenaline, dopamine and lysine as acyl acceptors, based on the occurrence of related phenolamides in tomato (Von Roepenack-Lahaye et al., 2003; Zacarés et al., 2007; Larbat et al., 2014), however no activity was detected.

2.3. Phenolamide distribution in tomato

Caffeoyl putrescine and feruloyl putrescine were the two main phenolamides accumulating in vegetative organs of plantlets and 28-day-old tomato plants (Fig. 3 A). Both metabolites were also found in flowers, although these organs contained a much higher diversity of phenolamides, the major ones being di- and tri-coumaroyl spermidines (Supplementary data 5). Caffeoyl putrescine was mainly detected in the upper parts of stems, with a concentration reaching $5.5 \pm 1.3 \mu\text{g g FW}^{-1}$. In the other plant parts, the concentration of these molecules was below $1 \mu\text{g g FW}^{-1}$. The distribution of feruloyl putrescine was different from caffeoyl derivatives. It mostly accumulated in roots of plantlet ($0.4 \pm$

$0.04 \mu\text{g g FW}^{-1}$), in roots of 28-day old plants ($1.2 \pm 0.1 \mu\text{g g FW}^{-1}$), and in flowers (from 1.3 ± 0.2 to $2.3 \pm 0.7 \mu\text{g g FW}^{-1}$, Fig. 3 A).

As previously reported (Royer et al., 2016), the foliar accumulation of caffeoyl putrescine dramatically increased upon the infection with *Pseudomonas syringae* (42-fold increase in the infected leaf compared to the control; Fig. 3 B). The infection with *P. syringae* also induced the foliar accumulation of feruloyl putrescine that was not detected in the control leaf (Fig. 3 B).

2.4. Gene expression in planta

The expression of the four SIPHT genes was measured, by RT-PCR, from the same set of tissues as for metabolic analysis (Fig. 3 C). SIPHT1 gene expression was detected in all plant parts. In the vegetative organs, the expression level was the highest in stems, then in roots and, to a lesser extent, in cotyledons and leaves. Interestingly, SIPHT1 was the only gene which expression was detected in the aboveground tissues of the 28-day old plants. SIPHT3 and 4 displayed a similar expression pattern and are restricted to roots and rootlets. Finally, SIPHT2 expression was not detected in any vegetative tissues. In flowers, we could show the expression of all four SIPHTs. SIPHT1 was expressed with the same order of magnitude in flower buds and opened flowers, whereas the expression level of SIPHT3 was increasing according to the development stage of the flowers. Finally, although detected, the expression level of SIPHT2 and 4 in flowers was low.

The inoculation of tomato leaves with *P. syringae* led to a strong modification of the expression pattern of the PHT genes. Indeed, while only SIPHT1 was detected in control leaves, the expression of all SIPHT genes but SIPHT3 was detected in inoculated leaf. Among them, SIPHT2 and SIPHT1 were markedly induced (51- and 809-fold increase respectively, Fig. 3 C)

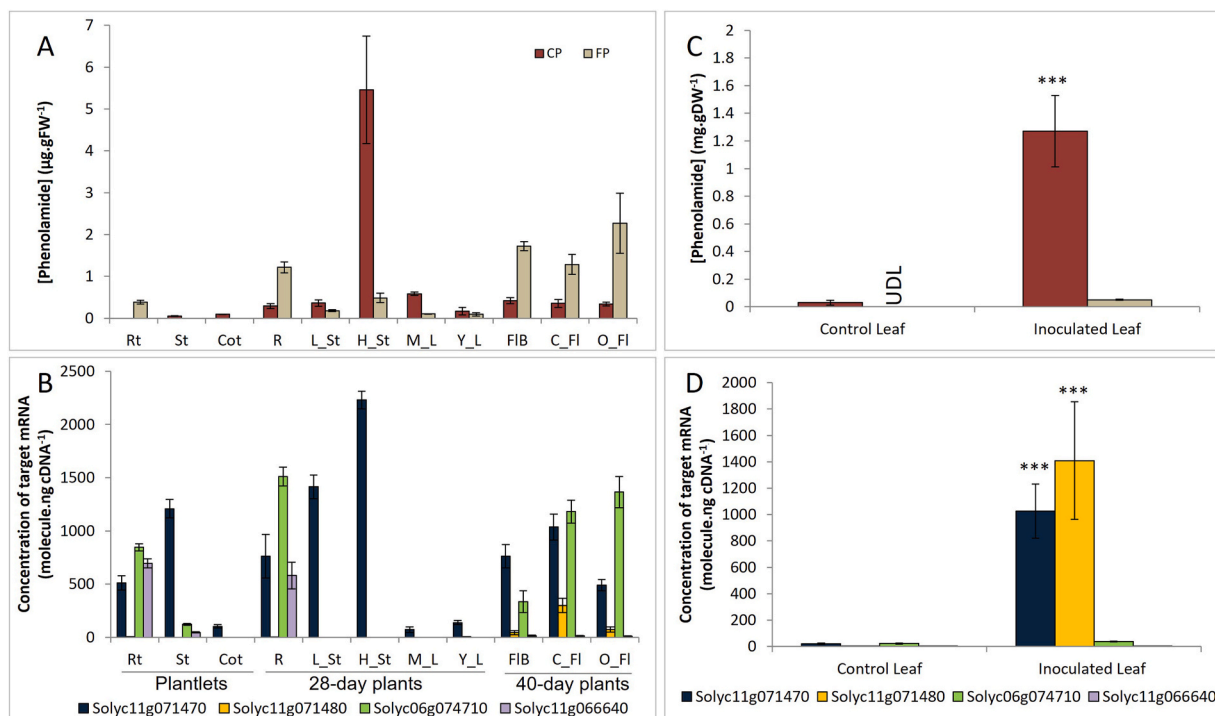


Fig. 3. Regio-specific and inducible accumulation of putrescine-associated phenolamides and the 4 SIPHT transcript abundance in tomato. The concentrations of caffeoylputrescine (CP) and feruloylputrescine (FP) were determined in different tomato organs (A) and in tomato leaves inoculated with *Pseudomonas syringae* for 72h (C). The absolute expression levels of the 4 genes coding for SIPHT were measured in the same tomato organs (B) and in tomato leaves inoculated with *Pseudomonas syringae* for 24h (D). Error bars represent the standard errors calculated from four biological replicates. Rt: Rootlet; St: Plantlet Stem; Cot: Cotyledon; R: Root; M_L: Mature Leaves; Y_L: Young Leaves; FIB: Flower Buds; C_FI: Closed Flower; O_FI: Open Flower. L_St: Lower Stem; H_Stem: High Stem; UDL: Under Detection Limit. $P < 0.001$ ***T. test.

3. Discussion

Despite their wide distribution among plants, their high structural diversity, and their involvement in several biological processes, the understanding on the phenolamide metabolism and regulation is far from being complete. This work provided insights on the accumulation of putrescine derivatives which are found constitutively and systemically in healthy tomato plants in addition to being strongly induced in response to pest attacks. The two new *N*-hydroxycinnamoyltransferases, in addition to the other two enzymes previously reported (Roumani et al., 2022), belong to a phylogenetic cluster of putrescine hydroxycinnamoyltransferases. The cluster contains NaAT1 putrescine transferase isolated from *N. attenuata* (Kaur et al., 2010). The two new identified enzymes are close to the rice transferases that uses putrescine and agmatine as acyl acceptors to synthesize *p*-coumaroyl and feruloyl derivatives (Tanabe et al., 2016).

The enzymatic characterization together with the expression pattern *in planta* of the four SIPHT indicated that the synthesis of putrescine containing phenolamides in tomato is controlled by a family of genes sharing a complementarity in function and spatial expression. This conclusion is in accordance to the observations made on rice (*O. sativa*) where the root and flower accumulation of agmatine and putrescine phenolamides were correlated to the spatial expression of three functionally characterized PHT (Tanabe et al., 2016). In this study, the combined analysis of substrate specificity and spatial expression allow to assume that the above-ground accumulation of caffeoylputrescine is principally controlled by SIPHT1. In roots, the high accumulation of feruloylputrescine correlates with the expression of SIPHT4 which exhibited a narrow specificity to feruloyl-CoA, and also the expression of SIPHT3 which had a wider range of substrates. In flowers, the accumulation of feruloylputrescine is not correlated to the SIPHT4 expression, but could be related to the expression of SIPHT2 and SIPHT3. Additional mechanisms may however contribute to better explain the specific ratio found between caffeoyl and feruloyl derivatives in the different plant parts, of which substrate availability appears as a major one. Moreover, the accumulation of other phenolamides in flowers could be partially explained by the specificity of SIPHTs (Supplementary data 5). Indeed, the ability of SIPHT3 to catalyse, *in vitro*, the synthesis of monoacylated spermidine, suggests that it could contribute to the accumulation of *p*-coumaroylspermidine and feruloylspermidine. The accumulation of mono-, di- and tri-acylated spermidine derivatives is more likely controlled by the activity of spermidine hydroxycinnamoyl transferase (SHT), which exhibits the ability to catalyse sequentially *N*-substitutions on both primary and secondary amino groups (Peng et al., 2016; Perrin et al., 2021). Interestingly, the expression patterns reported for the four SIPHT were confirmed and complemented by public gene expression databases such as Tomato Expressed Database (TED, <http://ted.bti.cornell.edu/cgi-bin/TFGD/digital/home.cgi>) and Tomato Expression Atlas (TEA, <https://tea.solgenomics.net/>). With respect to these databases, SIPHT3 appears as the main expressed PHT in the fruit tissues.

In accordance to their role in plant defense, phenolamides and among them, caffeoylputrescine and feruloylputrescine were significantly accumulated tomato leaves infested by *Pseudomonas syringae*. This accumulation appears correlated to the highly inducible expressions of SIPHT1 and moreover SIPHT2 which was barely detected in non-infested tissues. The high inducibility of these two genes was also observed in tomato leaves infested with larvae of the lepidoptera *Tuta absoluta* (Roumani et al., 2022). In addition, the inducibility of only these two SIPHT was demonstrated recently in tomato leaves treated with jasmonic acid. Other phytohormones (ethylene, salicylic acid) or the two elicitors flg15 and BP178 were however ineffective (Montesinos et al., 2021 Supplementary Table 2A).

4. Conclusions

In conclusion, the present study allowed the identification and the

biochemical characterization of a small family of hydroxycinnamoyltransferases catalyzing the biosynthesis of putrescine-containing phenolamides. The substrate specificity and the expression pattern of these four enzymes supported the spatial distribution of caffeoyl and feruloylputrescine distribution *in planta* in both healthy and infected plant.

5. Materials and methods

5.1. *In silico* investigations and phylogenetic analysis

The protein sequences of Solyc11g071470 and Solyc11g071480 previously functionally characterized as putrescine hydroxycinnamoyltransferase (Roumani et al., 2022) were blasted against a tomato genome protein sequence database (ITAG 4.0) using the BLASTp procedure. Proteins showing more than 60% identity were taken into account for the rest of the paper.

A phylogenetic tree was constructed with the protein sequences identified in tomato and a selection of BAHD acyltransferases functionally characterized, and representing the 8 clades of *N*-hydroxycinnamoyltransferases within this enzyme family (Roumani et al., 2020). Protein sequences were aligned with ClustalW and the tree was further constructed using neighbor-joining method with bootstrap values corresponding to 100 resampling.

The 3000 bp upstream sequence from the ATG initiation codon of each gene were used for promoter sequence analysis according to Chen et al. (2021). The genetic distance between the 4 promoters was calculated using MEGA 11 software. In addition, the four promoter sequences were searched for regulatory elements using the New PLACE database (<https://www.dna.affrc.go.jp/PLACE/?action=newplace>).

5.2. Plant material, growth conditions and treatments

Tomato plants (*Solanum lycopersicum*, var. Better Bush) were grown from seed in hydroponic conditions in a growth chamber (16 h photoperiod, 23 °C/18 °C day/night, 60 % air humidity). The nutrient solution, with a [NO₃] at 7 mM, was prepared with water and pure salts as described in supplementary data 6.

For the spatio-temporal study of the phenolamide accumulation and tomato gene expression, three harvests were realized. A first one on 10-day-old plantlets, which were separated into three organ classes: Rootlets, stem and cotyledons. A second harvest was done on 28-day-old vegetative plants, corresponding to 6 leaves-plants which were separated into 5 organ classes: Roots, "Low Stem" corresponding to a stem section between leaf 1 and leaf 4, "High Stem" corresponding to stem section between leaf 4 and leaf 6, "Mature Leaves" corresponding to the limb part of leaves 3 and 4, and "Young Leaves" corresponding to the limb of leaves 5 and 6. A last harvest on 40-day-old flowering plants was focused on flowers by discriminating "Flower Buds", "Closed Flowers" and "Open Flowers".

For the inducible study of the phenolamide accumulation and tomato gene expression, tomato plants were infected with *P. syringae* strain DC3000 isolated in 1960 in Gernsey (Buell et al., 2003) as described in (Royer et al., 2016). Leaves from control and infected plants were harvested 72h after the inoculation.

For each study, the harvested samples were frozen in liquid nitrogen, and then stored at −80 °C before being crushed in a fine powder using mortar and pestle. For each sample, two aliquots (100 mg each) were prepared, for RNA and phenolic extractions, and stored at −80 °C.

5.3. qPCR analysis

Total RNA extraction was realized from 100 mg frozen tissue using the E.Z.N.A. Plant RNA kit (Omega bio-tek, Norcross, GA, USA). A on-column-DNAse treatment (Qiagen, Hilden, Germany) was included during the RNA extraction. RNA quality was checked on a 1% agarose gel. RNA samples were quantified using absorbance at 260 nm, and their

purity were evaluated by the 260/280 nm absorbance ratio. A total of 200 ng of DNA-free RNA samples was reverse transcribed in a final volume of 20 μ L using the RNA to cDNA kit (Life technologies). cDNAs were then diluted ten times. Tomato gene expression was analysed by qPCR on a StepOnePlus RealtimePCRSytem (ThermoFisher Scientific, Bremen, Germany) using the primers described in [supplementary data 2](#). Each PCR reaction was realized in 20 μ L containing primer pairs at 0.3 μ M, 5 μ L of cDNA and 1x SYBR® Premix Ex Taq™ (Takara, Shiga, Japan) and followed amplification protocol consisting in a denaturing step at 95 °C for 30 s, 40 cycles at 95 °C for 5 s and 60 °C for 30 s. A melting curve stage was included in order to confirm the specificity of each reaction. Standard curves for absolute quantification were generated from purified pCR8 vectors containing the different targeted cDNA. Concentration of the standard targeted cDNAs, expressed as the number of copies per volume unit, were calculated using their absorbance at 260 nm (for dsDNA, 1.0 A_{260} = 50 μ g/ml), the average molecular weight of pCR8/targeted cDNA (1 bp = 660 Da) and the Avogadro constant (6.022×10^{23} molecules/mol). These standard solutions were serially diluted to obtain standard series ranging from 10^4 to 5 copies per μ L of standard, each step differing by 5-fold.

5.4. Expression and purification of recombinant proteins

The four full-length sequences (*Solyc11g071470*, *Solyc11g071480*, *Solyc06g074710* and *Solyc11g066640*) were cloned from tomato cDNA using the primers listed in [supplementary data 7](#). The PCR products were sub-cloned first in the pCR8 vector (Invitrogen, Carlsbad, California), prior to sequencing and then transferred into pET28b His-tag expression vector as a *Bam*HI-*Not*I fragment. The four expression vectors were introduced into *E. coli* strain BL21-DE3 by heat shock transformation and the transformants were selected on kanamycin. Protein expression was realized by growing 50 mL of culture at 37 °C until a DO_{600} comprised between 0.4 and 0.6. Then 1 mM of IPTG was added and the cultured was incubated for 22 h at 18 °C. The bacteria were then harvested by a 5000 g centrifugation for 15 min at 4 °C, then washed three times in 5 mL of PBS buffer (8.1 mM Na_2HPO_4 , 1.76 mM KH_2PO_4 , 2.7 mM KCl, 137 mM NaCl, pH 7.4) and finally resuspended in 1 mL of PBS. Bacterial lysis was realized by three 30 s runs of sonication (Bandelin Sonoplus HD, 2010 MS73 probe with an intensity of 200 W/cm²). After a final centrifugation at 16000g for 10 min at 4 °C, the supernatant containing the protein of interest was collected and stored at -20 °C. His-tag proteins were purified using a Ni-NTA purification system (Qia- gen) by following the specification of the manufacturer. Purified His-tag proteins, detected on SDS-PAGE ([Supplementary data 8](#)), were then used directly to perform enzymatic assays.

5.5. Enzymatic characterization

CoA esters of *p*-coumaric, caffeic, and ferulic acids were synthesized using the recombinant 4-CL enzyme from *N. tabacum*. Enzymatic assays were performed in a total volume of 100 μ L containing 40 μ M of potential acyl donors (*p*-coumaroyl-, caffeoyl- and feruloyl-CoA) and 500 μ M of polyamines (putrescine, cadaverine, agmatine, spermidine, spermine, tyramine, octopamine, dopamine, noradrenaline) as acyl acceptors in 100 mM Tris-HCl buffer, pH 9, containing 5 mM EDTA. For a given enzyme, the activity assays were realized with a same volume of purified enzyme (10 μ L) for all the substrate tested. This procedure allows comparing substrate specificity for each enzyme and the substrate preference between the four enzymes only on a qualitative basis. The reactions were initiated by the addition of purified enzymes, incubated at 35 °C for 5 min, and stopped by adding 50 μ L of acetonitrile containing 1% HCl. The reaction mix was then centrifuged for 10 min at 14000 g and filtered through a 0.2 μ m filter before being analysed on UPLC-DAD-ESI-MS according to ([Larbat et al., 2014](#)). The kinetic constants of the four enzymes were determined using a range of concentration of their respective preferential acyl donor (from 5 to 60 μ M for

p-coumaroyl-CoA, caffeoyl-CoA and feruloyl-CoA) and of their preferential acyl acceptor (from 20 to 400 μ M putrescine). Products were characterized according to their mass and mass fragmentation. Product accumulation was quantified by measuring the area under peak and converted with respect to caffeic, ferulic and *p*-coumaric acid standard curves. The apparent K_m was determined by Lineweaver-Burk extrapolation. The optimum pH was determined by measuring the caffeoyl-putrescine synthesis activity for all tomato genes, except *Solyc11g066640* which was determined for the feruloyl-putrescine synthesis activity, under a pH range from 5 to 13 by using different buffers at 100 mM in the reaction mix containing 40 μ M of acyl donor and 500 μ M of putrescine (Sodium phosphate from pH 5 to 8; Tris-HCl from pH 8 to 10 and Glycine from pH 10 to 13). The optimal temperature as determined on a range from 20 to 45 °C.

5.6. Phenolamide profiling by U-HPLC-MS analysis

Phenolic compounds including phenolamides were extracted from tomato plants as previously described ([Royer et al., 2016](#)). Briefly, about 100 mg FW of tissues were extracted with 1 ml 80% MeOH and blended (Ultra-Turrax, Polytron PT2100) for 30 s and centrifuged at 4000 g for 10 min. The extraction was repeated and the two supernatants were pooled in a new tube and vacuum dried. The dried residue was then dissolved in 1 mL of MeOH 70% and filtered (0.2 μ m) before analyses.

A qualitative analysis of phenolics from the different tomato organs was realized on a HPLC-DAD-LTQ Orbitrap MS (ThermoFisher Scientific, San Jose, CA, USA) as described in ([Larbat et al., 2014](#)). Briefly, 20 μ L of extracts were separated on a C18 LichroCART (250 mm \times 4.6 mm) column (Merck, Germany). The flow rate was set at 700 μ L min⁻¹ and mobile phases consisted of water modified with formic acid (0.1%) for A and methanol modified with formic acid (0.1%) for B. Phenolics were eluted using a first isocratic step at 1% of B for 2 min, then a linear gradient from 1% to 50% of B for 50 min and finally a linear gradient from 50% to 90% of B for 20 min. Mass analysis was carried out in electrospray ionization (ESI) positive mode (for details see [Larbat et al., 2014](#)). Quantitative data were obtained for caffeoylputrescine and feruloylputrescine by analyzing 5 μ L of each extract on a U-HPLC-DAD system (Shimadzu, Japan). The extract was separated on a C18 Kinetex (100 mm \times 2.1 mm) column (Phenomenex, USA) by using a gradient elution from 1 to 60% MeOH for 6.8 min, then 90% MeOH for 2.7 min with a flow rate of 300 μ L min⁻¹. The column was rinsed for 1 min with 90% MeOH and reequilibrated to 1% MeOH for 2 min prior to the next run. Compound quantification was based on measurement of area under each peak determined at 320 nm as already described in ([Larbat et al., 2014](#)). Caffeoylputrescine and feruloylputrescine concentrations were expressed as equivalent of ferulic and caffeic acids. The concentrations of other phenolamides were expressed as mass signal intensity per mg of fresh weight.

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CRedit authorship contribution statement

Marwa Roumani: Writing – original draft, Methodology, Formal analysis, Conceptualization. **Sébastien Besseau:** Writing – review & editing, Formal analysis. **Alain Hehn:** Writing – review & editing, Supervision. **Romain Larbat:** Writing – review & editing, Writing – original draft, Supervision, Funding acquisition, Data curation.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Romain Larbat reports financial support was provided by Regional Council of Lorraine. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.phytochem.2024.114271>.

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