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Abstract

Fully substituted phenolamide accumulation in the pollen coat of Eudicotyledons is a conserved evolutionary chemical trait. Interestingly, spermidine derivatives are replaced by spermine derivatives as the main phenolamide accumulated in the Asteraceae family. Here, we show that the full substitution of spermine in chicory (Cichorium intybus) requires the successive action of two enzymes, that is spermidine hydroxycinnamoyl transferase-like proteins 1 and 2 (CiSHT1 and CiSHT2), two members of the BAHD enzyme family. Deletion of these genes in chicory using CRISPR/Cas9 gene editing technology evidenced that CiSHT2 catalyzes the first N-acylation steps, whereas CiSHT1 fulfills the substitution to give rise to tetracoumaroyl spermine. Additional experiments using Nicotiana benthamiana confirmed these findings. Expression of CiSHT2 alone promoted partially substituted spermine accumulation, and coexpression of CiSHT2 and CiSHT1 promoted synthesis and accumulation of the fully substituted spermine. Structural characterization of the main product of CiSHT2 using nuclear magnetic resonance revealed that CiSHT2 preferentially catalyzed N-acylation of secondary amines to form N5,N10-dicoumaroyl spermine, whereas CiSHT1 used this substrate to synthesize tetracoumaroyl spermine. We showed that spermine availability may be a key determinant toward preferential accumulation of spermine derivatives over spermidine derivatives in chicory. Our results reveal a subfunctionalization among the spermidine hydroxycinnamoyl transferase that
was accompanied by a modification of free polyamine metabolism that has resulted in the accumulation of this new phe-
nolamide in chicory and most probably in all Asteraceae. Finally, genetically engineered yeast (Saccharomyces cerevisiae) 
was shown to be a promising host platform to produce these compounds.

**Introduction**

Plants accumulate a wide array of specialized metabolites. Historically defined as opposed to primary metabolites, these 
molecules have long been called secondary metabolites be-
cause they were often considered as evolutionary enigma or 
even waste products (Hartmann, 2007). Primary metabolites, 
that is, central metabolites, referred to molecules that are re-
quired for the survival of the host and which biosynthesis is 
fairly well conserved in all living organisms. Specialized metab-
olites include molecules that are dispensable for survival in an 
optimal environment but confer a great advantage in specific 
ecological niches and in the management of biological inter-
actions (Pichersky and Lewinsohn, 2011). Therefore, their syn-
thesis and accumulation are often distributed in a 
taxonomically restricted manner with few exceptions. These 
molecules are the basis of the extraordinary adaptability of 
plants to their environment as well as the remarkable chemi-
cal diversity occurring in the biosphere (Pichersky and 
Lewinsohn, 2011). Thus, terrestrialization of the green lineage 
is often associated with the emergence of these new mole-
cules (Weng, 2014). They are involved in various ecological 
functions notably plant interaction with its environment, for 
example, pollinator attraction, symbiotic microorganism at-
traction, defense against pathogens and herbivores or envi-
ronmental stresses (Ahmed et al., 2017). At the molecular 
level, the emergence of new metabolic pathways during plant 
evolution mainly relies on gene duplication followed by ran-
dom mutations (Chae et al., 2014). These genetic events oc-
curring in the open reading frames or/and in the regulatory 
parts of the genes can promote the appearance of new bio-
chemical paths leading to new metabolites associated with 
new functions. This evolutionary scenario is called neofunc-
tionalization if one gene retains its ancestral function, whereas 
the other evolves to promote a new function. If both genes 
experience mutations and participate in a new function while 
contributing together to the maintenance of their ancestral 
function, this is called subfunctionalization (Kroymann, 2011; 
Weng, 2014; Moghe and Last, 2015). Another model to ex-
plain the appearance of new metabolic pathways is escape 
from adaptive conflict (Kroymann, 2011; Moghe and Last, 
2015). In this model, a single gene evolved a new function at 
the expense of the ancestral function. This gives rise to an 
adaptive conflict that is solved through gene duplication 
(Kroymann, 2011). In contrast to primary metabolism, in 
which selection results in enzymes with high efficiency and 
strict substrate specificity, enzymes from specialized metab-
olism usually have broad substrate specificity but low catalytic 
specificity, which favors the emergence of multiple new path-
ways during evolution (Weng et al., 2012). This enzymatic 
promiscuity is thought to play a central role in evolution of 
specialized metabolic enzymes and generation of chemical di-
versity (Weng et al., 2012).

Phenolics are specialized metabolites that are widely distrib-
uted in the plant kingdom and contribute to all aspects of 
plant life. They are mostly derived from the phenylpropanoid 
pathway. Products of this pathway, hydroxycinnamonic acids, 
are recruited to form majority of phenolic compounds includ-
ing lignin, tannins, suberin, and flavonoids. Hydroxycinnamic 
acid moieties, for example, coumaric, caffeic, ferulic acid, or 
sinapic acids are rarely accumulated under their free forms 
but are often conjugated to acid alcohols, sugars or amines 
to form esters, glycosides, or phenolamides, respectively. The 
latter are also termed phenylamides or hydroxycinnamic acid 
amides (Edreva et al., 2007; Roumani et al., 2021). These 
polyamine-derived molecules are mostly known for their in-
volvement in floral induction or as defense compounds 
(Bassard et al., 2010; Roumani et al., 2021). Phenolic acids 
can be conjugated to either aromatic amines like tyramine, trypt-
amine, octopamine, or anthranilate or aliphatic polyamines 
like putrescine, spermidine, or spermine (Bassard et al., 2010; 
Roumani et al., 2021).

To date, all the enzymes involved in N-acylation of al-
phatic polyamines belong to the superfamily of the BAHD 
acyltransferases (Roumani et al., 2021). For instance, agma-
tine coumaroyl transferase (ACT) catalyzes N-acylation of 
agmatine in barley (Hordeum vulgare) and Arabidopsis 
(Arabidopsis thaliana; Burhenn et al., 2003; Muroi et al., 
2009), and hydroxycinnamoyl-CoA:putrescine acyltransferase 
(AT1) that of putrescine in Nicotiana attenuata 
(Onkokesung et al., 2012). The triamine spermidine was 
shown to be acylated by spermidine dinapoyl transferase 
(SDT) and spermidine dicoumaroyltransferase (SCT) in 
Arabidopsis (Luo et al., 2009), by hydroxycinnamoyl-
CoAspermidine acyltransferase (DH29) in N. attenuata 
(Onkokesung et al., 2012) and by spermidine hydroxycinna-
ymoyl transferase (SHT) in the tapetal cells of Arabidopsis 
and apple (Malus domestica; Grienenger et al., 2009; 
Elejalde-Palmett et al., 2015). We have previously identified 
and characterized two enzymes from chicory (Cichorium 
imybus), called spermidine hydroxycinnamoyl transferase-like 
proteins 1 and 2 (CiSHT1 and CiSHT2), closely related to 
AtSHT and MdSHT. Both chicory enzymes were shown to 
be involved in tetracoumaroyl spermine synthesis (Delporte 
et al., 2018). The only enzymes characterized so far that 
have the capability to acylate secondary amino groups to 
synthesize fully substituted polyamines are SHTs (Pichersky 
et al., 2006; Grienenger et al., 2009; Elejalde-Palmett et al., 
2015; Peng et al., 2016, 2019; Delporte et al., 2018; Perrin 

et al., 2021). The accumulation of fully substituted spermidine derivatives was shown to be restricted to the pollen coat of Arabidopsis and apple tree and suggested as a marker of the pollen exine of Eudicotyledons (Grienenberger et al., 2009; Elejalde-Palmett et al., 2015). We provided evidence that spermine-derived phenolamides constitute a chemical signature of the pollen coat of the Asteraceae family (Delporte et al., 2018). Both enzymes identified in chicory acylated either spermine or spermidine in vitro in the presence of hydroxycinnamoyl-CoA and all intermediates were detected, but, surprisingly the fully substituted polyamines were not the major products. Additionally, heterologous expression of CiSHT2 in sht Arabidopsis mutants which do not accumulate phenolamide in the pollen coat led to similar inconsistency that is, some phenolamides were accumulated, but fully substituted polyamines were not the main phenolamides (Delporte et al., 2018). Similar results were obtained when chicory hairy roots that did not accumulate phenolamides naturally were engineered to overexpress CiSHT2: no fully substituted phenolamides were detected whereas partially substituted phenolamides were present (Delporte et al., 2018). Moreover, heterologous and homologous expression of CiSHT1 did not induce any phenolamide accumulation.

In this report, we conducted in planta experiments in order to clarify the roles of CiSHT1 and CiSHT2. In order to know if both enzymes were required for the production of tetracoumaroyl spermine in the tapetal cells, we generated chicory plants silenced in the expression of either CiSHT1 or CiSHT2 or both. Interestingly, CiSht1 and CiSht2 single mutant plants have a different flower bud phenolamide pattern than the wild-type (WT). CiSht1 single mutant also exhibits a phenolamide profile different from that of the CiSht2 mutant. The data suggest that both enzymes act sequentially in vivo to synthesize tetracoumaroyl spermine. Heterologous expression of these genes in Nicotiana benthamiana confirmed this assumption. We provided evidence that CiSHT2 acylates preferentially secondary amines using free or monoacylated polyamines as a substrate, whereas CiSHT1 uses the already acylated polyamines to produce fully substituted amines. Moreover, while this enzymatic system is able to acylate either spermine or spermidine in vivo and in vitro, further investigation suggested that free polyamine availability may contribute to direct the flux toward either spermidine or spermine derivative production. Additionally, a bioconversion system using yeast (Saccharomyces cerevisiae) as a chassis to produce spermine derivatives was successfully established but needs further improvement for the efficient production of different isomers of acylated spermine.

Results

Generation of CiSht1 and CiSht2 chicory mutants from genome-edited hairy roots

In order to decipher the functional roles of CiSHT1 and CiSHT2 and try to understand the meaning of this apparent biochemical redundancy, mutants have been generated. The genes CiSHT1 and CiSHT2 are 1,356-bp and 1,359-bp long, respectively, and do not contain any introns (Delporte et al., 2018). The target sequences were selected to induce breaks at positions 550 and 663 for CiSHT1 and at positions 235 and 351 for CiSHT2 (Figure 1A). Three binary vectors were prepared but only two gave rise to mutant plants (pYLKCRISPR-sgRNA1–sgRNA2-CiSHT2 was ineffective). The first, named pYLKCRISPR-sgRNA1–sgRNA2-CiSHT1, was designed to generate a double mutation in CiSHT1 and the second, termed pYLKCRISPR-sgRNA1–sgRNA2-SHT1/sgRNA1–sgRNA2-SHT2, to generate a double mutation in both genes, thus expressing four sgRNAs (Figure 1, B and C). Through Agrobacterium rhizogenes-mediated transformation, 12 and 7 glufosinate-resistant hairy root lines were obtained with pYLKCRISPR-sgRNA1–sgRNA2-CiSHT1 and pYLKCRISPR-sgRNA1–sgRNA2-SHT1/sgRNA1–sgRNA2-SHT2 vectors, respectively. Genomic DNA was extracted from each resistant line in order to determine the presence of mutations in the target loci by high-resolution melting (HRM) curve analysis. PCR products from plants with divergent melting curves compared to the WT, were further sequenced in order to confirm mutations and to characterize them. Seven lines with biallelic mutations for each transformation event have been identified and used for further experiments (Supplemental Figure S1). Three independent sht1 mutant lines (8, 17, and 19; Supplemental Figure S1A) arising from transformation with pYLKCRISPR-sgRNA1–sgRNA2-CiSHT1, three independent sht2 mutant lines (21, 22, and 23; Supplemental Figure S1B), and one sht1/sht2 double mutant line (7; Supplemental Figure S1C) arising from transformation with pYLKCRISPR-sgRNA1–sgRNA2-SHT1/sgRNA1–sgRNA2-SHT2 were generated. Most of the mutations occurred at a position about 3-bp upstream the protospacer adjacent motif (PAM) sequence, as reported in other studies (Jinek et al., 2012). Editing types were either small deletion or insertions, but sometimes the inter-guide fragment was completely deleted as for the line SHT2 (23; Supplemental Figure S1B), which is consistent with what was shown in our previous study (Bernard et al., 2019). The target 1 of CiSHT1 was not effective in editing the target sequence with both binary vectors. The fact that some targets are less effective than others was already observed in apple (Nishitani et al., 2013). The frame shifts induced by the genome-editing introduced an early stop on mRNA translation in all the mutated hairy root lines (Supplemental Figure S2).

Metabolic analysis of genome-edited plants

To investigate whether mutations generated by the CRISPR/Cas9 system had an impact on the phenolamide content of chicory flower buds, plants were regenerated from hairy roots. Flower buds from sht1 mutant lines, sht2 mutant lines, sht1/sht2 double mutant line, and from plants transformed with A. rhizogenes WT strain (thereafter these plant lines are called WT) were collected. As shown previously (Delporte et al., 2018), tetracoumaroyl spermine was the main phenolamide in WT flower buds and both tricoumaroyl spermine and tricoumaroyl spermidine were
accumulated at a lower level (Figure 2). In the flower buds of sht2 mutant lines and of the sht1/sht2 double mutant, no phenolamides were detected and both genotypes exhibited identical metabolic profile (Figure 2). In the flower buds of the sht1 mutant lines, only partially substituted spermine that is, dicoumaroyl spermine and tricoumaroyl spermine, were detected instead of tetracoumaroyl spermine. All together, these data suggest that both CiSHT1 and CiSHT2 are required to promote the synthesis of the fully substituted phenolamides in chicory flower buds. CiSHT2 would catalyze the first N-acylation steps, whereas CiSHT1 would fulfill the N-acylation to produce the fully substituted phenolamides.

**Transient expression of CiSHT1 and CiSHT2 in N. benthamiana leaves**

In order to confirm the previous results, CiSHT1 and CiSHT2 were transiently expressed in leaves of *N. benthamiana* where phenolamide-like compounds are not expected to accumulate. Leaves of *N. benthamiana* were infiltrated with *Agrobacterium tumefaciens* containing constructs of CiSHT1 or CiSHT2 or with a mixture of both strains. As a control, leaves were infiltrated with the void vector, pEAQ-HT. Four days after infiltration, leaves were collected, and the phenolic compounds were extracted before analysis. As expected, no spermine or spermidine derivatives were detected in *N. benthamiana* leaves infiltrated with bacteria containing either the empty vector, pEAQ-HT and more interestingly with the pEAQ-HT-DEST1-SHT1 (35S-CiSHT1) vector (Figure 3). In leaves transiently expressing SHT2 (35S-CiSHT2), a mixture of phenolamides was detected, notably, mono- and di-coumaroyl spermidine as well as mono- and di-coumaroyl spermine. However, no fully substituted phenolamides could be detected or only in trace amounts. Finally, leaves agroinfiltrated with the vector pEAQ-HT-DEST1-SHT1 together with the vector pEAQ-HT-DEST1-SHT2 (35S-CiSHT1 + 35S-CiSHT2) accumulated tricoumaroyl spermidine, tricoumaroyl spermine, and tetracoumaroyl spermine. The main fully substituted phenolamide was tricoumaroyl spermidine (Figure 3). Interestingly, the peaks present in the chromatogram obtained with an extract of leaf infiltrated with pEAQ-HT-DEST1-SHT2 alone disappeared. To deepen our knowledge about the reaction catalyzed by both enzymes, an experiment was conducted in order to characterize the different isomers and determine their relative abundance. Eight independent *N. benthamiana* plants were agroinfiltrated as already described and the metabolite profiles analyzed by hierarchical clustering (Figure 4; Supplemental Table S1). Three monoucumaroyl spermidine...
Figure 2 Stacked HPLC chromatograms of extracts obtained from flower buds at stages 13–15 isolated from WT, sht1 mutant, sht2 mutant or sht1/sht2 mutant. The identity of the numbered peaks was confirmed by mass spectrometry: (1) tetracoumaroyl spermine, (2) tricoumaroyl spermidine, (3) tricoumaroyl spermine, and (4) dicoumaroyl spermine.

Figure 3 Stacked HPLC chromatograms of extracts obtained from agroinfiltrated N. benthamiana leaves. Plants were infiltrated with either the empty plasmid, CiSHT1 (35S-CiSHT1), CiSHT2 (35S-CiSHT2), or CiSHT1 plus CiSHT2 (35S-CiSHT1 + 35S-CiSHT2). The identity of the numbered peaks was confirmed by mass spectrometry: (1) monocoumaroyl spermine, (2) monocoumaroyl spermidine, (3) dicoumaroyl spermine, (4) dicoumaroyl spermidine, (5) tricoumaroyl spermine, (6) tricoumaroyl spermidine, and (7) tetracoumaroyl spermine.
isomers, two dicoumaroyl spermidine isomers, and one dicoumaroyl spermine were detected when *N. benthamiana* leaves were agroinfiltrated with *CiSHT2*. Traces of dicoumaroyl spermidine were also detected. The main monocoumaroyl spermidine (MonoCSpd isomer 1) had a RT of 2.5 min in our chromatographic conditions (Figure 3) as also shown in the right panel. DiCSpd, dicoumaroyl spermidine; MonoCSpd, monocoumaroyl spermidine; DiCSpm, dicoumaroyl spermine; TriCSpm, tricoumaroyl spermine; TetraCSpm, tetracoumaroyl spermine; TriCSpd, tricoumaroyl spermidine.

Figure 4 Heatmap of spermine and spermidine derived phenolamides in *N. benthamiana* agroinfiltrated with *CiSHT1, CiSHT2*, or *CiSHT1* plus *CiSHT2*. Pick area means for each condition (eight plants) and for each metabolite are expressed per mg of dry material (DM). Natural logarithm of the means for each condition was used to conduct the heatmap hierarchical clustering with the Ward’s method. Colors in the heatmap are related to the sequential logarithmic scale presented in the right panel. DiCSpd, dicoumaroyl spermidine; MonoCSpd, monocoumaroyl spermidine; DiCSpm, dicoumaroyl spermine; TriCSpm, tricoumaroyl spermine; TetraCSpm, tetracoumaroyl spermine; TriCSpd, tricoumaroyl spermidine.

Conversion of N\(^{-}\)-monocoumaroyl spermidine into N\(^{1}\)-monocoumaroyl spermidine. In our analysis, ESI-MS-HRMS spectra were similar to those reported by Hu et al. (1996) confirming identical behavior toward fragmentation of the isomers (Supplemental Figure S3). A structure has been assigned to the three isomers and N\(^{-}\)-monocoumaroyl spermidine was shown to be the main isomer accumulated in *N. benthamiana* leaves (MonoCSpd isomer 1; Supplemental Table S1). The identity of the two dicoumaroyl spermidine was not investigated further. Indeed it was already shown that their discrimination based on tandem mass spectrometry (MS/MS) data was not conclusive (Bigler et al., 1996). The main spermine-derived products accumulated in *N. benthamiana* leaves expressing *CiSHT2* (Figures 3 and 4; Supplemental Table S1) and accumulated in chicory sht1 mutants (Figure 2) has a RT of 8.5 min in our chromatographic conditions. The same dicoumaroyl spermine isomer was synthesized by *CiSHT2* in vitro as well as accumulated in chicory hairy roots overexpressing *CiSHT2* (Delporte et al., 2018). It was identified as one of the dicoumaroyl spermine isomers by mass spectrometry. This compound was purified from hairy root overexpressing *CiSHT2* and precisely characterized. The purified compound analyzed by ESI-HRMS gave a [M + H] \(^{+}\) ion at m/z of 495.2971 and the molecular formula predicted from the accurate mass measurement was C\(_{28}\)H\(_{38}\)N\(_{4}\)O\(_{4}\) as expected for a dicoumaroyl spermine isomer (Figure 5A). In addition, nuclear magnetic resonance (NMR) analysis revealed \(\text{\(^1H\)}\) and \(\text{\(^{13C}\)}\) characteristic signals consistent with a spermine derivative substituted with coumaroyl groups (Table 1; Supplemental Figure S4). It should be pointed out that the number of \(\text{\(^1H\)}\) and \(\text{\(^{13C}\)}\) signals observed on the spectra were surprisingly halved indicating a symmetry in the structure. Thus, spermine could be acylated either on N\(^1\) and N\(^{16}\) positions or on N\(^5\) and N\(^{10}\) positions. Long range \(\text{\(^1H\)}\)-\(\text{\(^{13C}\)}\) correlations were investigated by 2D NMR using HMBC experiments to get more information about the connectivity of the coumaroyl groups on the spermine. \(\text{\(^3J_{HC}\)}\) cross-peaks were observed between the protons H-4/H-11 (\(\delta\) 3.57 ppm) and H-6/H-9 (3.59 ppm) of the spermine and the equivalent quaternary carbons from coumaroyl groups (\(\delta\) 169.9 ppm) corresponding to carbonyl of an amide function. These results suggest the grafting of coumaroyl units on secondary amines of spermine to give N\(^{-}\), N\(^{10}\)-dicoumaroyl spermine (Figure 5C). In the ESI-HRMS spectrum, typical fragments were observed at m/z 147.0441, 204.1019, 275.1754, and 349.2600 confirming the identity of this compound (Figure 5, A and B). From the precursor ion at m/z 495.2709, a first loss of NH\(_3\) (−17 Da) gave a fragment with a moderate intensity at m/z 478.2709. Loss of NH\(_3\) from the ions at m/z 349.2600 and 275.1754 gave ions with very low intensities at m/z 332.2321 and 258.1490, respectively. All these NH\(_3\) losses are in accordance with the presence of free primary amine functions in the dicoumaroyl spermine under investigation. Moreover, the very low intensity fragment at m/z 421.2117 confirm our structural determination. Indeed, it can only arise from the precursor ion at m/z 495.2709 by
Figure 5 ESI-MS-HRMS spectra of purified N₅,N₁₀-dicoumaroyl spermine. A, Fragmentation of purified N₅,N₁₀-dicoumaroyl spermine. The position of the coumaroyl moieties was deduced from RMN data (see Table 1; Supplemental Figure S4). B, Fragmentation scheme of N₅,N₁₀-dicoumaroyl spermine. C, Chemical structure of N₅,N₁₀-dicoumaroyl spermine as deduced from NMR data.
ary amines of spermine, while CiSHT1 would catalyze the N-acylation of primary amines of spermine. This observation prompted us to determine whether the pools of available polyamines in a specific tissue could be responsible for the production of a particular compound rather than the acyl acceptor specificity of the enzymes. In Arabidopsis flower buds, the ratio of spermidine over spermine is clearly in favor of spermidine (around 5 times more spermidine than spermine; Fellenberg et al., 2012). We quantified free polyamines in chicory flower buds as well as in N. benthamiana leaves. In N. benthamiana leaves, spermidine levels (1 μmol g⁻¹ DW) were higher than that of spermine (0.12 μmol g⁻¹ DW). The ratio spermidine/spermine was close to 8. In contrast in chicory flower buds, this ratio was close to 0.8. Spermidine and spermine levels were 0.5 and 0.7 μmol g⁻¹ DW, respectively. These data suggest that the plant pool of available polyamine (and more specifically their relative abundance) likely plays a main role in determining SHTs product synthesis and accumulation.

### Bioproduction of tetracoumaroyl spermine in S. cerevisiae

The elucidation of CiSHTs coordinated and sequential activities requirement for spermine derivative phenolamide synthesis prompted us to initiate metabolic engineering in yeast for the bioproduction of tetracoumaroyl spermine. To this aim, a precursor-directed synthesis strategy was employed. A yeast strain was first transformed with At4CL5 allowing CoA ester production from hydroxycinnamates added to the external medium as required for BAHD acyltransferases activities (Perrin et al., 2021). Then CiSHT1 and CiSHT2 alone or combined were introduced in the yeast chassis. The engineered yeasts were incubated on medium supplemented with coumarate. After 48 h, yeasts were pelleted, phenolic compounds extracted, and analyzed by liquid chromatography-ultraviolet (LC-UV) (Figure 6). As expected, no phenolamide could be detected when yeasts were transformed with At4CL5 alone and in yeast coexpressing At4CL5 and CiSHT1. In yeast transformed with At4CL5 and CiSHT2, accumulations of monoucumaroyl spermidine and tricoumaroyl spermidine were evidenced. Partially substituted spermine derivatives was only effective when spermine was added. In S. cerevisiae grown in liquid medium, the spermidine/spermine ratio was shown to be around 6 (Marshall et al., 1979). Thus, the endogenous pools of spermidine were too important to support spermine-derived phenolamide production which confirms what was evidenced above, that is free polyamine pools determine the nature of the end product. When At4CL5 and CiSHT2 were coexpressed and yeasts incubated on medium supplemented with both coumarate and spermine, accumulation of monoucumaroyl spermine, dicoumaroyl spermine, and tricoumaroyl spermine occurred. In these conditions, yeasts accumulated also the spermine derivatives but the main phenolamide was the N⁵,N¹⁰-dicoumaroyl spermine eluting at 8.5 min confirming the data presented above. Finally, as expected, the expression of At4CL5, CiSHT2, and CiSHT1 together promotes accumulation of tetracoumaroyl spermine especially when coumarate and spermine were added in the medium. Identity of tetracoumaroyl

### Table 1 ¹H and ¹³C-NMR data of N⁵,N¹⁰-dicoumaroyl spermine in methanol-d₄

| Position | δ_C | δ_H (J in Hz) | HMBC |
|----------|-----|---------------|------|
| 1        | –   | –             | –    |
| 2        | 37.6 | 2.88 (t, J = 6.7) | 3, 4 |
| 3        | 26.7 | 1.94 (t, J = 6.7) | 2, 4 |
| 4        | 43.7 | 3.57 (m)      | 2, 3, 6 |
| 5        | –   | –             | –    |
| 6        | 48.6 | 3.59 (m)      | 4, 8, CONH' |
| 7        | 27.6 | 1.77 (m)      | 8, 9 |
| 8        | 27.6 | 1.77 (m)      | 4, 7 |
| 9        | 48.6 | 3.59 (m)      | 7, 11, CONH'' |
| 10       | –   | –             | –    |
| 11       | 43.7 | 3.57 (m)      | 9, 12, 13 |
| 12       | 26.7 | 1.94 (t, J = 6.7) | 11, 13 |
| 13       | 37.6 | 2.88 (t, J = 6.7) | 11, 13 |
| 14       | –   | –             | –    |
| 1'       | 127.4 | –            | 3', 5', β' |
| 2'       | 130.8 | 7.47 (d, J = 8.4) | 4', 6', β' |
| 3'       | 116.6 | 6.79 (d, J = 8.4) | 1', 4', 5' |
| 4'       | 160.8 | –            | –    |
| 5'       | 116.6 | 6.79 (d, J = 8.4) | 1', 3', 4' |
| 6'       | 130.8 | 7.47 (d, J = 8.4) | 2', 4', β' |
| α'       | 113.8 | 6.87 (d, J = 15.7) | 1', CONH' |
| β'       | 145.0 | 7.59 (d, J = 15.7) | 2', 6', 3', CONH' |
| CONH'    | 169.9 | –            | –    |
| 1''      | 127.4 | –            | 3'', 5'', x'' |
| 2''      | 130.8 | 7.47 (d, J = 8.4) | 4'', 6'', β'' |
| 3''      | 116.6 | 6.79 (d, J = 8.4) | 1'', 4'', 5'' |
| 4''      | 160.8 | –            | –    |
| 5''      | 116.6 | 6.79 (d, J = 8.4) | 1'', 3'', 4'' |
| 6''      | 130.8 | 7.47 (d, J = 8.4) | 2'', 4'', β'' |
| α''      | 113.8 | 6.87 (d, J = 15.7) | 1'', CONH' |
| β''      | 145.0 | 7.59 (d, J = 15.7) | 2'', 6'', x'', CONH'' |
| CONH''   | 169.9 | –            | –    |

Position refers to the position indicated in Figure 5C. d, doublet; δ, chemical shift; HMBC, heteronuclear multiple bond correlation; J, coupling constant; m, other multiples; t, triplet.
Spermine was confirmed by MS (Supplemental Figure S5). In these conditions partially acylated spermine derivative accumulation decreased accordingly, and accumulation of spermidine derivatives was also effective. Two additional peaks were detected when yeasts were engineered with \( \text{CiSHT2} \) or \( \text{CiSHT1} \) and \( \text{CiSHT1} \). They correspond to a dihydrocoumaroyl spermidine derivative and a dihydrocoumaroyl spermine derivative (i.e. \( N\)-dihydrocoumaroyl, \( N' \), \( N''\)-dicoumaroyl spermidine and \( N\)-dihydrocoumaroyl, \( N' \), \( N''\)-dicoumaroyl spermine, Supplemental Figures S6 and S7). The yeast enoyl reductase \( \text{TSC13} \) (temperature sensitive \( \text{CSG2} \) suppressor protein 13) is known to reduce coumaroyl-CoA into dihydrocoumaroyl-CoA which is further used, in our case, as a substrate by CiSHTs and competed with coumaroyl-CoA (Perrin et al., 2021). This heterologous system confirms our findings, that is both enzymes are needed for tetracoumaroyl spermine accumulation in vivo and provide an efficient tool for the bioproduction of phenolamides derived either from spermidine or from spermine.

**Discussion**

The presence of fully substituted phenolamides in the outer surface of male gametophytes is a common feature in Eudicotyledons (Elejalde-Palmett et al., 2015). Two enzymes \( \text{AtSHT} \) and \( \text{MdSHT} \) belonging to the BAHD acyltransferases family were characterized and shown to be directly responsible for the synthesis of trisubstituted spermidine in the tapetum of anthers (Grienengerber et al., 2009; Elejalde-Palmett et al., 2015). Thereafter the synthesized compounds have to be exported out of the tapetal cells to be deposited on the pollen surface. The mechanism responsible for this deposition still needs to be described. A recent study has shown that in the Asteraceae family the major phenolamides found in the pollen wall are fully substituted spermine conjugates, and that the presence of these unusual compounds is linked to the activity of two new members of the SHT family in chicory: \( \text{CiSHT1} \) and \( \text{CiSHT2} \) (Delporte et al., 2018). The biochemical and functional characterization of these two enzymes has provided evidence of their involvement in the biosynthesis of tetracoumaroyl spermine. Nevertheless, in vitro and in vivo experiments suggested that there might be some particular features in the tapetum of chicory that act in favor of fully substituted spermine accumulation. Here, additional experiments were conducted to deepen our knowledge of this biochemical pathway.

The CRISPR/Cas9 system was used to knockout \( \text{CiSHT1} \) and \( \text{CiSHT2} \) using the protocol set up by Bernard et al. (2019). The metabolic analysis of mutant plants derived from transformed hairy roots has highlighted that both \( \text{CiSHT1} \) and \( \text{CiSHT2} \) are required and sufficient to promote tetracoumaroyl spermine biosynthesis in chicory. The \( \text{sht2} \) mutants did not produce phenolamides, whereas the \( \text{sht1} \) mutants only produced dicoumaroyl and tricoumaroyl spermine but not the fully acylated spermine. These data indicate a role for \( \text{CiSHT2} \) in the first \( N\)-acylation steps and for \( \text{CiSHT1} \) in the last steps. The results obtained with these mutants are in accordance with the experiments done with chicory hairy roots overexpressing either \( \text{CiSHT1} \) and \( \text{CiSHT2} \) (Delporte et al., 2018).

The requirement of both enzymes to synthesize the fully substituted phenolamides was further confirmed using \( \text{N. benthamiana} \) and yeast heterologous systems. Thus, no phenolamides were produced when expressing only \( \text{CiSHT1} \) while \( \text{CiSHT2} \) expression only promoted partially substituted spermine accumulation. The co-expression of both genes led to the expected fully acylated phenolamide synthesis and accumulation. The main molecule produced in vitro or in vivo by \( \text{CiSHT2} \) is a dicoumaroyl spermine as determined by...
mass spectrometry. To determine the preferred N-acylation sites of CiSHT2, this compound was purified and analyzed by NMR. It was identified as N5,N10-dicoumaroyl spermine. These data suggest that CiSHT2 may have a higher affinity for secondary amines of spermine and N5- or N10-monocoumaroyl spermine, whereas CiSHT1 would prefer primary amines of di- or tri-coumaroyl spermine.

Taken together these results suggest that CiSHT2 would more efficiently promote dicoumaroyl spermine synthesis, whereas CiSHT1 would perform the last N-acylation steps toward fully substituted amine biosynthesis. A similar biochemical mechanism was suggested in N. attenuata (Onkokesung et al., 2012). An acyltransferase (DH29) was shown to catalyze the first N-acetylation step in dicaffeoyl spermidine formation, whereas a second enzyme named CV86 was suggested to act on monoaacoyl spermidine synthesized by DH29 to promote dicaylated spermidine synthesis. Position of caffeoyl moieties was not further investigated but both enzymes belong to the BAHD acyltransferase superfamily.

More detailed in vitro biochemical experiments are necessary to assess this assumption, but this task is hampered by the lack of commercial standards. Purification or chemical synthesis of these compounds may be considered but their high degree of diversity (number and position of substitutions and nature of the amines) and their low amounts in plant tissues render this work extremely challenging. Optimization of the yeast bioproduction system described in this study could help reaching this goal. The use of standards would also be of interest for absolute quantification and to confirm structure assignments. For this last point, very robust analytical experiments were conducted and we are therefore quite confident in our structure determination. For instance, for monocoumaroyl spermidine derivatives, ESI-MS-HRMS fragmentation patterns revealed to be very similar to those obtained with chemically synthesized standards (Hu et al., 1996). Likewise, for N5, N10-dicoumaroyl spermine, NMR evidenced unambiguously the grafting position of coumaroyl residues on secondary amines, the symmetry of the molecule being confirmed by the number of signals halved. Moreover, ESI-MS-HRMS fragmentation study was in agreement with this structural hypothesis. To conclude, ESI-MS-HRMS and NMR experiments conducted were proven to be very reliable analytical techniques for the structure assignments of spermine or spermine-derived phenolamides.

Recently an enzyme able to acylate spermine has been identified in Solanum richardii, a wild relative of eggplant (Peng et al., 2019). This enzyme named Spermine hydroxycinnamoyl transferase (SrSpmHT) was shown to use exclusively spermine as the acyl acceptor substrate and promote monohydroxycinnamoyl spermine synthesis. The product of the reaction was tentatively identified as N4-coumaroyl spermine based on comparison with an authentic standard with LC-UV. Thus, SrSpmHT would acylate primary amine of spermine. SrSpmHT belongs to the BAHD family and is phylogenetically close to AtSDT and AtSCT and seems to have emerged and evolved independently of SHT family (Roumani et al., 2021). Like AtSDT and AtSCT, SrSpmHT lacks the FYGN motif, which was shown to be a specific motif in amino acid sequences of the enzymes belonging to the SHT family such as AtSHT, MdSHT, CiSHT1, and CiSHT2 (Delporte et al., 2018). Taken together, our data suggest that in chicory fully acylated spermine synthesis relies on a two-step mechanism. Furthermore, they confirm that the enzymes belonging to the SHT subfamily of the BAHD acyltransferase superfamily are the only enzymes identified to date, that are capable of acylating secondary amine groups of polyamines.

CiSHT1 and CiSHT2 are both able to use spermine or spermidine as acyl acceptors in vitro and in vivo. Nonetheless in chicory flower buds, the main phenolamide is tetracoumaroyl spermine. With no apparent preference for the substrate, it was tempting to assume that the supply of free polyamines could determine which phenolamides would be the main accumulated conjugate. It was shown that in Arabidopsis flower buds, the content of spermidine was 5 times higher than that of spermine (Fellenberg et al., 2012). We have previously shown that in sht Arabidopsis, mutant expressing CiSHT2 in the tapetal cells, spermidine derivatives were the main phenolamides (Delporte et al., 2018). Furthermore, in N. benthamiana leaves transiently expressing CiSHT1 and CiSHT2 as well as in S. cerevisiae transformed with both genes, tricoumaroyl spermidine was the main product (this study). The measurement of free polyamines has shown that the ratio spermidine/spermine were 8 and 0.8 in N. benthamiana leaves and in chicory flower buds, respectively. This shows that substrate availability or substrate relative availability could determine the identity of the final product. This observation suggests that in the Asteraceae there are at least two evolutionary events that led to the accumulation of these unusual spermine derivatives in the pollen coat: modification of polyamine metabolism and modification of catalytic properties of BAHD-acyltransferases. An extended analysis of polyamine contents in species belonging to the Asteraceae family and close families may help to strengthen this hypothesis. It was previously shown that spermine conjugates were absent in Stylidiaceae, Argophyllaceae, and Calyceraceae (Delporte et al., 2018). This additional analysis should provide an overview of the chronology of the evolutionary events mentioned above. It will be interesting to decipher if modification of polyamine metabolism drove evolution of SHTs, if SHTs evolution led to polyamine metabolism changes or if both events occurred independently. Ultimately whatever the chain of events, this has led to metabolomic diversification that could be responsible for an evolutionary advantage that still deserves to be uncovered. In any case, the functions of these fully acylated polyamines, deposited on the pollen surface, are still under debate, as is the benefit of accumulating spermine derivatives instead of spermidine derivatives. Many hypotheses have been made about
their biological roles (Grienenberger et al., 2009; Elejalde-Palmett et al., 2015; Vogt, 2018). The most obvious function of these compounds is the protection of male gametophytes harboring sperm cells (i.e. the cells responsible for transmitting half part of the genetic material to offspring) against UV radiation. It is possible that the advantage of switching from spermidine- to spermine-conjugates comes from the decoration of all the four nitrogen atoms of the spermine which increases the UV absorbance of a single molecule by about 30% compared to spermidine derivatives, without affecting the concentration of the metabolites, and therefore increases the protection capacity (Vogt, 2018).

In Arabidopsis, the lack of phenolamides in the pollen coat was shown to have no effect on pollen viability or fertility (Grienenberger et al., 2009). Nevertheless, the impact of UV radiation on DNA has not been studied and it seems relevant to investigate UV damage at the molecular level. In this context, the analysis of pollen collected from the Arabidopsis sht mutant and the chicory sht double mutant compared to pollen collected from their WT counterparts could provide interesting clues. These compounds could also have some effect on plant interaction with pollinator (Lin and Mullin, 1999). Insect feeding studies using pollen collected from the mutants or from the WT could be considered. Moreover, the conjugates of hydroxycinnamic acids with polyamines have been proposed to have numerous biological activities such as, antifungal, antimicrobial, and antiviral effects (Walters et al., 2001; Fixon-Owoo et al., 2003; Kyselka et al., 2018).

The evaluation of biological activity is a difficult task due to the poor availability of pure compounds. The elucidation of the biochemical pathway of tetracoumaroyl spermine and its reconstruction in the heterologous system S. cerevisiae, implemented in this work, is an important step toward providing sufficient amounts of molecules to support these biological tests. Nevertheless, this bioproduction system still needs to be optimized to increase the production of spermine derivatives. Indeed, main compounds produced were not the fully substituted amines, and spermidine derivatives. Indeed, main compounds produced were modification of polyamines have been proposed to have numerous biological activities such as, antifungal, antimicrobial, and antiviral effects. These compounds could provide interesting clues. These compounds could also have some effect on plant interaction with pollinator (Lin and Mullin, 1999). Insect feeding studies using pollen collected from the mutants or from the WT could be considered. Moreover, the conjugates of hydroxycinnamic acids with polyamines have been proposed to have numerous biological activities such as, antifungal, antimicrobial, and antiviral effects (Walters et al., 2001; Fixon-Owoo et al., 2003; Kyselka et al., 2018).

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The vectors pDRf1-4CL5-GW, pDRf1-4CL5, and pRS423-GW were generated by BP reaction with pDONR-SHT1 and pDONR221. pDONR-SHT1 and pRS423-FJTAL and pDONR221. pDONR-SHT2 were used to generate pDRf1-4CL5-SHT2, pDRf1-4CL5-SHT1, and pRS423-SHT1 by LR recombination.

Expression in S. cerevisiae

The vectors pDRF1-4CLS-GW, pDRF1-4CLS, and pRS423-FJTAL were provided by Dominique Loqué (Eudes et al., 2011, 2016). pRS423-GW was generated by BP reaction with pRS423-FJTAL and pDONR221. pDONR-SHT1 and pDONR-SHT2 were used to generate pDRF1-4CLS-SHT2, pDRF1-4CLS-SHT1, and pRS423-SHT1 by LR recombination with pDRF1-4CLS-GW or pRS423-GW. The S. cerevisiae pad1 knockout (MATa his3D1 leu2D0 met15D0 ura3D0 Δ pad1, ATCC 4005833) were transformed using the freeze–thaw method (Connelly et al., 1999; Gietz and Schiestl, 2007). Yeasts were selected on solid medium containing yeast nitrogen base (YNB) without amino acids supplemented with 2% glucose (w/v) and 1× dropout-uracil, 1× dropout-histidine, or 1× dropout-uracil–histidine. Overnight cultures from four independent single colonies of recombinant yeast harboring pDRF1-4CLS, pDRF1-4CLS-SHT2, pDRF1-4CLS-SHT1, or pDRF1-4CLS-SHT2 plus pRS423-SHT1 constructs were mixed and grown in 1× YNB medium supplemented with 2% glucose and the required yeast synthetic drop-out medium supplement. These cultures were used to inoculate 2 mL of fresh medium to reach an OD600 of 0.15. The liquid cultures were then incubated at 30°C at 200 rpm for 5 h before adding substrates. Coumarate, spermidine, and spermine were added at a final concentration of 1 mM. The cultures were further grown for 24 h at 200 rpm at 30°C in the presence or absence of the different precursors.
Plant phenolic acid extraction
For chicory and *N. benthamiana*, lyophilized plant material was powdered, and 25 mg were resuspended in 1 mL of a methanol/water/acetic acid mixture (75/23/2, v/v/v). The mixture was then incubated in the dark under agitation at 4°C for 1 h. Homogenate was clarified by centrifugation (14,000 g, 4°C, 10 min) and passed through 0.45-μm filters. For *N. benthamiana* used for the statistical analysis, eight 4-g samples were separately collected by centrifugation (20,000 g, 5 min, 4°C). The supernatants were passed through a 0.45-μm filter prior to centrifugation (20,000 g, 5 min, 4°C). The supernatants were clarified by centrifugation (14,000 g, 4°C, 10 min) and passed through a 0.45-μm filter before HPLC-UV analysis.

Metabolite analyses
Metabolite analysis by liquid chromatography-diode array detector (LC-DAD) was carried as described in Delporte et al. (2018). ESI-HRMS analysis, was performed according to the procedure described in our previous work (Delporte et al., 2018). For NMR analysis, pure compound was dissolved in 0.75 mL of solvent (methanol-d₄ or DMSO-d₆). Six hundred microliters of the solution were then transferred into a 5-mm NMR tube. NMR spectra were acquired at 300 K on a Bruker Avance III 600 spectrometer (600.13 MHz for proton frequencies, Wissenbourg, France) equipped with a z-gradient inverse probe head (TXI, 5-mm tube). The TOPSPIN (V3.2; Bruker, Billerica, CA, USA) software was used. The 1D proton spectra were acquired using 32 scans of 128 K data points, using spectral widths of 8,403 Hz. The 1D carbon spectra were acquired using 24 K scans of 64 K data points, using spectral widths of 37,878 Hz. The 2D COSY and 2D TOCSY spectra were acquired using 8 scans per 256 increments that were collected into 2 K data points, using spectral widths of 12,019 Hz in both dimensions. For the TOCSY, a mixing time of 100 ms were employed. The NUS-based 2D HSQC (non uniform sampling-based 2D heteronuclear single quantum coherence) spectra were acquired using 16 scans per 8 K increments that were collected into 4 K data points, using spectral widths of 12,019 Hz in F2 and 26,412 Hz in F1. The NUS-based 2D HMBC spectra were acquired using 64 scans per 8 K increments that were collected into 4 K data points, using spectral widths of 12,019 Hz in F2 and 37,732 Hz in F1. The number of NUS sampling points was 256 complex points (3.125% sampling density of 8 K points; LeGuennec et al., 2015). All nonzero filled obtained spectra were manually phased and baseline-corrected, calibrated with solvent signal (¹H (3.31) and ¹³C (49.0) for methanol-d₄; Fulmer et al., 2010).

Extraction and analysis of free polyamines
Polyamines were extracted and analyzed by liquid chromatography as described previously (Jubault et al., 2008). The HPLC design consisted of a thermoelectric pump (SpectraSystem P1000 XR, Thermo Fisher, San Jose, CA, USA) and (Spectra- Series AS100) autosampler with a 20-μL injection loop, and detection through an FP-2020 Plus fluorometer (Jasco, Inc., Easton, MD, USA). Signals were computed and analyzed using Azur software (Datalys, St Martin d’Hères, France).

Dicoumaroyl spermine purification
Hairy roots overexpressing CiSHT2 generated in our previous study were used (Delporte et al., 2018). These lines were shown to accumulate high amounts of dicoumaroyl spermine when fed with exogenous spermine. Five grams of lyophilized material, cultivated as described, were powdered and extracted with 200 mL of a methanol/water/acetic acid mixture (75/23/2, v/v/v) for 2 h at 4°C in the dark under agitation. Homogenate was then clarified by centrifugation (5 min at 4,600 g) and the supernatant was passed through a 0.45-μm filter. The pellet was re-extracted a second time following the same procedure. Then, the filtrates were pooled and washed with ethyl-acetate (v/v) and the aqueous phase was collected and passed through a cation exchanger column (50 mg, CM-Sephadex C-50, PHARMACIA, Uppsala, Sweden) equilibrated with water. The resin was washed with 2 L of water and eluted with 20 mL of a mixture of methanol/8M acetic acid (v/v). The eluted fraction was concentrated under rotary evaporation to get a final volume of 1 mL. Dicoumaroyl spermine was purified after separation by HPLC using the protocol described above. The collected fractions containing dicoumaroyl spermine were then pooled and concentrated under rotary evaporation to get a final volume of 2 mL. This concentrated fraction was further purified using a preparative flash 4250 (Interchim, Montluçon, France). Purification was performed on a 250 × 21 mm Interchrom 2 mm preparative column (RP18). The chromatographic separation was performed using water (solvent A) and methanol (solvent B), both acidified with 0.1% formic acid. The solvents were delivered at a flow rate of 22 mL min⁻¹. The column was equilibrated with 20% solvent B. The separation conditions were as follows: start at 20% solvent B, 10 min gradient to 70% solvent B followed by 2 min gradient to 100%, and 3 min isocratic 100% solvent B, then a 2 min gradient to return to 20% solvent B and 5 min of isocratic re-equilibration at 20% solvent B. The collected fractions were concentrated under rotary evaporation. A 1-mL concentrated fraction was frozen and lyophilized in order to obtain a dried powder. Approximately 2.5 mg of dicoumaroyl spermine were purified by this procedure. The purified compound was analyzed by NMR and ESI-HRMS.

Statistical analysis
For heatmap construction, pick area for each quantification ion were evaluated and expressed per milligram of dry materials. Natural logarithm of the means for each condition
(eight plants) was used to conduct the heatmap hierarchical clustering with Ward’s method. The heatmap was visualized using the heatmap-package, R software (version 4.0.3, company Foundation for Statistical Computing, Vienna, Austria).

**Accession numbers**

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers MG457243 (CiSHT1) and MG457244 (CiSHT2).

**Supplemental data**

The following materials are available in the online version of this article.

- **Supplemental Table S1.** Relative quantification of phenolamides in *N. benthamiana* agroinfiltrated with *CiSHT1, CiSHT2*, or *CiSHT1 + CiSHT2*.
- **Supplemental Table S2.** Sequence of primers used in this study.
- **Supplemental Figure S1.** Genotypes of *sht1, sht2*, and *sht1/sht2* mutants.
- **Supplemental Figure S2.** Predicted amino acid sequences of *SHT1* and *SHT2* of mutants used in this study.
- **Supplemental Figure S3.** Characterization of monocoumaroyl spermidine isomers.
- **Supplemental Figure S4.** ^1^H NMR spectra of N⁵, N¹⁰-dicoumaroyl spermine.
- **Supplemental Figure S5.** Extracted ion chromatograms and fragmentation of tetracoumaroyl spermine produced by engineered yeast.
- **Supplemental Figure S6.** HRMS spectra in ESI⁺ of trisubstituted spermidine derivatives produced by engineered yeast recorded in HDMS² mode.
- **Supplemental Figure S7.** HRMS spectra in ESI⁺ of trisubstituted spermine derivatives produced by engineered yeast recorded in HDMS³ mode.
- **Supplemental Figure S8.** *CiSHT1* DNA sequence, sgRNA target sites, and the primer sites for sequencing and HRM analysis.
- **Supplemental Figure S9.** *CiSHT2* DNA sequence, sgRNA target sites, and the primer sites for sequencing and HRM analysis.

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**Conflict of interest statement.** None declared.

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