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Metabolism and roles of hydroxycinnamic acids in *Cichorium intybus* L.

Julie Buges

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UNIVERSITÉ DE LILLE

Doctoral School **ED SMRE 104**

University Department **UMRt 1158 BioEcoAgro**

Thesis defended by **Julie BUGES**

Defended on **3rd March, 2022**

In order to become Doctor from Université de Lille

Academic Field **Plant Sciences**

Speciality **Biological Engineering**

Metabolism and Roles of Hydroxycinnamic acids in *Cichorium intybus* L.

Thesis supervised by Jean-Louis HILBERT Supervisor
David GAGNEUL Co-Supervisor

Committee members

<i>Referees</i>	Nathalie GUIVARC'H	Professor at Université de Tours	
	Alain HEHN	Professor at Université de Lorraine	
<i>Examiners</i>	Nathalie JULLIAN-PAWLICKI	Professor at Université de Amiens	Committee President
	Eric COUROT	HDR Associate Professor at Université de Reims	
<i>Supervisors</i>	Jean-Louis HILBERT	Professor at Université de Lille	
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Métabolisme et Rôles d'acides hydroxycinnamiques chez *Cichorium intybus* L.

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Keywords: *cichorium intybus* l., specialized metabolism, molecular engineering, acyltransferases, hydroxycinnamic acids, heterologous expression

Mots clés: *cichorium intybus* l., métabolisme spécialisé, ingénierie moléculaire, acyltransférases, acides hydroxycinnamiques, expression hétérologue

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Human-induced climate change is here and now. It's not just something we need to think about for our grandchildren.

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Dr. José Graziano Da Silva

The security of people and nations rests on four pillars - food, energy, water and climate

Tom Burke

METABOLISM AND ROLES OF HYDROXYCINNAMIC ACIDS IN *CICHORIUM INTYBUS* L.
Abstract

Industrial chicory, *Cichorium intybus* L. var. *sativum*, accumulates a wide array of potent specialized metabolites. These metabolites exhibit very diverse structures, activities and functions that are remarkable from a physiological standpoint, but also for their applications such as in human and animal health, agronomy and other diverse industries.

This work focused on two specialized metabolites of *C. intybus*: an unusual phenolamide tetracoumaroyl-spermine (TetraSpm) and 3,5-isochlorogenic acid (diCQA). This research contributes to the biochemical and physiological characterization as well as the investigation of the evolutive history of the enzymes involved in the biosynthesis of these two hydroxycinnamic acid conjugates. We used computational analysis and molecular engineering approaches.

We demonstrated the sequential action of the two CiSHTs (Spermine Hydroxycinnamoyl Transferases) *in vivo* in the biosynthetic pathway of TetraSpm. We investigated the influence of free polyamine availability and structural features on the catalytic activities of SHT proteins. Furthermore, we engineered a chimeric protein able to catalyze the production of TetraSpm in a single step.

The study of diCQA metabolism in *C. intybus* involved the identification and preliminary characterization of target genes belonging to the GDSL-lipase like enzymatic family. Moreover, it emphasized the diversity of plant acyltransferases involved in specialized metabolism.

The physiological roles and functions of TetraSpm and diCQA are still to be determined. However, the conservation of TetraSpm among the *Asteraceae* family suggests that it plays a significant role in their evolution, and the therapeutic properties of diCQA are of great interest in human health. Thus, deciphering these biosynthetic pathways is crucial for their reconstitution in more efficient systems *via* molecular engineering and consequently the valorization of these compounds.

Keywords: *cichorium intybus* l., specialized metabolism, molecular engineering, acyltransferases, hydroxycinnamic acids, heterologous expression

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MÉTABOLISME ET RÔLES D'ACIDES HYDROXYCINNAMIQUES CHEZ *CICHORIUM INTYBUS* L.**Résumé**

La chicorée industrielle, *Cichorium intybus* L. var. *sativum*, accumule une grande diversité de métabolites spécialisés. Ces métabolites montrent une grande variété de structures, activités et fonctions qui sont considérables d'un point de vue physiologique, mais aussi pour des applications humaines telle qu'en santé humaine et animale, en agronomie et autres diverses industries.

Ce travail se concentre sur deux métabolites spécialisés de *C. intybus* : une phénolamide inhabituelle, la tétracoumaroyl-spermine (TetraSpm), et l'acide isochlorogénique (3,5-diCQA). Il constitue une contribution aux caractérisations biochimique, physiologique ainsi qu'à l'étude de l'histoire évolutive d'enzymes impliquées dans la biosynthèse de deux acides hydroxycinnamiques conjugués. Nous avons utilisé à ces fins des approches bioinformatiques et d'ingénierie moléculaires.

Concernant la TetraSpm, nous avons démontré l'action successive des deux CiSHTs (Spermine Hydroxycinnamoyl Transférases) *in vivo* dans sa voie de biosynthèse. Nous avons exploré l'influence du ratio de polyamines libres disponibles et des caractéristiques structurales des protéines SHT sur leurs activités catalytiques. En outre, nous avons produit une protéine chimérique capable de catalyser la production de TetraSpm en une seule étape.

L'étude du métabolisme du diCQA chez *C. intybus* a impliqué l'identification et la caractérisation préliminaire de gènes cibles appartenant à la famille enzymatique des GDSL-lipases like. De plus, cela a mis en évidence la diversité des acyltransférases impliquées dans le métabolisme spécialisé des plantes.

Les rôles physiologiques et fonctions de la TetraSpm et du diCQA restent à déterminer. Cependant, la conservation de la TetraSpm au sein de la famille des Astéracées suggère son importance dans leur histoire évolutive, et les propriétés thérapeutiques du diCQA sont d'un grand intérêt en santé humaine. Ainsi, la compréhension de leurs voies de production permettra de leurs reconstitutions dans des systèmes hétérologues plus efficaces grâce à l'ingénierie moléculaire et par conséquent à la valorisation de ces composés.

Mots clés : *cichorium intybus* l., métabolisme spécialisé, ingénierie moléculaire, acyltransférases, acides hydroxycinnamiques, expression hétérologue

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I mentioned that I began my journey in Plant Sciences during my Bachelor's degree in Lille. For that, I must thank Dr. Marie-Christine Quillet, who agreed to provide a month-long internship to a curious first-year student and who followed her progress over the years. Thank you also Dr. Anne-Sophie Blervacq who created the opportunity for many students, including myself, to develop their scientific english skills and in an international context within the many programs she instigated. Pr. Simon Hawkins and Pr. Ivan Couée are also thanked for their guidance and recommendations. I would like to thank Pr. Lukas Schreiber and Dr. Charlotte Petruschke for introducing me to the German way of working, which I particularly appreciated.

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I have so many people to thank. So many wonderful people in my life, I feel really grateful to have you all in my life and in my support system. I will switch to french just a minute for my family, hold on.
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Alright, back to English.
My friends, my loves, my pillars, my team, my second family. First of all I love

you so damn much and I am so, so grateful to have you in my life.

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I would like to make a few peculiar thanks, so I decided to make it a whole paragraph because why not.

The first one is to taxol or paclitaxel, a specialized metabolite that I mention in my literature review for its anti-cancer properties. I'd like to thank it for possibly saving my mother's life this year. Even if it did make her feel like she was 95 years old for a few months. But now she feels like she's 75, so I guess all is forgiven.

The second shout out I'd like to make is to my therapist, Dr Delannoy. I have grown so much with your help. Worked on issues I knew I had, unraveled some I did not know I had. But overall I discovered my true self. And I'm starting to like her, she's sassy and strong. More seriously, I would not have never make it this far without your help, be it for my overwhelming anxiety or my burn-out. Now I know that, even if it can get out of hand sometimes, I have the tools you taught me on how to tame it and use it to be productive, cautious and rigorous. I

will never be able to thank you enough for these past three years but this is my attempt at it.

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And on that note, to science ! Because that is why we are here after all.

Acronyms

A | B | C | D | E | F | G | H | I | L | M | P | R | S | T | U | W

A

AA Amino Acid.

B

BAHD named after the initials of the first characterized enzymes of the family. **BEAT**: Benzyl alcohol O-acetyltransferase; **AHCT**: anthocyanin O-hydroxycinnamoyltransferase; **HCBT**: N-hydroxycinnamoyl/benzoyltransferase; **DAT**: deacetylvindoline 4-O-acetyltransferase.

C

cDNA coding DNA.

CoA Coenzyme A.

CQA Caffeoylquinic Acid.

CTA Caffeoyltartric acid.

D

diCQA dicaffeoylquinic Acid.

diCTA dicaffeoyltratric acid or chicoric acid.

DNA Desoxyribonucleic Acid.

E

EAC Escape from Adaptative Conflict.

F

FAO Food and Agriculture Organization of the United Nations.

FWE Nexus Food-Water-Energy Nexus.

G

gDNA genomic DNA.
GELP GDSL esterase/lipase proteins.

H

HAL Histidine Ammonia Lyase.
HCA Hydroxycinnamic Acid.
HCAA Hydroxycinnamic Acid Amide.
HCT Hydroxycinnamoyl-CoA shikimate/quinic hydroxycinnamoyl Transferase.
HPLC High-Performance Liquid Chromatography.
HQT Hydroxycinnamoyl-CoA quinic hydroxycinnamoyl Transferase.

I

IPCC Intergovernmental Panel on Climate Change.
IPP Isopentenyl PyroPhosphate.
IUPAC International Union of Pure and Applied Chemistry.

L

LINE Long Interspersed Nuclear Element.

M

MeJA Methyljasmonate.
mRNA Messenger RNA.
MS Mass Spectrometry.
MYA Million Years Ago.

P

PAL Phenylalanine Ammonia Lyase.
PCR Polymerase Chain Reaction.
pH potential of Hydrogen.

R

RCP Representative Concentration Pathways.
RNA Ribonucleic Acid.
ROS Reactive Oxygen Species.
RT-qPCR Reverse Transcription quantitative PCR.

S

SCPL Serine Carboxypeptidase Like.

SDG30 Sustainable Development Goals for 2030.

sgRNA Single Guide RNA.

SHT Spermidine or Spermine Hydroxycinnamoyl Transferase.

SSP Shared Socio-economic Pathway.

T

T-DNA Transfer DNA.

TE Transposable Elements.

U

UV Ultraviolet.

W

WGD Whole Genome Duplication.

WT Wild-Type.

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Chapter 1

Literature Review

Plants have demonstrated great adaptability throughout their evolution. This is largely due to their sessile lifestyle, as they do not have the option of fleeing when faced with adversity. The changing climate is having and will continue to have numerous deleterious effects on them, affecting greatly their fitness and consequently human survival in mid- to long-term. Plant scientists play a critical role in answering arduous questions such as how plants acclimate to their environment and whether they will be able to overcome the challenges that climate change will bring them in the near future. To provide decision makers with the most accurate data on the situation, these answers will necessitate interdisciplinary collaboration (modeling, statistics, climate science, plant biology).

Climate change and its effects have been studied for decades: global temperature rising, more frequent and intense extreme weather events, habitat destruction, species extinction, social and geographical injustice are some of its current and future effects. Heat waves will be extremely difficult to deal with for societies, but some will suffer more than others due to their heightened vulner-

ability. Vulnerability was defined during the fourth IPCC ¹ as the ability and susceptibility of a system to cope with unfavorable climate change impacts and it is usually divided into three components: exposure, sensitivity and adaptive capacity (Parry 2007; Preston et al. 2008).

At a global level, it is acknowledged that the regions that have contributed the least to greenhouse gases and human activities which have exacerbated climate change will be the ones to bear the brunt of its consequences because of their tropical climates: Amazonia, Southern Africa, Sahel, India, and Southeast Asia (Bathiany et al. 2018). At the European level, injustice for freshwater access will be observed in the face of increasing climate variability, but this varies depending on the different proposed scenarios. Nevertheless, all scenarios converge to similar conclusions regarding the impacts Northern and Southern Europe will experience: Northern countries will be less vulnerable due to higher precipitation and better socioeconomic resources, whereas Mediterranean countries will be impacted to a greater extent. The situation in Central Europe is less predictable and depends on the scenarios (Koutroulis et al. 2018). This is why experts in the field recommend using unified RCP ²/SSP ³ frameworks to obtain more consistent predictions on the impact of climate change on various factors such as freshwater vulnerability.

In a thesis on Plant Biology, talking about a country's freshwater vulnerability may seem out of place. However, the Food-Water-Energy Nexus (FWE Nexus) concept binds these two research fields together (Byers et al. 2018 ⁴). There are intrinsic links between these three natural resources (food, water and energy) which can be quantitative (direct and material link) and qualitative (indirect links through resources management and local legislation about their usage). The FWE nexus is well considered on an academic research level but much less among policymakers (Ghodsvali et al. 2019). The United Nations' latest report considers the FWE nexus in the establishment of the SDG30 ⁵

¹ IPCC: Intergovernmental Panel on Climate Change ² RCP: Representative Concentration Pathways (Temperature change; Precipitation change; Sea ice extent) ³ SSP: Shared Socio-economic Pathway (Population; Urbanization; Rates of technological change Income; Human Development Index; Income distribution) ⁴ Video abstract can be found at this link: <https://iopscience.iop.org/article/10.1088/1748-9326/aabf45/meta> ⁵ Sustainable Development Goals for 2030

(Figure 1.1; Nations 2019).



Figure 1.1 – Sustainable Development Goals for 2030 established by the United Nations in their 2019 report. (<https://sdgs.un.org> consulted on December 2nd 2021)

In the United Nations SDG, goals 2 (zero hunger), 3 (good health and well-being), 12 (responsible consumption and production), 13 (climate action) and 15 (life on land) concern Plant Biologists. The FAO ⁶ emphasizes the importance of sustainable agriculture from an ecological as well as a socioeconomic standpoint in helping to adapt to and mitigate the effects of climate change, in addition to ensuring food security for all. Adaptation measures aim to reduce the vulnerability of human and natural systems to climate change, by altering exposure, lowering sensitivity, and increasing societal adaptability. Mitigation measures include reducing greenhouse gases concentrations in the atmosphere by reducing, avoiding and/or removing greenhouse gases emissions. FAO offers resources for agricultural actors, scientists, and policymakers to stay informed about climate-smart agriculture principles and the need for tailor - made solu-

⁶ FAO: Food and Agriculture Organization of the United Nations

tions based on environmental and societal conditions. Their objectives are to increase agricultural productivity and incomes in a sustainable manner, to adapt and build resilience to climate change, and reduce and/or eliminate greenhouse gases emissions where possible ⁷.

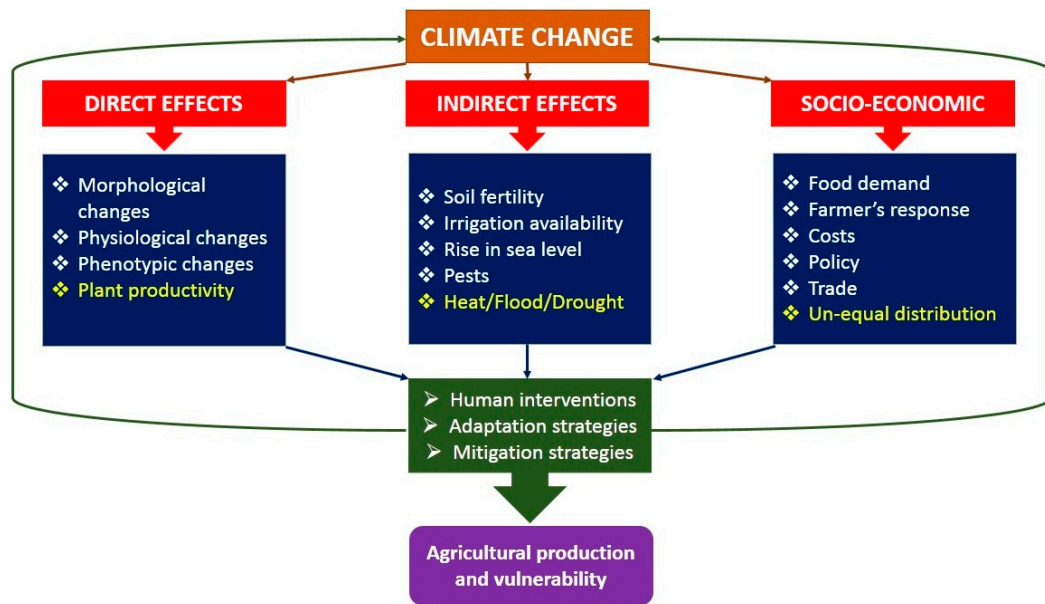


Figure 1.2 – Direct, indirect and socio-economic effects of climate change on agricultural production. Figure from Raza et al. 2019.

Climate Change leads to these direct, indirect on socio-economic effects. Those different factors impact on agricultural production.

Plant scientists have a significant role to play in all of these areas. It is critical to investigate the effects of combined constraints on crop models in order to predict the most appropriate and long-term solutions (Figure 1.2) (Buges et al. 2018; Raza et al. 2019; Zandalinas et al. 2021). At the molecular and physiological levels, combined stresses and environmental cues can result in neutrality, additivity, synergy, antagonism, idiosyncrasy, or dominance of the effects of one of the applied constraints (Buges et al. 2018).

As a result, tailored studies for each crop in their cultivation area must be es-

⁷ <https://www.fao.org/climate-smart-agriculture/en/> consulted on 19 November 2021

established. Understanding molecular response pathways is a major focus of this research area (Figure 1.2) (Raza et al. 2019; Zandalinas et al. 2021). This also applies to specialized metabolites that modulate plant responses to their environment, as well as biotic and abiotic constraints. Previously regarded as waste products of primary metabolism (Hartmann 2007), their importance in plant defense and acclimation is now recognized. Their functions and roles in responses of plants to combined constraints are critical in the current circumstances.

The presented work contributes to this global effort to improve our understanding of plant molecular pathways involved in the production of desirable natural products.

1.1 Characteristics of specialized metabolism

1.1.1 Evolutionary mechanisms leading to the emergence of specialized metabolites

1.1.1.a Emergence of enzymes of the specialized metabolism

Specialized metabolites are not ubiquitous as primary or central metabolites. Their name reflects their specialized role in environmental molecular acclimation as well as their limited presence to specific lineage or even species. The enzymes involved in their synthesis derive from primary metabolism enzymes. Plants have a higher rate of WGD⁸ and polyploidization. These events are also more recent than in other kingdoms (for instance the last genome duplication event occurred approximately 450 MYA⁹ in the lineage leading to humans whereas it occurred 200 MYA in the angiosperms lineage) (Panchy et al. 2016). There are other mechanisms that lead to gene duplication as presented in Figure 1.3. They are classified as subgenomic duplication events: tandem duplication, segmental duplication, transposon-mediated duplication and retroduplication. Tandem duplication leads to clusters of genes, very common among specialized metabolism genes. The other three mechanisms are associated with TE¹⁰. All these mechanisms only explain 70 % of the observed duplications in plant genomes, the mechanisms for the last 30 % remain unclear. Two main fates can fall upon a duplicated gene: being lost or being retained.

A lost duplicated gene can either be deleted completely from the genome or become a pseudogene, meaning it lost its function through mutations and later on will be physically lost from the genome through negative selective pressure. Usually, duplicates obtained *via* TE-associated mechanisms are more likely to be lost. TE-mechanisms are highly error inducing in the gene sequence or in parts regulating its expression, and can lead to non-functional duplicated genes but also insertion in an essential gene and thus non-survival of the individual. After WGD, the genetic content can be reduced and a whole set of duplicates lost (this

⁸ WGD: Whole Genome Duplication ⁹ MYA: million years ago ¹⁰ TE: Transposable Elements

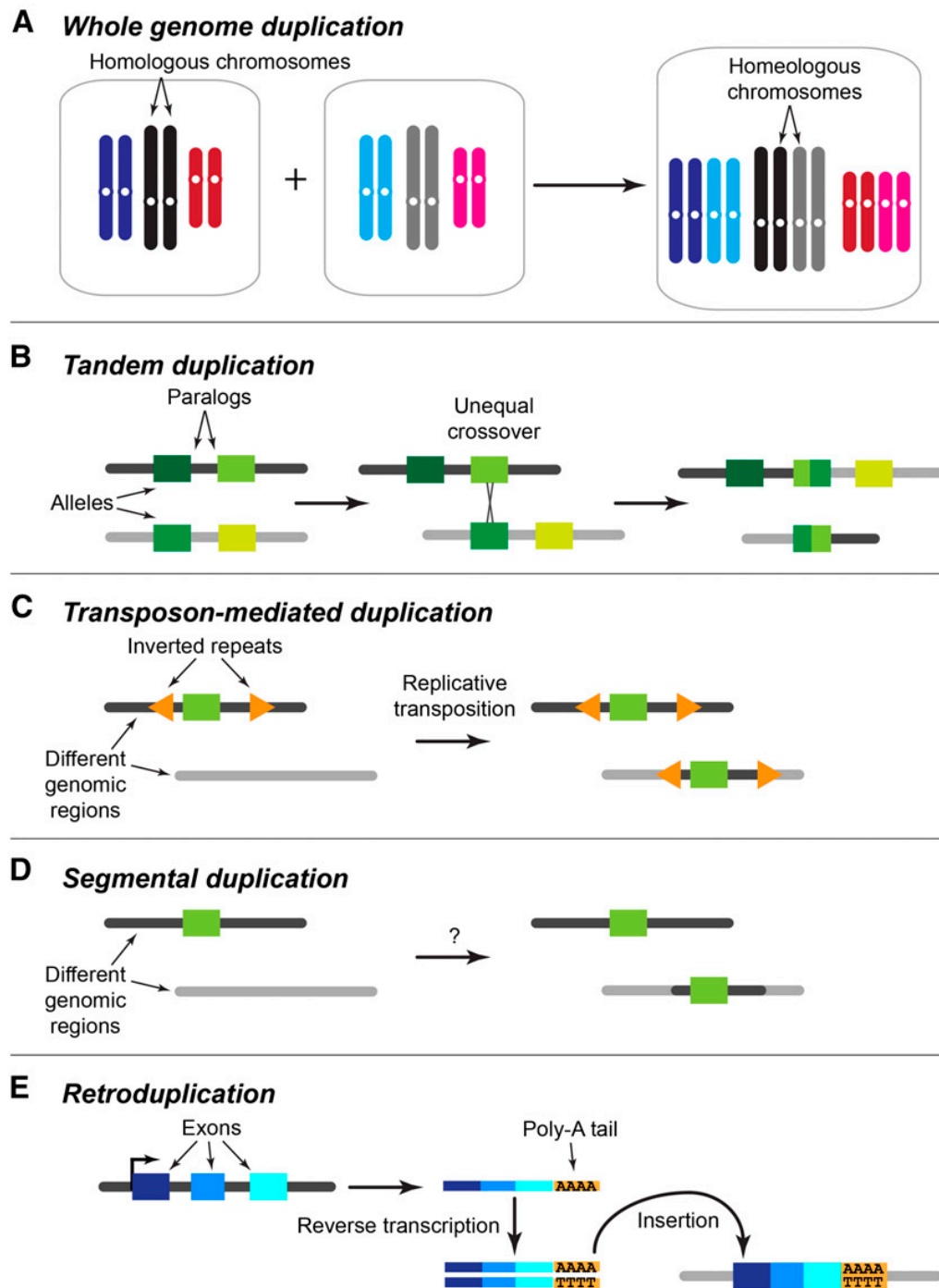


Figure 1.3 – Plant genome duplication mechanisms. Figure and legend from [Panchy et al. 2016](#).

A. WGD, or duplication of genes *via* an increase in ploidy. **B.** Tandem duplication, or duplication of a gene *via* unequal crossing over between similar alleles. **C.** Transposon-mediated duplication, or duplication of a gene associated with a TE *via* replicative transposition. **D.** Segmental duplication, or duplication of genes *via* replicative transposition of LINE elements in the human genome. The extent and causative elements of segmental duplication are ill defined in plants. **E.** Retroduplication, or duplication of a gene *via* reverse transcription of processed mRNA.

phenomenon is called fractionation).

Mechanisms leading to retention of a gene duplicate are numerous and not mutually exclusive (Figure 1.4). In the case of pseudogenes (nonfunctional retained gene) occurrence in plant genomes when they should be lost, a potential explanation is that they have not yet been negatively selected by evolution. As mentioned, the duplication events they arise from are too recent.

For functional genes, different mechanisms may be at hand. Genetic drift can be a dominant factor for allele retention of mutations which accumulate truly randomly, without selection pressure. However, there is a selection for genetic redundancy, for instance as a fail-safe in the case of the production of an essential metabolite. If duplicates do not acquire new functions, their retention may be due to one (or more) of four phenomena: gene dosage increase, subfunctionalization, gene balance or paralog interference. Gene dosage increase can also serve as a fail-safe for a crucial function in the plant. Subfunctionalization results in divided functions of the ancestral genes that are implemented unequally in both duplicates and must thus be retained in order to maintain the ancestral function. Gene balance can occur after WGD to keep molecular interactions (DNA-protein or protein-protein) balanced: if duplicates were lost, a decrease in one of the interactors would lead to an imbalance. Paralog interference is similar to gene balance in one aspect: keeping the interactions between proteins balanced. However, it is only relevant in the case of homomultimerization, such as in the case of transcription factors, where the loss of the degenerate copy would result in an imbalance.

Concerning duplicates retained thanks to a gain of function, two mechanisms exist: neofunctionalization or EAC¹¹ (Figure 1.4). In the neofunctionalization model, one duplicate retains the ancestral function while the other gains a new function after mutation. To establish that a neofunctionalization of a paralog occurred, function of the ancestral gene needs to be assessed first. EAC involves two of the precedent mechanisms. The first mechanism is subfunctionalization

¹¹ Escape from Adaptive Conflict

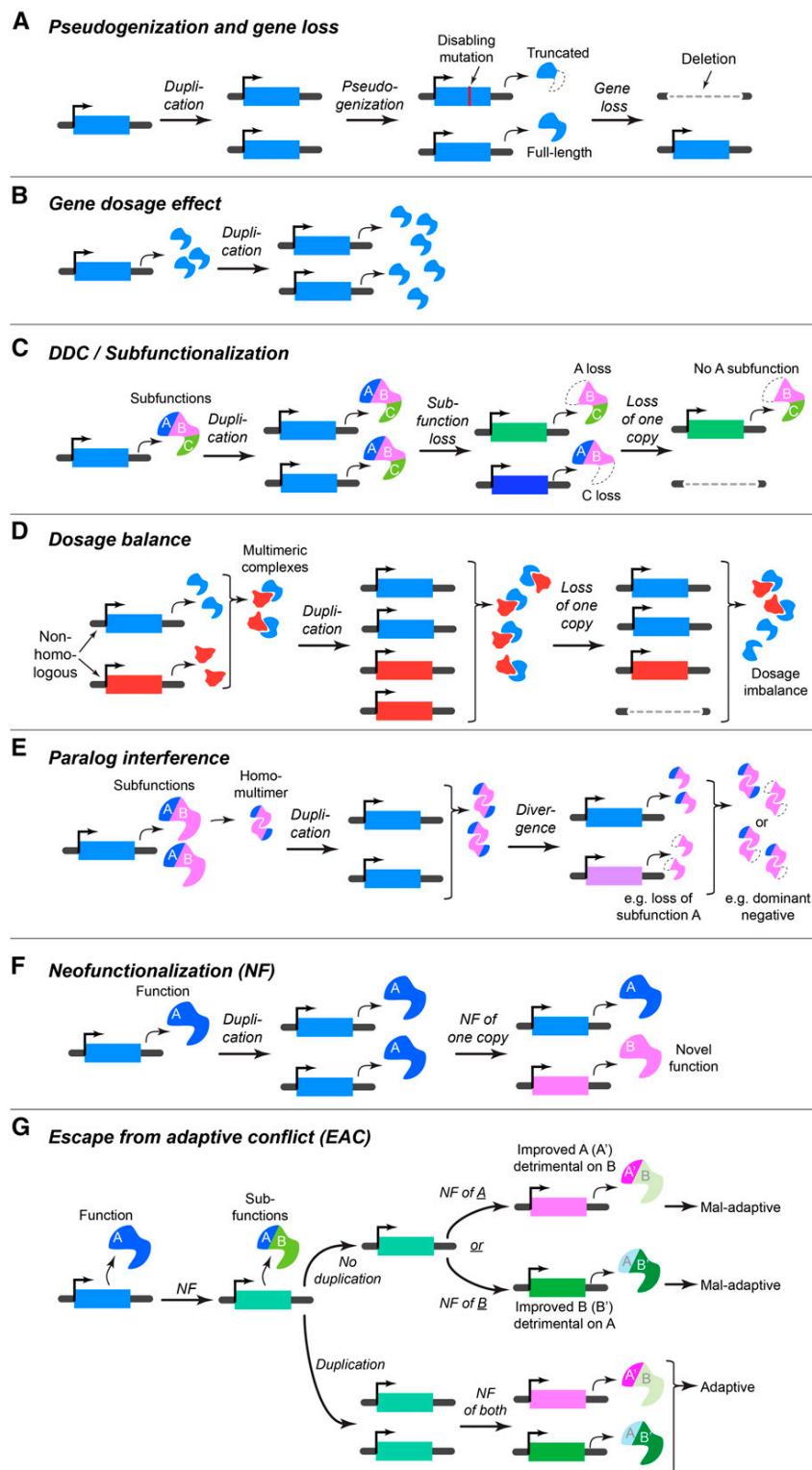


Figure 1.4 – Fates of duplicated genes. Figure and legend from [Panchy et al. 2016](#). Duplicate genes can be pseudogenized/lost (A), retained by selection on existing functions (B–E), or retained by selection on novel functions (F and G). Models of selection on existing function include the following: gene dosage, or retention of both duplicates because of a beneficial increase in expression (B); duplication-degeneration-complementation (DDC)/subfunctionalization, or retention of both duplicates to preserve the full complement of ancestral functions (C); dosage balance, or retention of both duplicates to maintain the stoichiometric balance (D); and paralog interference, or retention of both duplicates to prevent interference between the products of each paralog (E). Models of selection on novel functions include the following: neofunctionalization, or retention of both duplicates because of a gain of function post duplication (F); and escape from adaptive conflict (EAC), or retention of both duplicates that allows for the independent optimization of conflicting ancestral functions (G).

that occurs before the duplication of the gene. After the duplication, the second mechanism is the neofunctionalization of both duplicates in order to keep balance in the molecular system (Figure 1.4).

1.1.1.b Features of the genes and enzymes of the specialized metabolism

Enzymes of the specialized metabolism are known to often be more promiscuous than their primary metabolism counterparts (Figure 1.5). Some can either perform reactions on a wider array of substrates, produce different products from the same set of substrates or even catalyze different reactions when placed in a different environment or when fed with a different substrate (Bar-Even et al. 2011; Kruse et al. 2020; Moglia, Lanteri, et al. 2014). These advantages in chemodiversity are undermined by a lower catalytic efficiency (around 30 fold) than central metabolism enzymes (Figure 1.5 B.). It has been suggested that these slower kinetic parameters were due to their more recent emergence under more specific and less stringent selection pressure. Another hypothesis is that this would prevent substrate availability competition with primary metabolism pathways (Jing-Ke Weng, Lynch, et al. 2021).

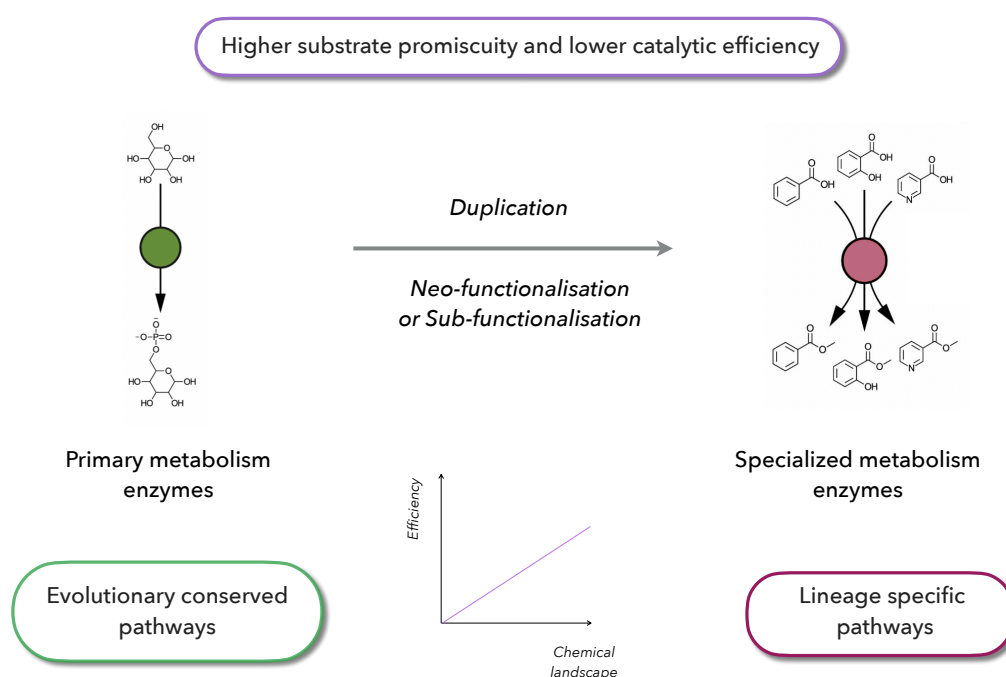


Figure 1.5 – Comparison between the characteristics of primary and specialized metabolism enzymes. Figure adapted from J.-K. Weng et al. 2012.

Specialized metabolism genes are sometimes organized in clusters, due to the duplication events they emerge from (Jing-Ke Weng, Lynch, et al. 2021). This participates in the co-regulation of the genes implicated in the same pathway when they form a physical cluster in the genome (Figure 1.6 A.). A recent study highlights that gene clusters can share a chromatin signature which could facilitate co-regulation of genes even if physically far apart on the chromosome (Nützmänn, Doerr, et al. 2020). Moreover, this system would allow for easier modulations of specialized genes expression regulation of specific pathways when unneeded or potentially nefarious for the plant.

On a protein level, enzymes involved in the same biosynthetic pathway can also be organized in a structural and functional complex called metabolon (Figure 1.6 B.). This structural organization allows for higher efficiency and yield of the biosynthetic pathway. However, the techniques for correctly identifying the partners of a metabolon are costly, can result in false positives, and their interpretations must be cross-checked with those obtained by other methods for greater accuracy (Bassard and Halkier 2018).

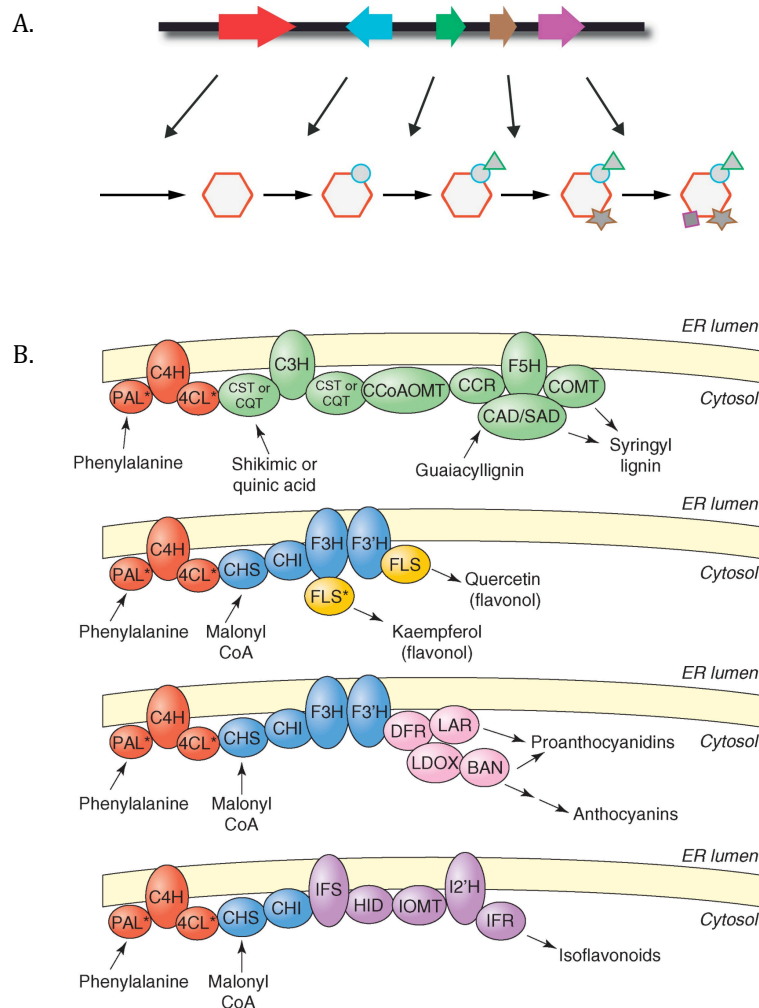


Figure 1.6 – Representations of genetic clusters and metabolons.

A. Cluster organization of genes encoding the biosynthesis of a specialized metabolite. Red: gene encoding the enzyme catalyzing the building block molecule. Other colors: gene encoding the successive modifications of the precursor to produce the final product. Figure adapted from [Nützmann and Osbourn 2014](#)

B. Metabolons identified in phenylpropanoid pathways. Figure from [Jørgensen et al. 2005](#)

1.1.2 Main classes of specialized metabolites and their biosynthetic pathways

Specialized metabolites (formerly named secondary metabolites) are divided into three main classes according to their chemical structures: nitrogen-containing metabolites, terpenoids (or isoprenoids) and phenolics. Despite their diversity in structure and function, their biosynthetic pathways are intertwined as they all take their roots in primary metabolism (Figure 1.7).

Numerous biosynthetic steps leading to specialized metabolites occur in different location, spatially separating intermediates to prevent disastrous effects for the producing plant. The infamous glucosinolates are great example of this in *Brassicaceae* (Schenck et al. 2020) This is also seen for other more classical pathways like the phenylpropanoid pathways of which the steps are divided in three different subcellular compartments: plastids, cytosol and vacuole. Some species have even evolved the ability to produce specific organelles through the dedifferentiation of chloroplasts cells. For instance the phenyloplasts particular plastids accumulate a major phenol ¹² in the vanilla fruit (Brillouet, Verdeil, et al. 2014). A less specific phenomenon is the presence of tannosomes (modified thylakoids) in some vascular plants, in which tannin polymerization, trafficking and storage occur (Brillouet, Romieu, et al. 2013).

¹² 4-O-(3-methoxybenzaldehyde) β -d-glucoside

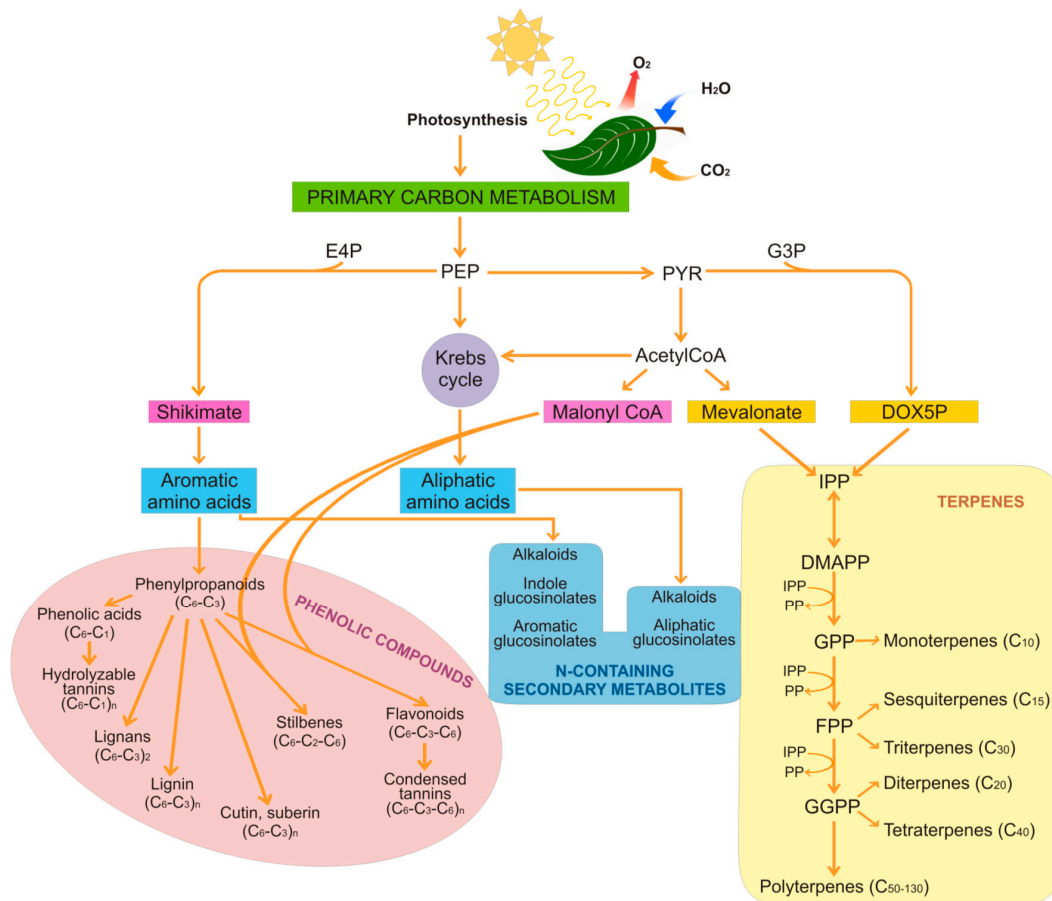


Figure 1.7 – Schematic representation of the biosynthetic pathways of the three classes of specialized metabolites. Figure from [Borrelli et al. 2016](#).

E4P: erythrose 4-phosphate; G3P: glyceraldehyde 3-phosphate; PEP: phosphoenolpyruvate; PYR: pyruvate; DOX5P: deoxyxylulose 5-phosphate; IPP: isopentenyl pyrophosphate; DMAPP: dimethylallyl pyrophosphate; GPP: geranyl pyrophosphate; FPP: farnesylpyrophosphate; GGPP: geranylgeranyl pyrophosphate.

1.1.2.a Nitrogen-containing metabolites

Specialized metabolites containing nitrogen are comprised of alkaloids, glucosinolates, non-proteinogenic amino acids, betalains and cyanogenic glycosides. Alkaloids encompasses molecules containing a nitrogen atom but no sulfur atom. Alkaloids have several subclasses. «True» alkaloids contain their nitrogen atom inside their heterocyclic ring. This nitrogen atom originates from amino acids such as tyrosine, tryptophan, lysine, or arginine. «Pseudo» alkaloids have their nitrogen atom that is outside of the heterocyclic ring. «Proto» alkaloids possess a nitrogen-containing heterocycle but the atom does not come from an amino acid (steroid-like, terpene-like, purine-like alkaloids) (Ahmed et al. 2017). Sulfur and nitrogen containing metabolites encompass GSL¹³, GSH¹⁴ and thionines (Pagare et al. 2015). They are some of the most represented molecules of this class.

GSLs are the most diverse and are composed of a glucoside moiety (β - D-thioglucoside), a (Z)-N-hydroxyminosulfate moiety and a lateral chain (R-amino acids with R being of various nature) (M. D. Mikkelsen et al. 2004 ; Miękus et al. 2020). They are particularly found in *Brassicaceae*. Non-proteinogenic amino acids such as canavanine, azetidine-2-carboxylic acid or mimosine do not compose peptides, but are structural homologs of proteinogenic amino acids. Their are found mostly in *Fabaceae* seeds (Jan et al. 2021). Betalains originate from tyrosine present in the Caryophyllales order where they replace anthocyanins as pigments in the tissues of the plant (Brockington et al. 2015). Cyanogenic glycosides also derive from amino acids and are involved in their hydrolyse releasing compounds with a nitrile group, including cyanide (Jan et al. 2021).

Famous metabolites of this class include nicotine, morphine, vinblastine, betacyanin, linamarin, caffeine, papaverine, sinigrin, most of great importance for health applications.

¹³ GSL: glucosinolates ¹⁴ GSH: glutathione derivatives

1.1.2.b Terpenoids

Terpenoids are the largest and the most diverse class of specialized metabolites with 40 000 identified molecules (Tholl 2015). They are produced in the aerial and flower tissues but can also be found in some species roots. Their other name, isoprenoids, originates from their common precursor IPP¹⁵. Their basic structure is the 5 carbon molecule, isoprene. IPP arises from two pathways: the melanovate pathway localized the cytosol and the MEP/DOXP¹⁶ pathway (or MEP pathway), a plastidic pathway (Lichtenthaler et al. 2006). IPP is isomerized into DMAPP¹⁷ and the two C5 molecules are conjugated to form terpenes of different carbon chain length.

GPP¹⁸ (C10) , FPP¹⁹ (C15) , GGPP²⁰ (C20) , squalene (C30) and phytoene (C40) are the 5 original building blocks for the synthesis of linear and cyclic terpenes. According to the number of carbons, terpenoids are termed hemiterpenes (C5), monoterpenes (C10) sesquiterpenes (C15), diterpenes (C20), triterpenes (C30), tetraterpenes (C40) and polyterpenes (C50-C130) (Gershenzon et al. 2007). IPP and some terpenoids are involved in the synthesis of essential primary metabolites such as chlorophyll and phytohormones (gibberellins and cytokinins), demonstrating that the frontiers between specialized and primary metabolisms are weak (Vranová et al. 2013).

Famous metabolites in this class include artemisinin (anti-malarial), taxol (anticancer), various VOCs (used in fragrances or essential oils), and linalol (used in cosmetics).

1.1.2.c Phenolics

Phenolics are ubiquitous in the plant kingdom. They are characterized by the presence of a hydroxylated benzene ring or phenol moiety and can go from a simple six-carbon aromatic ring with one or more hydroxyl groups attached to complex polymeric molecules (Vogt 2010; Pagare et al. 2015). There are different

¹⁵ IPP: Isopentenyl PyroPhosphate ¹⁶ MEP/DOXP: 2-C-methylerythritol-4-phosphate / 1-desoxy-D-xylulose-5-phosphate ¹⁷ DMAPP: dimethylallyl pyrophosphate ¹⁸ GPP: geranyl pyrophosphate ¹⁹ FPP: farnesyl pyrophosphate ²⁰ GGPP: geranylgeranyl pyrophosphate

types of phenolics: flavonoids, lignans and hydroxycinnamic acids... Flavonoids are a major class of phenolics and around 9 000 molecules have been identified so far (Yechun Wang et al. 2011). They are composed of two aromatic rings and are best known for their pigmentation and protective properties (B. Singh et al. 2017) Monolignols (coumarylic alcohol, coniferyl alcohol and sinapyl alcohol) after polymerization compose lignin in a species-dependent manner. Lignin is the second most abundant biopolymer on Earth after cellulose (Q. Liu et al. 2018). Hydroxycinnamic acids are most often found in a conjugated form, through esterification to sugars or acids (glycosides) or amidation to polyamines (phenolamides). Phenolamides (or phenylamides) are discussed in details in section 1.3.1.

Phenolics are mostly synthesized from the shikimate pathway since their amino acid precursors are either tyrosine or phenylalanine. Some derive directly from shikimate such as non hydrolyzable tanins. Most of them originate from phenylalanine after its oxidative deamination by PAL²¹ to form *trans*-cinnamic acid. PAL is the first step of the phenylpropanoids (or polyketides) pathway. It is at the origin of the emergence of phenolic compounds in plants and has evolved from HAL²², a primary metabolism enzyme found in eukaryotes as well as prokaryotes (Emiliani et al. 2009). The phenylpropanoid pathway is exclusive to the plant kingdom. It produces C6-C3 backbone phenylpropanoid molecules and most essentially coumaroyl-CoA. Coumaroyl-CoA is a crucial metabolite in specialized metabolism and is a precursor for most phenolic compounds. Phenolics are involved in physiological processes such as pollination and against biotic and abiotic constraints (Cheynier et al. 2013).

Famous metabolites of this class include lignin, suberin, anthocyanins, tannins or salicylic acid (precursor of acetylsalicylic acid commonly known as aspirin).

Specialized metabolites have a wide range of activities and properties that are crucial to decipher in two aspects. They offer tremendous potential for valorization in human applications ranging from agronomic improvement to

²¹ PAL: Phenylalanine Ammonia Lyase ²² HAL: Histidine Ammonia Lyase

industrial uses, as well as medicinal and cosmetic industries. Nevertheless, their physiological roles must not be overlooked. Furthermore, a better understanding of the functions *in planta* in molecular responses to their environment, defense, growth, development, reproduction and communication is a powerful tool for future crop improvement.

1.1.3 Roles of specialized metabolites in plants

Land plants have evolved for over 470 million years, and metabolic diversity has been essential for colonizing new environments alongside the development of adapted phenotypes. During terrestrialization and colonization, there was an increase in selection pressure for potent molecules involved in plant defense. The emergence of specialized metabolites is thus thought to be an adaptive strategy of land plants to increase their fitness in new environmental conditions (Bateman et al. 1998). Indeed, they were involved in multiple stages of the evolution of land plants. For instance, phenylpropanoids would have promoted UV protection; lignins confer mechanical strength to plant cell walls and thus promoted upright stand of vascular plants and are involved in resistance to mechanical and herbivore damages; ... (Figure 1.8) (Lowry et al. 1980; Bateman et al. 1998; Jing-Ke Weng 2014).

There are many environmental constraints that plants must cope with due to their sessile lifestyle, and specialized metabolites play many roles in the interaction of the plant with its environment (Figure 1.9). The different functions and specificities of specialized metabolism management in land plants will be discussed in this section.

Acclimation to their environment and molecular stress responses

Molecular cues and responses are critical for a the ability of plants to thrive, or even just survive. Contrary to adaptation traits, acclimation refers to non-heritable metabolic or phenotypic changes. Because acclimation and stress responses use the same molecular signaling pathways, the concentrations of produced specialized metabolites in response to these cues must be finely tuned to provide an adequate response.

High or low temperatures, UV exposition, water availability or excess and salt excess are the main abiotic constraints land plants may encounter. All these constraints, as well as biotic ones, usually also lead to oxidative stress as an indirect effect. Specialized metabolites take part in the molecular responses

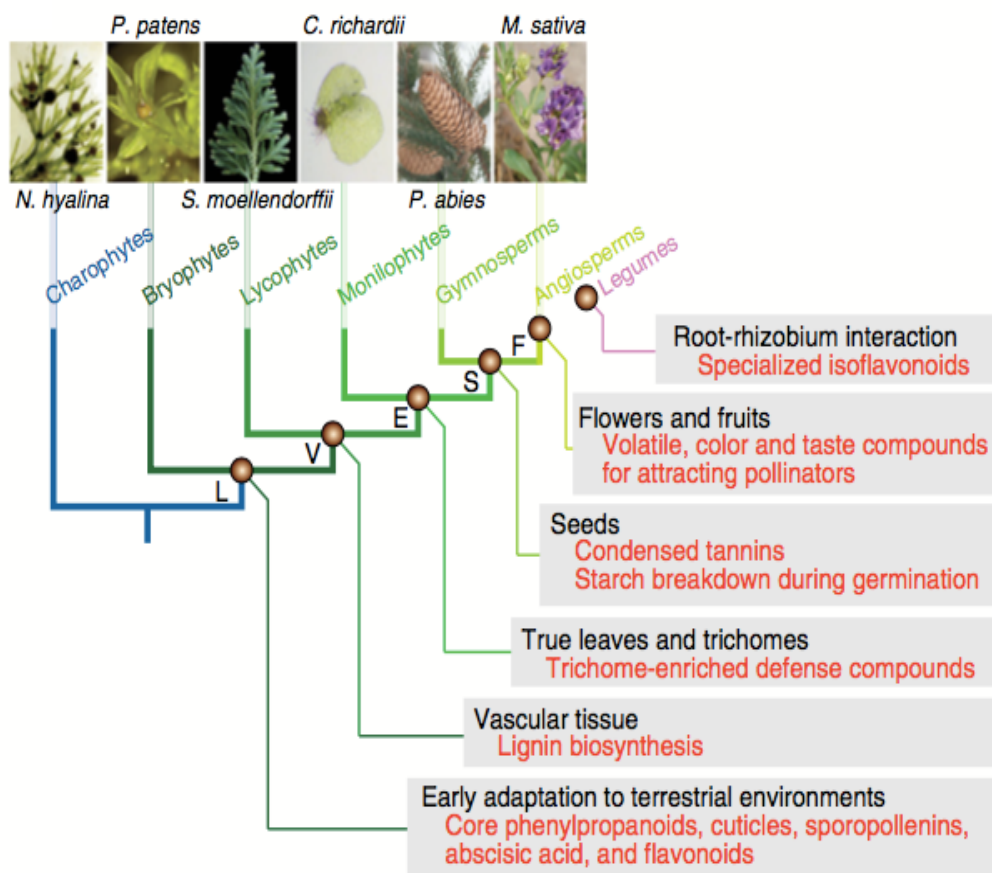


Figure 1.8 – Involvement of specialized metabolites during land plants evolution. Figure from [Jing-Ke Weng 2014](#).

induced by these environmental cues (Figure 1.9). Phenolics are well known radical scavengers and antioxidants. They participate in the regulation of ROS²³ accumulation ([Aboul-Enein et al. 2007](#)). Flavonols are majorly involved in UV-defense, in protection of reproductive tissues and hence fertility, in pollen tube germination and in the protection of the genetic material of plants ([Yuting Liu et al. 2021](#)). Phenylacylated-flavonols, kaempferol, and quercetin are known to be implicated in UV tolerance ([Hofmann 2000](#); [Tohge et al. 2016](#)).

²³ Reactive Oxygen Species

Wink, M. (2016) eLS, John Wiley & Sons, Ltd (Ed.)

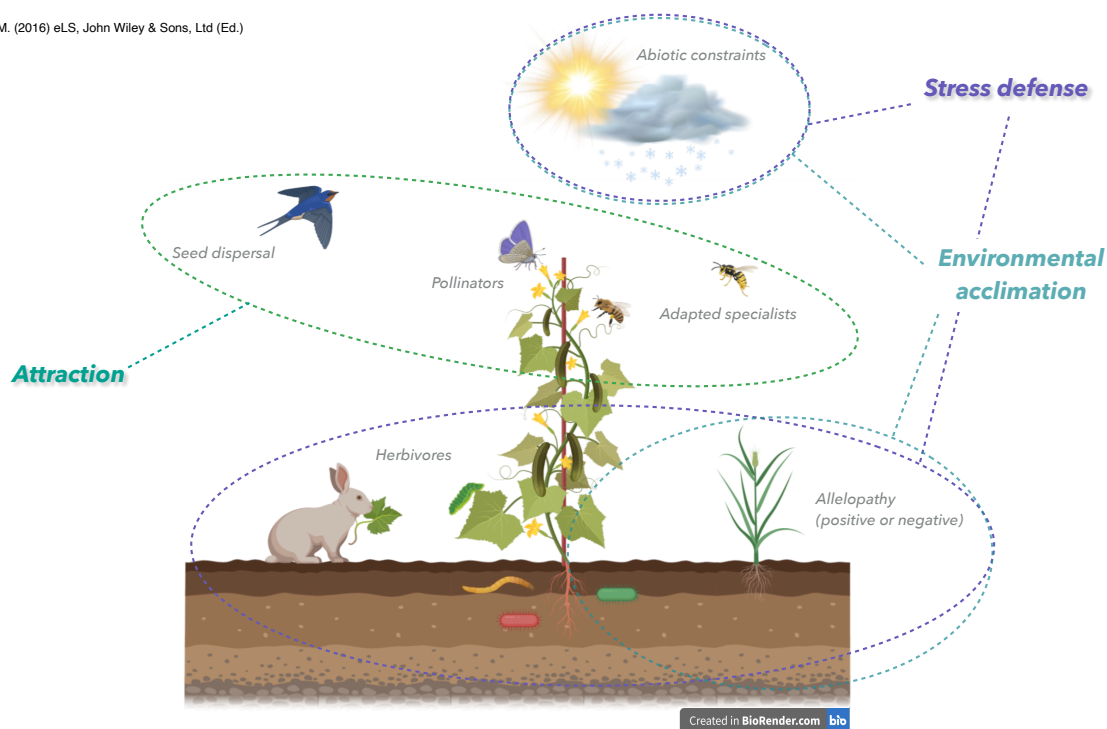


Figure 1.9 – Roles of specialized metabolites in plants.

Terpenes have been proved to be involved in temperature change tolerance, especially when combined to herbivory attacks. For instance sesquiterpene lactone taraxinic acid β -D glucopyranosyl ester protects the roots of *Taraxacum officinale* when temperatures fluctuate underground and the plant has already received molecular cues indicating a potential attack by one of its common herbivores is incoming (*Melolontha melolontha*) (W. Huang et al. 2020). Terpenoids contents are also increased in dwarf birch (*Betula nana*), native of the Arctic tundra, in response to the increase in temperature due to climate change and herbivory attacks (T. Li et al. 2019). This is an example of a synergic response to combined constraints that provokes a specialized metabolism response.

Major activities exhibited by specialized metabolites and their breakdown products are anti-microbial and anti-oxidant. As a result, they are investigated in the context of biotic stresses. When plants are subjected to such constraints, their

contents increase. Furthermore, they meet the requirement for a specific molecular response to the wide range of putative pathogens, insects, and herbivores that a plant may encounter in its environment (Figure 1.9). Plant defenses are activated through phytohormones, but the dichotomy with specialized metabolism is not as strong as previously perceived: they are now also recognized as regulators in plant defense molecular responses. For instance, many VOCs activate jasmonic acid signaling and so plant defenses. Glucosinolates are catabolized into benzoxazinoids that is a positive regulator of callose accumulation (Erb et al. 2019). More specifically, isoflavonoides, terpenes, alcaloïdes and polyphenols are elicited during pathogen attacks and some members of these classes possess antimicrobial activities (Sharma et al. 2017 ; Y. Chen et al. 2018 ; García-Calderón et al. 2020). Moreover, specialized metabolites can be involved in the HR²⁴ of plants that prevents further propagation of pathogens around the infected tissues (Heath 2000; Edreva et al. 2008).

Some specialized metabolites are well known as insecticides, herbicides or for other repulsive properties such as nicotine, benzoxazinoids or glucosinolates (Erb et al. 2019). They can also have more indirect effects against biotic attacks such as reducing the plant digestibility for the insect, attracting predators through the emission of VOCs (a natural mechanism of insect regulation revalorized in organic agriculture for biological control; Roumani 2021).

Communication and attraction

Plants must communicate with the living organisms around them, whether they are other plants (current or future), symbiotic organisms, pollinators, etc (Figure 1.9). They do it to either inform their partners (plants, herbivore predators, microorganisms) about their current status or to repel pathogens and herbivores, as discussed above using two ways: above and below ground communication (D. Kessler et al. 2015).

Plants commonly use VOCs to prime other plants in order to overcome both abiotic and biotic constraints (Figure 1.10). Stress priming is the process of

²⁴ HR: hypersensitive response

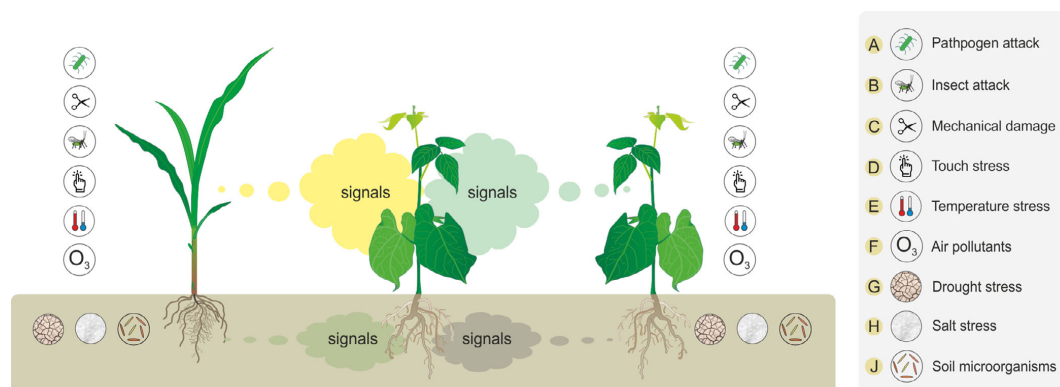


Figure 1.10 – Communication of plants through VOCs. Figure and legend from [Ninkovic et al. 2021](#).

A. pathogen attack; B. insect attack; C. mechanical damage; D. touch stress; E. temperature stress; F. air pollutants; G. drought stress; H. salt stress or J. soil microorganisms.

inducing moderate stress or eliciting the production of defense molecules in an individual so that they can defend themselves more effectively when faced with a more severe constraint (biotic or abiotic). This can have an effect on nearby plants by causing them to flower earlier in order to increase their chances of survival. VOCs composition differs according to the type of stress the emitting plant underwent: nerolidol, other volatile compounds such as geraniol, linalool and methyl salicylate in the case of cold stress; monoterpenes and isoprene in the case of oxidative and thermal stress; increase in hydrophilic compounds and decreased in of 2-decanone and α -ionone and other VOCs in the case of salt in *Solanum lycopersicum*; different groups of VOCs are involved in the response to herbivory, pathogen attack, mechanical damages ([Ninkovic et al. 2021](#)).

One of VOCs primary function is to chemically attract mutualists (beneficial microbes, seed dispersers) and pollinators to the plant (Figure 1.10). They can be used in a competition context with neighboring plants to increase their individual reproductive success ([Ninkovic et al. 2021](#)). Another important parameter in pollinator attraction, particularly for self incompatible species, is the attractiveness of the reproductive organs to pollinators in terms of floral structure and size, scent, pigmentation patterns and nectar. Hydroxycinnamates, phenolamides, and flavonoids all have roles in these aspects ([Yuting Liu et al.](#)

2021). Flavonoids are renowned pigments of petals in angiosperms (notably anthocyanins) that will attract pollinators to the reproductive organs. VOCs (often terpenoids) are important for nectar quality and scent, that both attract pollinators. The antioxidant and antimicrobial properties of phenolics help maintain, in combination with other classes of specialized metabolites, levels of toxicity and bitterness in nectars suitable for both pollinator attraction and repulsion of undesirable microbes (Schmitt et al. 2021).

Plants also emit molecules in the rhizosphere that can serve different purposes (Figure 1.10). Flavonoids are involved in most of the following processes, but are not the only molecules involved. In general, specialized metabolites contribute to allelopathic interactions, meaning they can influence developmental steps in other individuals like growth or seed germination in a positive or a negative way. Root exudates can influence the microbiome composition by attracting mutualists such as symbiotic mycorrhizal fungi or beneficial microbes while repelling phytopathogens and parasitic nematodes (Jing-Ke Weng, Lynch, et al. 2021). Natural product emissions in the ground can benefit future plants by priming them to specific constraints or by establishing a beneficial microbiome composition in the ground for the next generation (L. Hu et al. 2018). Priming through root exudates of neighboring plants has also been observed in the case of drought stress or under short day conditions to accelerate the flowering process (Figure 1.10; Falik et al. 2014). Nonetheless, root exudates are not always beneficial and can also inhibit growth to avoid competition. Flavonoids are involved in those allelopathic interferences, yet the mechanisms and the exact nature of the molecules involved in these communications are still not well understood (Jing-Ke Weng, Lynch, et al. 2021).

Plants benefit greatly from these above- and below-ground communications in terms of individual and communal survival and overall fitness. Nonetheless, these metabolic pathways can also be exploited by insects, phytopathogens and phytoparasites bringing nuance and balance into the whole biome (Jing-Ke Weng, Lynch, et al. 2021; Ninkovic et al. 2021).

Plant development

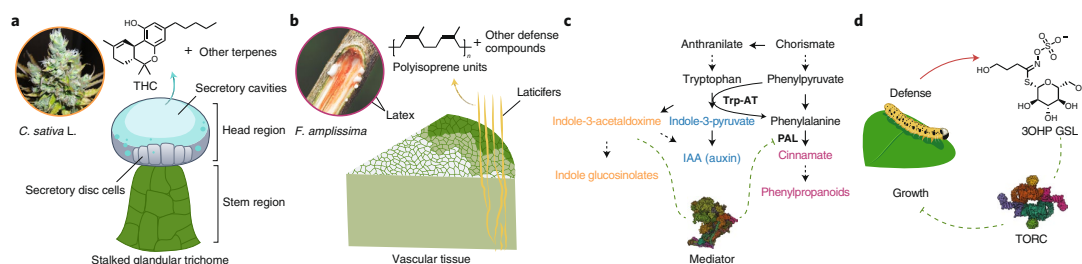


Figure 1.11 – Specialized metabolism roles in plant development. Figure and legend from [Jing-Ke Weng, Lynch, et al. 2021](#).

A. The stalked glandular trichome is a highly adaptive tissue in *C. sativa* L. that biosynthesizes cannabinoids and other terpenes and stores them in the secretory cavities in the head region. THC, tetrahydrocannabinol. **B.** Laticifers, specialized secretory cells in the vascular tissues of many plants, including *Ficus amplissima*, accumulate latex and ooze it upon injury. Latex contains polyisoprene units and other lineage-specific defense compounds. **C.** An example of interconnection of metabolic and signaling networks, where the indole glucosinolate and phenylpropanoid pathways are linked to production of auxin, regulating plant growth according to the metabolic status of these pathways. Trp-AT, tryptophan aminotransferase. **D.** Certain glucosinolates produced in response to insect herbivory in Brassicales can regulate plant growth through the TOR signaling pathway. 3OHP GSL, 3-hydroxyl-propyl glucosinolate; TORC, target of rapamycin complex.

The roles of all the classes of specialized metabolites in plant reproduction for attraction, structure and phenotypes of the reproductive organs of Angiosperms particularly was already mentioned ([Yuting Liu et al. 2021](#)). The interconnection of specialized metabolites and phytohormones through their precursors can influence the plant molecular responses when their contents are increased while undergoing stresses ([Jing-Ke Weng, Lynch, et al. 2021](#)). Moreover, specialized metabolites or their breakdown products can have direct effects on hub regulators like TOR²⁵ or on phytohormones like phasic acid on ABA²⁶, modulating seed germination and responses to long-term drought (Figure 1.11; [Malinovsky et al. 2017](#); [Jing-Ke Weng, Ye, et al. 2016](#)). Their roles in signaling cascades should thus not be overlooked.

Moreover, the accumulation of specialized metabolites demonstrate other clear influences on the development of plants. The most visible are the dedicated

²⁵ TOR: target of rapamycin ²⁶ ABA: abscisic acid

organs some species possess to store those potent molecules such as glandular trichomes, latex-producing laticifers, pigment-accumulating petal epidermal cells and root tubers (Figure 1.11). Another level of organization around specialized metabolites is subcellular compartmentalization in either already existing structures and different cell types or in dedicated organelles to avoid unwanted interaction and auto-toxic effects (Erb et al. 2019; Ninkovic et al. 2021). The sequestration of specialized metabolites in the vacuole is a strategy widely used across land plants (Shitan et al. 2020).

Those strategies of regulation, be it compartmentalization, dedicated organs, breakdown of biosynthesis steps in different compartments or cell types and transports are essential. As demonstrated above, specialized metabolites are very potent molecules and plants need to protect themselves from the negative side effects of hyperaccumulating such compounds in their tissues. Nevertheless, it is because of their high potential as active compounds and their great diversity in structure, activities, functions that they are of such interest in human applications. Their valorization can be rendered in human health through therapeutic or agronomic applications, in cosmetics as well as other industries. The quest for these natural products across the world is not a new phenomenon. However, the focus of plant biology on model plants, as well as the time required to develop efficient tools for studying specialized metabolite pathways, slowed their deciphering.

1.1.4 Valorization of specialized metabolites in human activities

Ethnobotanical studies know a renewed interest because we are now understanding the importance of studying non-model species in order to discover new potent natural products and potential remedies (Figure 1.12). Traditional medicine from all over the world is leading the quest for species of interest and the active molecules that confer them their therapeutic properties (Figure 1.13). For instance, malaria cases were reduced by 40 % in the African continent thanks to the exploitation of artemisinin, isolated from the *Artemisia annua* L. (*Asteraceae*). because its anti-inflammatory properties were reviewed in chinese studies (Tu 2011). Many *Asteraceae* members are known for their use in traditional medicine, such as *Arnica montana*, *Sylibium marianum*, *Calandula arvensis* and *Aster tataricus* L. (Panero et al. 2016; H. Singh et al. 2014; Bessada et al. 2015; Rho et al. 2020). It is one of the most represented families in ethnobotanical and pharmaceutical studies.

The health benefits of specialized metabolites are numerous (Figure 1.13). In the terpenoids class, we find artemisinin already mentioned above. In alkaloids, we find the analgesics morphine and codeine. They have a huge human and economic importance in that field (De Luca et al. 2012), but their properties are used in other domains as well. Phenolics are especially studied in the cosmetic industry for their antioxidant activities. Latex is of great use in several industries. Taxol, currently used in chemotherapies, is classified as an alkaloid, a terpene and a phenol all at once.

One could not forget the most obvious fields in which specialized metabolites are very profitable: agronomy and horticulture. The selection of varieties with higher amounts of these compounds is of interest to improve the tolerance of plants against a variety of constraints hence improving yields, but also to enhance their aesthetic and organoleptic qualities (Verpoorte et al. 2000).

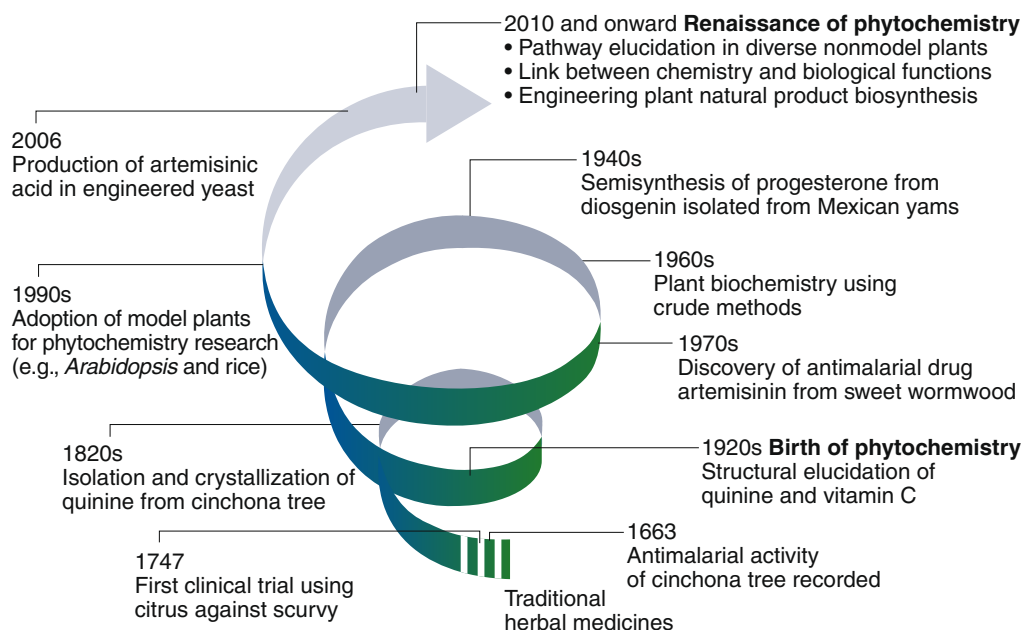


Figure 1.12 – Main steps in Phytochemistry history. Figure from [Jing-Ke Weng, Lynch, et al. 2021](#).

Despite their numerous potential usages, a lot of challenges are being met to develop production of those natural products at an industrial level. The first is their low concentrations in plant tissues, which hampers their extraction at sufficient levels to test their activities and do not allow the use of plants as a primary source of the metabolites. Moreover, as mentioned in the precedent section, specialized metabolites are finely regulated in plants spatially and temporally. Finding the optimal culture conditions to maximize yield is a difficult task. Another challenge is their complex structures that make them difficult or sometimes impossible to synthesize *in vitro* ([Feher et al. 2003](#)). The most cost-effective and feasible option for their production is *in vivo* thanks to biotechnologies. Models such as prokaryotes (*E. coli*), or eukaryotes (yeasts, hairy roots, cals, plant cells) when metabolically engineered with a whole reconstructed biosynthetic pathway of the molecule of interest can be used in bioreactors and yield high contents of the metabolite of interest, the last challenge being their proper extraction.

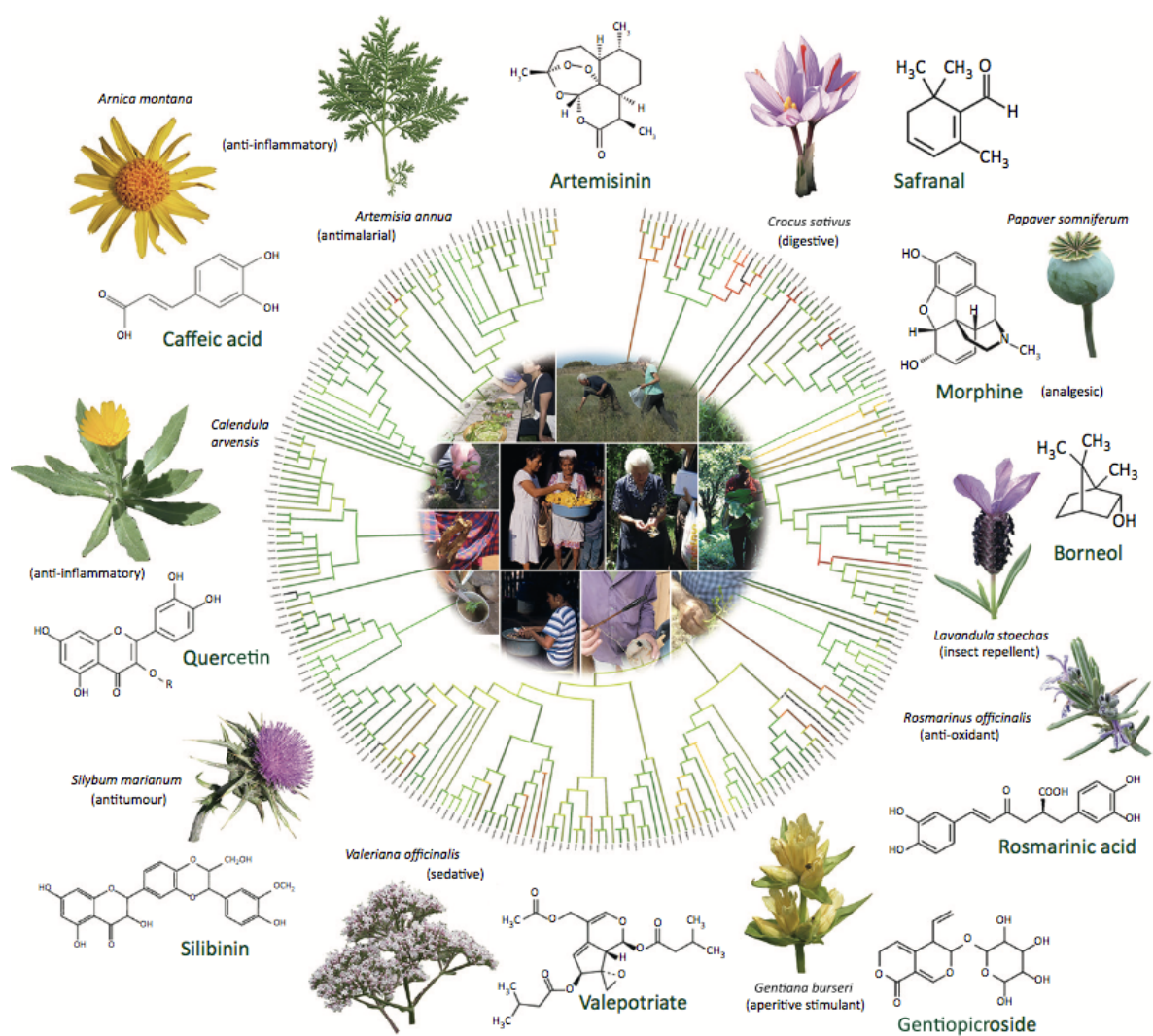


Figure 1.13 – Examples of medicinal plants accumulating specialized metabolites of interest. Figure from Garnatje et al. 2017.

To alleviate some of these challenges, elucidating the biosynthetic pathways of these molecules of interest in accumulating non-model species is crucial. This is nowadays facilitated by the emergence of *-omics* methods and of more efficient sequencing methods. Those data will enable plant scientists to unravel biosynthetic pathways of specialized metabolites and recreate them in more suitable heterologous systems *via* metabolic engineering.

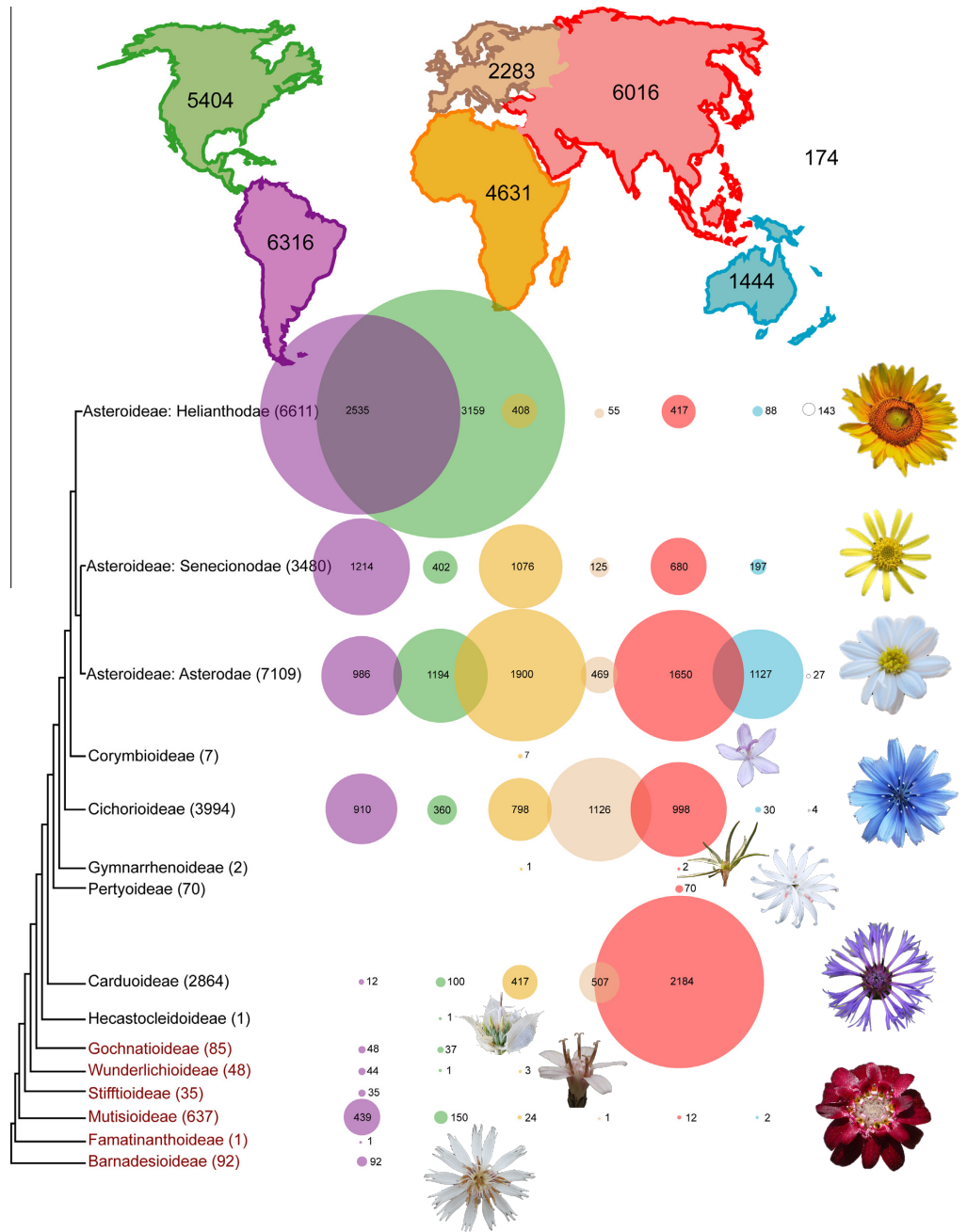


Figure 1.14 – Repartition of sub-families of *Asteraceae* across the world. Figure adapted from Barreda et al. 2015 and Delporte 2017.

1.2 *Cichorium intybus* and its benefits

Chicory has a long history of use in various human civilizations. Its wild relatives can be found throughout Western Europe, North America, parts of South America, the Middle East, and Asia (Figure 1.14). In ancient Egypt (1600 BC) it was consumed before the main meal for its already known digestive properties (Short et al. 2015). These properties are now attributed to inulin (Pouille et al. 2020). The majority of healing properties of chicory are attributed to its root.

A great number of chicory legends, on the other hand, mention its pale blue flowers, used in ophthalmic treatments. The faithfulness of young women with beautiful blue eyes to their departed fiancés is a common denominator of these folklore, which can be found in Italy, Germany, and Jerusalem. Ovide tells the story in *Metamorphoseon libri* of Clytie, who is seduced by the Sun (Apollo or Helios for the Greeks) but is then abandoned by him (Ovide 2017; translation from latin to french of Ovide's work). She transformed into a heliotropic plant so she could keep looking at her lost love during the day. Inspired by Ovide work's, other legends gave chicory the surname of Sun's Fiancée. Indeed, chicory flowers open in the morning with dark blue petals that lighten throughout the day before closing at sunset. In these legends, the young women standing in the same spot for so long, awaiting the return of their fiancés, saw their feet and legs transformed into roots, and their watery blue eyes into chicory's flowers (de Gubernatis 2004).

All this folklore linking chicory flowers to the blue eyes of young women might take its roots in the ophthalmic healing properties of the decoction of its blue flowers, probably due to the presence of vitamin E, as in carrots, a remedy that was used during Middle Ages (de Gubernatis 2004). *C. intybus* was used in many traditional remedies against various diseases and symptoms across the world (Table 1.1), showcasing its natural accumulation of potent molecules with therapeutic properties.

Table 1.1 – Traditional medicinal uses of *Cichorium intybus*. Table from [Street et al. 2013](#).

Country	Traditional use(s)	Plant part(s)	Preparation(s)
Afghanistan	Malaria	Root	Aqueous extract
Bosnia and Herzegovina	Diarrhea, strengthening the prostate and other reproductive organs, pulmonary cancer, hangover, and purification of biliary tract	Aerial part, flowers, roots	Not stated
	Liver disorders, spasmolytic, cholesterol, antiseptic	Aerial	Decoction
Bulgaria	Cholagogue stimulant for gastric secretion, hypoglycemic	Roots, aerial parts	Decoction
India	Liver disorders	Seeds	
	Diabetes	Whole plant	Not stated
	Jaundice, liver enlargement, gout, and rheumatism	Root	Decoction
	Cough relief	Not stated	
Iran	Eupeptic, stomachic, depurative, choleric, laxative, hypotension, tonic, and antipyretic	Whole plant	Not stated
Italy	Blood cleansing	Leaves	Not stated
	High blood pressure	Leaves	Decoction
	Blood purification, arteriosclerosis, antiarthritis, antispasmodic, digestive	Leaves/roots	Decoction
	Depurative	Whorls	Decoction
	Choleric, hepatoprotective against jaundice, mild laxative, hypoglycemic	Leaves	Decoction, squashed fresh leaves
Jordan	Internal hemorrhage, sedative in typhoid	Whole plant	Cooking
Morocco	Renal disease	Aerial/roots	Not stated
	Kidney disorders, diabetes	Whole plant	Decoction
Pakistan	Diabetes	Roots	Decoction
Poland	Digestive complaints and lack of appetite	Roots	Tea
Serbia	Diarrhea	Flower	Infusion
	Diuretic, digestive, laxative, anti-inflammatory, liver complaints, reducing blood sugar	Roots	Decoction/tea
	Cholagogue, digestive, hypoglycemic	Aerial part/root	Not stated
South Africa	Jaundice, tonic	Leaves, stems, roots	
Turkey	Cancer, kidney stones	Roots	Decoction
	Wound healing	Leaf	Ointment
	Hemorrhoids, urinary disorders	Aerial	Tea

Centuries later, we reach the pinnacle of chicory popularity in Europe, particularly in the north of France. In 1806, Napoleon the First imposed the Continental Blockade, which prohibited all commercial exchanges with the Great Britain, depriving France (among other countries) of imports of sugar, cotton, and, most pertinently here, coffee, from the colonies. Chicory is an endemic plant of northern France, and its culture grew large enough to be used as a coffee substitute (Kandeler et al. 2009). The beverage made from its torrefied roots grew in popularity over time and France is still the largest European producer of *Cichorium intybus* L. var. *sativum* and var *foliosum* (Figure 1.15).



Figure 1.15 – Botanical representation of *Cichorium intybus* (left) and publicity for coffee substitute from torrefied chicory roots after the World Wars (right).
Right : “Chicorée la Renommée des Flandres” (une jeune femme ravitaille un soldat français et un soldat belge avec de la chicorée) from Jean-Pierre Verney.

The species of the genus *Cichorium* belong to the *Asteraceae* family (Figure 1.16), of which evolutive advantages allowed them to conquer the terrestrial world and represent 10 % of the identified Angiosperms species. They emerge in South America 70 MYA and colonized the rest of the globe. The three major subfamilies of *Asteraceae* are *Cichorioideae* (with *Cichorium* and *Lactuca* genera), *Carduoideae* (with *Cynara* genera) and *Asteroideae* (with *Helianthus* genera) (Barreda et al. 2015).

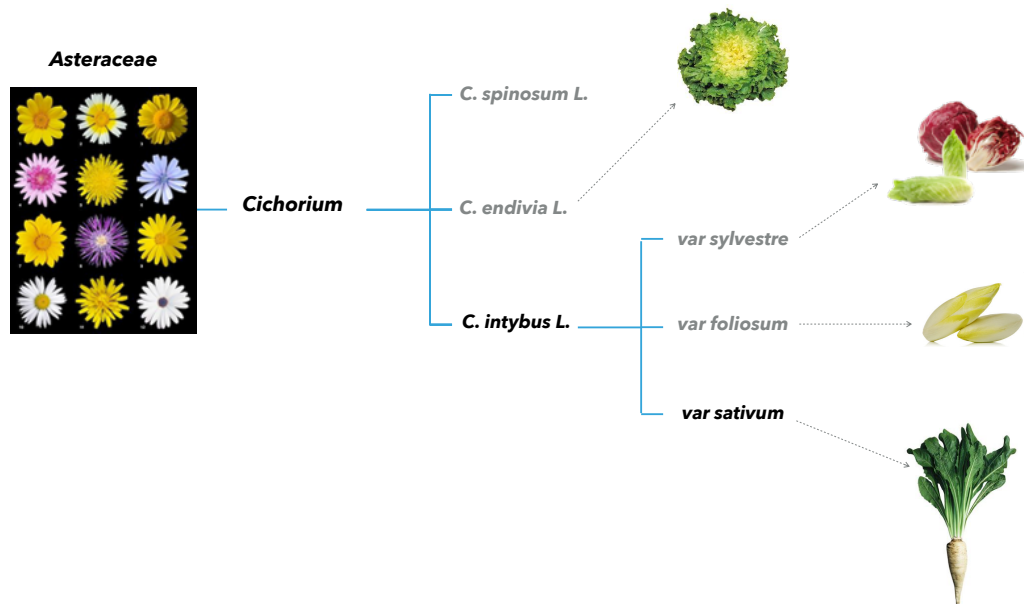


Figure 1.16 – Phylogenetic tree of cultivated *Cichorium* species.

The *Cichorium* genus encompasses six species: *C. intybus* L., *C. endivia* L., *pumilum* Jacq., *C. calvum* Sch. Bip. ex Asch., *C. spinosum* L., *C. bottae* Deflers, found principally in Europe and Asia. *C. intybus*, *C. spinosum*, and *C. bottae* are perennial species in the wild whereas the others are annual.

Wild *Cichorium intybus* L., as all *Asteraceae*, is characterized by having a capitulum as an inflorescence (also known as umbel). The height of the floral stem can reach 1 meter above ground. Its root can measure 20 cm in length and 3 cm in diameter. Its leaves range from 10 to 30 cm in length, are stalked, lanceolate and unlobed. Wild chicory grows on the roadsides and in the margins of fields and flowers from July until October. Their flowers are sporophytic self-incompatible and are pollinated by insects. The fruits are akenes (L. Gonthier, Blassiau, et al. 2013). Chicory varieties have been selected over the years to produce longer and larger roots enriched in inulin. Inulin content can reach 20 % in the fresh root and up to 90 % of the dry weight. It is a polymer of fructose that has many usages, such as sugar substitute (H. P. I. Bais et al. 2001) (Figure

1.17).

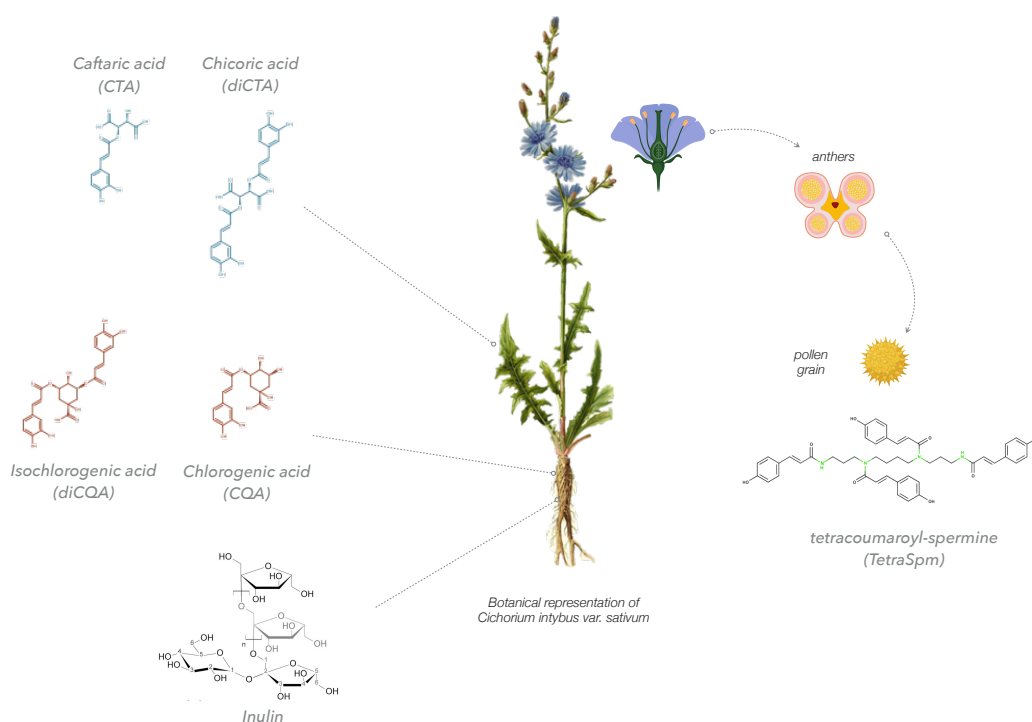


Figure 1.17 – Molecules of interest accumulating in *Cichorium intybus* var. *sativum*.

Numerous natural products have been identified in chicory, mostly in its root. Sesquiterpene lactones are abundant, notably lactucine, 8-deoxylactucine and lactucopicrine, and data suggest they are responsible for chicory bitterness so of great interest in agronomical studies (Rees et al. 1985; Willeman et al. 2014; Van Beek et al. 1990; de Kraker et al. 1998). Chicory also accumulates phenolic compounds such as cichoriine or flavonoïdes like derivatives of quercetine, kampferol and epigenine (Carazzone et al. 2013). However, the major phenolic compounds are HCA²⁷ derivatives such as quinic acid esters: chlorogenic (caffeoylquinic acid or CQA) and isochlorogenic acids (dicafeoylquinic acid or diCQA); or tartaric acid esters: caftaric acid (caffeoyltartric acid or CTA) and chicoric acid (dicafeoyltartric acid or diCTA) (Legrand et al. 2016; Street et al. 2013) (Figure 1.17). CQAs are reviewed in length in section 1.4.1. In the aerial

²⁷ HCA: Hydroxycinnamic Acid

parts of chicory, more specifically the flowers, phenolic compounds of interest also accumulate. Unusual phenolamides have been identified and characterized (Figure 1.17). These natural products of interest are also reviewed in length in section 1.3.1.

Chicory is an ideal species for the study of these specialized metabolism. It is an unusual non-model plant of agronomical interest with available genomic data (Dauchot et al. 2009; Ghedina et al. 2015; Cadalen et al. 2010; L. Gonthier, Bellec, et al. 2010), facilitating the identification of target genes by sequence homology. Moreover, it can easily be transformed and cultivated through the production of protoplasts or of hairy roots from which mutant plant lines can be regenerated (C. Rambaud et al. 1992). Recently, the CRISPR-Cas9 technology was optimized in this species, giving us even more tools to elucidate the biosynthetic pathways of all the mentioned natural products of interest chicory accumulates (Bernard, Gagneul, et al. 2019).

During my thesis, I worked on the deciphering of the biosynthetic pathways of two molecules of interest in *C. intybus*. Both are derivatives of HCA and are synthesized through acylation reactions. The first compound is a unusual conjugate of HCA with an amine, tetracoumaroyl-spermine or TetraSpm. This phenolamide or HCAA²⁸ accumulates in the pollen coat of chicory. The second molecule of interest in this work is diCQA. The biosynthetic pathway of this root-accumulated compound was previously investigated in chicory but unsuccessfully. Known functions and roles of phenolamides and CQAs are presented in the following sections.

²⁸ hydroxycinnamic acid amide

1.3 Roles and functions of phenolamides

1.3.1 Definition of phenolamides and their characteristics

Phenolamides, also known as phenylamides or hydroxycinnamic acid amides are conjugates of hydroxycinnamic acid derivatives with aromatic or aliphatic amines.

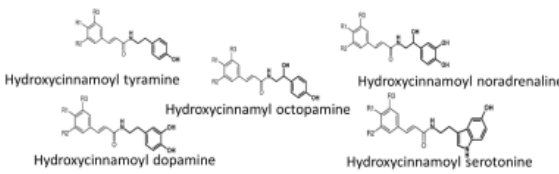
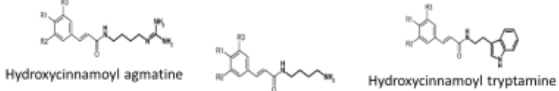
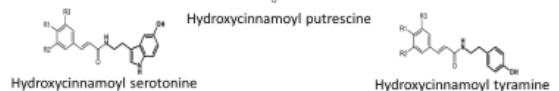
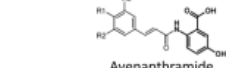
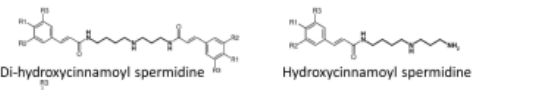
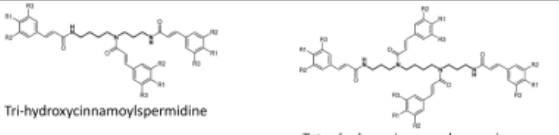
Classes	Enzymes	Plantes	Phenolamides
Serotonine/Tyramine Derivates	CaTHT,SiTHT,NtTHT10/30,NtTHT4/11 SiTHT1-3,SiTHT7-1 SiTHT7-8,CaSHT	Solanaceae	 Hydroxycinnamoyl tyramine Hydroxycinnamoyl octopamine Hydroxycinnamoyl noradrenaline Hydroxycinnamoyl dopamine Hydroxycinnamoyl serotonin
Agmatine/Tryptamine /Putrescine Derivates	AtACT1	Brassicaceae	 Hydroxycinnamoyl agmatine Hydroxycinnamoyl tryptamine
	HvACT,OsAHT1 OsPHT3,OsPHT NaAT1,HvTHT2 HvTHT7,HvTHT8 OsTHT1,OsTHT2 OsTBT1,OsTBT2	Gramineae/ Solanaceae	 Hydroxycinnamoyl putrescine Hydroxycinnamoyl serotonin Hydroxycinnamoyl tyramine
Anthranilate Derivates	DcHCBT	Carnation	 Avenanthramide
	AsHHT1,AsHHT4	Oat	
Spermidine Derivates mono or di substituted	AtSCT,AtSDT	Rosids	 Di-hydroxycinnamoyl spermidine Hydroxycinnamoyl spermidine
	Os12g27220 Os12g27254	Poaceae	
	NaCV86,NaDH29 SmSpmHT, SrSpmHT	Solanaceae	
Spermidine Derivates Tri substituted	AtSHT,MdSHT NtSHT,SiSHT VvSHT,RicSHT RocSHT,SmSHT1 SrSHT1,CiSHT1 CiSHT2	Eudicotylédons	 Tri-hydroxycinnamoylspermidine Tetra-hydroxycinnamoyl spermine
	CYP98A8/A9, AtTSM1	Brassicaceae	

Figure 1.18 – Classification of phenolamides according to their origin. Figure adapted from Roumani, Besseau, et al. 2021.

R1 = H or OH; R2 and R3 = H, OH or OMe

The phenolic moiety of the hydroxycinnamic acid derivatives is covalently linked through amide bonds to an aromatic monoamine or to an aliphatic amine. Five classes of phenolamides can be considered according to the type of amine constituting their backbone: derivatives of tyramine and serotonin, derivatives

of agmatine, derivatives of tryptamine and putrescine, derivatives of spermidine, derivatives of spermine and derivatives of anthranilate. These classes are represented in Figure 1.18 (Roumani, Besseau, et al. 2021). In this work, we are more interested in the derivatives of two polyamines: spermine and spermidine. While spermidine is considered to be a contributor to primary metabolism for plant growth and longevity (Eisenberg et al. 2009; Nambeesan et al. 2010), increases in spermine contents activate plants defense genes against abiotic and biotic constraints (Hasan et al. 2021; Sreenivasulu et al. 2000; Roychoudhury et al. 2011; Zrig et al. 2011; Radhakrishnan et al. 2013; Duan et al. 2008; Mitsuya et al. 2009; Gonzalez et al. 2011). These phenomena demonstrate the crucial importance of polyamine homeostasis in plants fitness and defense (Tiburcio et al. 2014).

A theoretical number of over 5 000 phenolamides compounds is predicted across the plant kingdom. However their roles and biosynthetic pathways are not entirely understood (Roumani, Besseau, et al. 2021). The last step of their synthesis is catalyzed by very diverse N-hydroxycinnamoyltransferases which explains the variety of phenolamide structures. If their functions in plants are still unknown, their structure and their tissue distribution are well documented and are discussed in section 1.5 in relation to our metabolite of interest. (Martin Tanguy et al. 1978; Luo, Fuell, et al. 2009 ;Kaur et al. 2010; Xuekui Dong et al. 2015; Elejalde-Palmett et al. 2015).

1.3.2 Physiological roles of phenolamides

Typically, conjugated molecules are thought to be the end of a storage pathway, and this was the main hypothesis for a long time when it came to phenolamides. Some can be stored as hexose conjugates and remobilized on demand after the phenolic rings have been hydroxylated and methylated, for example. The intracellular accumulation of hydroxycinnamoyl-spermidines was also associated with their glycosylation (Bassard, Ullmann, et al. 2010). However, several studies illustrate that they can act also as metabolite intermediates, especially because they are at the crossroad of the phenylpropanoid and polyamines pathways (Bassard, Ullmann, et al. 2010).

1.3.2.a Plant defense against biotic and abiotic constraints

Defense against abiotic constraints

The studies that link specialized metabolites to plant defenses against abiotic constraints are scarce and show more indirect correlations than those that link specialized metabolites to defense against biotic constraints. Polyamines, the building blocks of phenolamides, have been shown to have indirect antioxidant, protective, and stabilizing properties in plant cells (Bassard, Ullmann, et al. 2010). The free polyamines of interest of this work, spermine and spermidine were shown to prevent leakage of electrolytes or amino acids for instance (Chattopadhyay et al. 2002). Conversely, the correlation of free polyamines contents with plant defense against abiotic stress has mentioned above, but not for their conjugated form as phenolamides.

Their role in plant defense is still hypothetical, but this hypothesis is worth considering based on their scavenging, antioxidant, or putative UV-B protective properties (Langebartels et al. 1991; A. Edreva et al. 1998; Choi et al. 2007; Georgiev et al. 2012; Abe et al. 2017). Such properties are of particular interest, particularly in light of climate change and the increasing frequency and intensity of abiotic and biotic constraints. Furthermore, several studies show

that certain abiotic constraints have an effect on phenolamide content. Following the application of the constraints listed in this section, the content of phenolamides can thus be increased. Mineral deficiencies, high ozone levels, drought, UV-B exposure, and even heat have all been shown to increase the content of putrescine and spermidine derivatives in wheat and tobacco (Klapheck 1983; Aglika Edreva et al. 1995; A. Edreva et al. 1998; Königshofer et al. 2002; Demkura et al. 2010). Mechanical injuries increase the contents of derivatives of tyramine, octopamine and putrescine in *Solanaceae*, more specifically *Solanum lycopersicum* and *Solanum tuberosum* (Jonathan Negrel, Pollet, et al. 1996; Pearce et al. 1998; Matsuda et al. 2005). In a study on long-term impacts of a combination of abiotic constraints linked to climate change, hence heat, elevation of CO₂ and O₃ concentrations, phenolamides were specifically elicited (B. Mikkelsen et al. 2015). However, phenolamides can also be downregulated by salt stress or nitrogen deficiency (Shevyakova et al. 2006 ; Larbat et al. 2014). This downregulation is correlated to the accumulation of phenolic compounds, precursors of phenolamides, in nitrogen-deficient tissues, which could be due to a decrease in free polyamine contents or a downregulation of the N-hydroxycinnamoyl-transferase involved in conjugates formation. (Larbat et al. 2014 ; Royer et al. 2016).

Defense against biotic constraints

Similarly to studies on the relationship between phenolamides and responses to abiotic constraints, the role of phenolamides in responses to biotic constraints has yet to be fully elucidated. Numerous studies show that they accumulate after a pathogen attack or herbivory wounds. Again, their presumed activities in these situations would be related to their scavenging abilities (Figure 1.19).

Except for fully substituted spermidine or spermine, which are only found in the pollen coat, discussed in more length in section 2.1.1 (Elejalde-Palmett et al. 2015; Delporte 2017), all types of phenolamides can be associated with pathogen defense. It has been demonstrated that the accumulation of phenolamides is

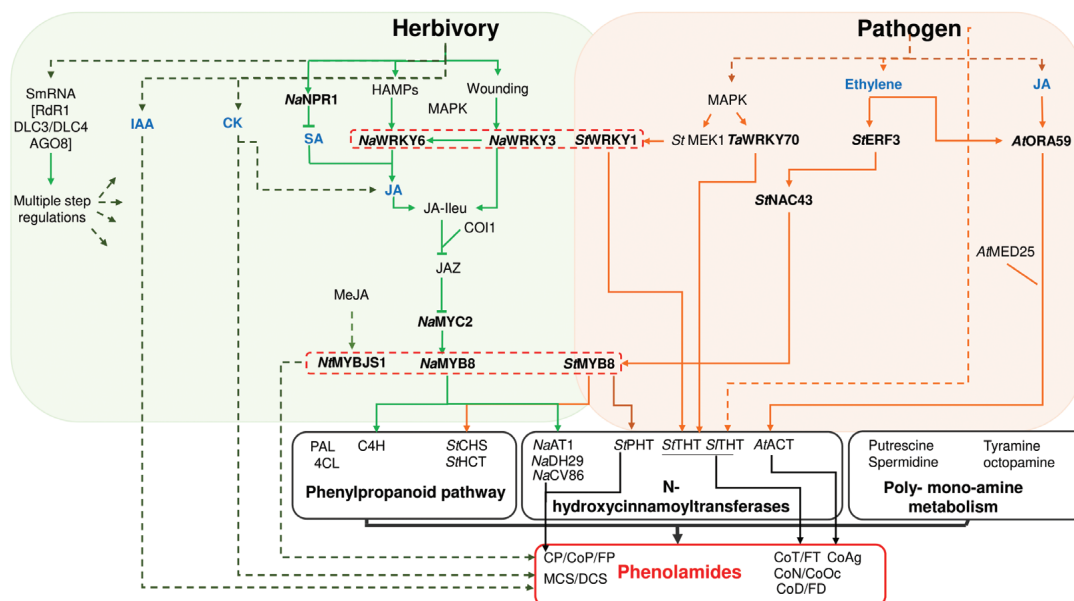


Figure 1.19 – Phenolamide accumulation under biotic constraints. Figure and Legend from Roumani, Besseau, et al. 2021.

The orange box represents the mechanisms described in response to pathogen infection and the green box those in response to herbivory. Transcription factors are in bold and phytohormones in blue. In the case of pathogen infection, phenolamide accumulation relies on ethylene (ET) and jasmonic acid (JA) signaling pathways. ET activates different transcription factors (ERF and NAC) and ends by activating MYB factors that induce the expression of genes of the phenylpropanoid pathway, together with acyltransferases. In *Arabidopsis*, JA activates the AtORA59 factor, which leads immediately to overexpression of the AtACT enzyme. Finally, a third pathway implies the activation of WRKY transcription factors through the MAPK pathway. In the case of herbivory, the JA signaling pathway is mainly activated. Once JA is accumulated, Coronatine Intensive1 (COI1) degrades the Jasmonate Zim-Domain (JAZ) proteins and releases transcription factors belonging to the bHLH family MYC. The release of MYC2 activates the MYB transcription factor. Herbivory also induces the accumulation of other phytohormones and SmRNA, which can modulate the JA-dependent response and/or contribute to a systemic accumulation of phenolamides. The mechanisms for such aspects of the regulation are still unclear. Solid lines represent a direct relationship between these elements, whereas dotted lines represent relationships between elements with probable intermediates.

PAL, phenylalanine ammonia lyase; 4CL, 4-coumarate:CoA ligase; C4H, cinnamate-4-hydroxylase; CP, caffeoylputrescine; CoP, *p*-coumaroylputrescine; FP, feruloylputrescine; MCS, monocaffeoylspermidine; DCS, dicaffeoylspermidine; CoT, *p*-coumaroyltyramine; FT, feruloyltyramine; CoN, *p*-coumaroylnoradrenaline; CoOc, *p*-coumaroyloctopamine; CoD, *p*-coumaroyldopamine; FD, feruloyldopamine; IAA, indole acetic acid; CK, cytokinin; SA, salicylic acid.

related to increased plant defense through studies of KO mutant lines of genes encoding for enzymes involved in phenolamide production pathways or through external application of phenolamides (Roumani 2021). Their protective role was explained by two courses of action. The first is a direct antibacterial or antifungal activity demonstrated in *in vitro* assays (Newman et al. 2001; Morimoto et al. 2018; von Röpenack et al. 1998; Zacarés et al. 2007; Stoessl et al. 1970; Ube et al. 2017; Muroi et al. 2009; Morimoto et al. 2018). The second effect of phenolamides accumulation is a reinforcement a plant cells secondary wall during pathogen infection by suberization, notably by tyramine, agmatine, anthramide, putrescine and tryptamine derivatives (Roumani 2021). By incorporating polymerized phenolamides, the cell wall becomes less digestible by pathogens hydrolytic enzymes thus protecting the plant from a fungi infection and proliferation (King et al. 2005). The roles of phenolamides against herbivory (mainly by insects) was studied particularly in *Solanaceae* and *Poaceae* species. Most of the accumulated phenolamides are derivatives of putrescine, spermidine and sometimes tyramine (Roumani, Besseau, et al. 2021). In *Nicotiana attenuata*, the presence of caffeoylputrescine and of dicaffeoylspermidine was demonstrated to be detrimental to *Manduca sexta* larvae (Kaur et al. 2010). Caffeoylputrecine is also an inhibitor of the oviposition of *Liriomyza trifolii* in *Capsicum annuum* (*Solanaceae*) (Tebayashi et al. 2007). This activity isn't ubiquitous across all studied species. A study from Onkokesung and colleagues (2012) suggests that higher protection can be obtained when a wide array of phenolamides is accumulated.

1.3.2.b Phenolamides putative roles in reproduction

The high accumulation of derivatives of putrescine, spermidine and spermine in the pollen grain is at the origin of the hypothesis that those metabolites might play a role in floral initiation and reproduction. Indeed, they have a putative link with development and growth regulation, cell proliferation, cell differentiation, embryogenesis, organogenesis and cal formation (Martin Tanguy 1997; A. M. Edreva et al. 2007; Bassard, Ullmann, et al. 2010; Guo et al. 2003). Another proposed role for phenolamides in some species is that they serve as polyamine

and hydroxycinnamoyl storage in pollen grains, which can be remobilized at specific metabolic points such as germination (Figure 1.20) (Bassard, Ullmann, et al. 2010). A recent study in *Glycine max* (*Fabaceae*) shows that phenolamides act as ROS scavenger during floral development to avoid pollen abortion (Ding et al. 2019). The roles of phenolamides during floral initiation has been established in several families (*Asteraceae*, *Araceae*, *Brassicaceae*, *Solanaceae*). Phenolamides accumulate in the leaves of apical meristems and in the apex just before floral initiation, and are absent afterwards. Mutants not accumulating phenolamides were unable to bloom (Bassard, Ullmann, et al. 2010). However, they are not always essential for fertility (Cabanne et al. 1981; Martin Tanguy 1985; Martin Tanguy 1997).

The pollen grain wall is constituted of several layers: intine, exine which can be subdivided into nexine and sexine, and finally the pollen coat (or tryphine, not always present) (Figure 1.20). Intine is a pecto-cellulosic layer and is the most intern layer. Its constituents are synthesized by the microspores. Exine and its two sublayers form the gaps and apertures that confer their specific characteristics to each species pollen grain. The pollen coat is present in most Angiosperms with zoophile pollination (J. Jiang et al. 2013; H. Ma 2005) and is constituted of long chains lipids, glycoproteins and oleosines as well as sterol esters. Sterol esters would stabilize the semi-rigid structure of the pollen coat more efficiently (Rejón et al. 2016).

The pollen coat has roles in pollen/stigmate recognition or rejection in cases of auto-incompatibility, but also in pollination via the mechanistic of adhesion to the pollinators as well as in the protection of the genetic material against abiotic and biotic constraints (Pacini et al. 2005; H. Lin et al. 2013; Murphy 2006).

The constituents of the exine and pollen coat layers are synthesized in the tapetum (Shi et al. 2015). The tapetum is one of the four layers of the anthers wall. Tapetum cells are nutritive cells in the anthers that possess two specific organelles: elaioplasts and tapetosomes. These organelles have not been fully characterized to this day, biochemically or in terms of function but it is supposed

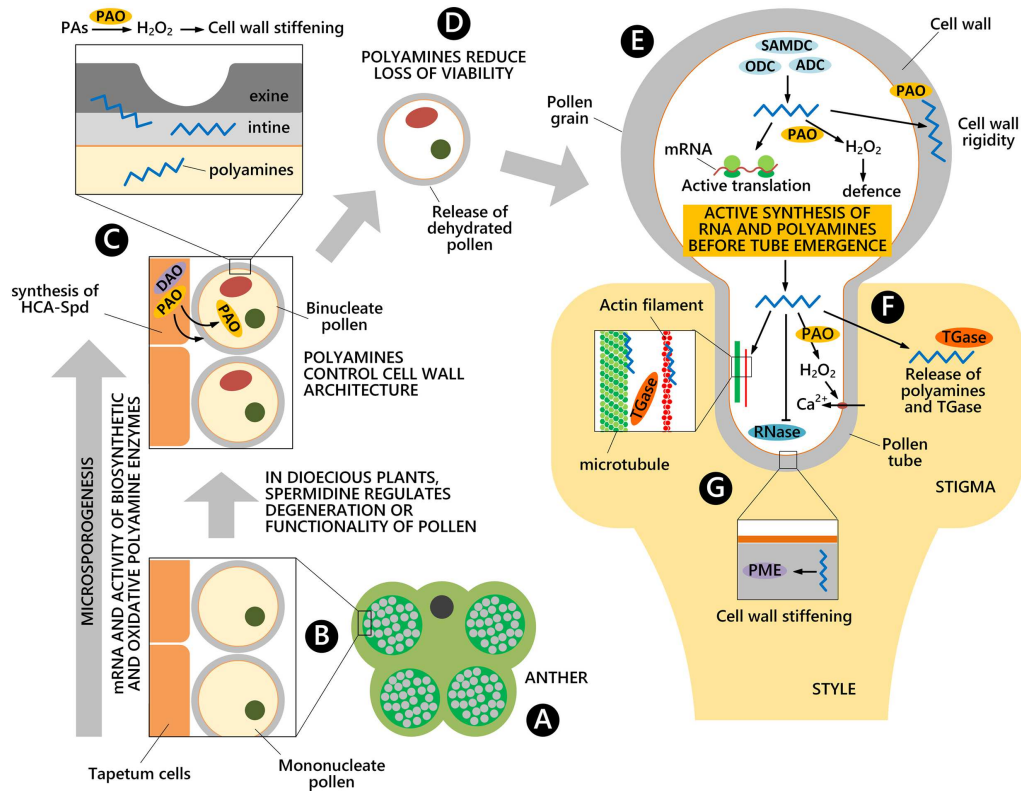


Figure 1.20 – Involvement of polyamines in pollen development. Figure and legend from Aloisi et al. 2016.

PA (polyamine) biosynthetic and oxidative metabolisms occur from the early stage of pollen formation inside the anthers (A), when both microspores and the tapetal cell layer of the anther contribute to microspore cell wall architecture (B). Pollen accumulates high levels of free PAs and HCAs, mainly localized in the cell wall. PA catabolism by PAO and DAO modulates the rigidity of the cell wall (C). Once dehydrated, pollen grains are released and PAs contribute to maintain pollen viability (D). During germination on a stigma (E), PAs promote the translation of transcripts and they are also released in the extracellular space, together with TGase (F). During pollen tube growth in a compatible style, PAs take part in the cytoskeleton organization, in cell wall deposition and remodeling by the PME enzyme as well as in the regulation of ion transport through the plasma membrane. PAs also exert an inhibitory effect on RNase enzymes (G).

they are involved in the biosynthesis of sporopollenine, a hydrophobic molecule that coats the outer layer of the pollen grains, and of which the precursors constitute the exine layer (S. S. Kim et al. 2013). This transport of specialized metabolites from their production tissue to the tissue in which they play their role is quite common and involves excretion, secretion as well as storage structure but is not well understood. (Jing-Ke Weng, Lynch, et al. 2021). The transport (active or passive) of sporopollenin or other specialized metabolites in the pollen grain has not yet been elucidated.

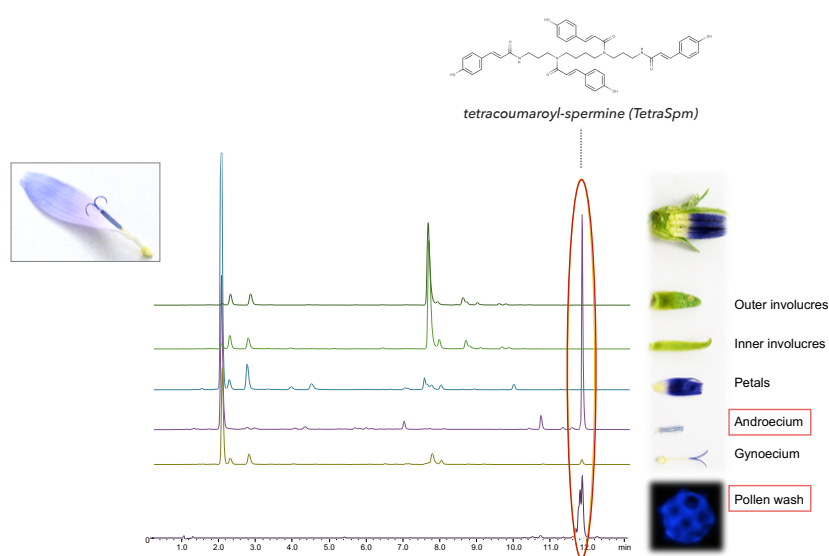


Figure 1.21 – Identification of phenolamides present in chicory floral buds. Figure and legend adapted from Delporte 2017. Stacked HPLC chromatograms of methanolic extract obtained from different parts of floral buds and of methanol pollen wash.

In this work, I focused on specific polyamine derivatives, fully substituted spermine derivatives found in *C. intybus* pollen exine (Delporte 2017). The particularity of fully substituted spermidine and spermine is that they are found only in the pollen coat. In *C. intybus* it was demonstrated that the fully substituted spermine accumulation was precisely localized in the pollen grain (Figure 1.21). This seems common to only *Asteraceae* members, in which the fully substituted tetrahydroxycinnamoyl-spermine is the major phenolamide

accumulated (Delporte, Bernard, et al. 2018). While trihydroxycinnamoyl-spermidine is considered a marker of Eudicotyledons pollen exine (Figure 1.22 A.; Elejalde-Palmett et al. 2015), TetraSpm has already been detected in flower or pollen extracts of other *Asteraceae* species such as *Matricaria chamomilla*, *Coreopsis tinctoria* and *C. intybus* (A. Yamamoto et al. 2002; S.-C. Lam et al. 2016; Božičević et al. 2017; Delporte, Bernard, et al. 2018). It is however absent in families close to *Asteraceae* (*Calyceraceae*, *Stylidiaceae* and *Argophylliceae*) or any other plant families (Delporte, Bernard, et al. 2018; Figure 1.22 B.).

This unusual pollen HCAA is the first molecule of interest of this work. The second molecule of interest is found in the root of *C. intybus* and is a HCA conjugate of a different nature. Instead of a polyamine, the HCA is the substituent of quinic acid here. The following section reviews the biosynthetic pathway of such compounds known as caffeoylquinic acids. The current knowledge available concerning the biosynthetic pathways of the specific molecules of interest of this work, TetraSpm and diCQA, is reviewed in the introductions of Chapters 2 and 4 respectively.

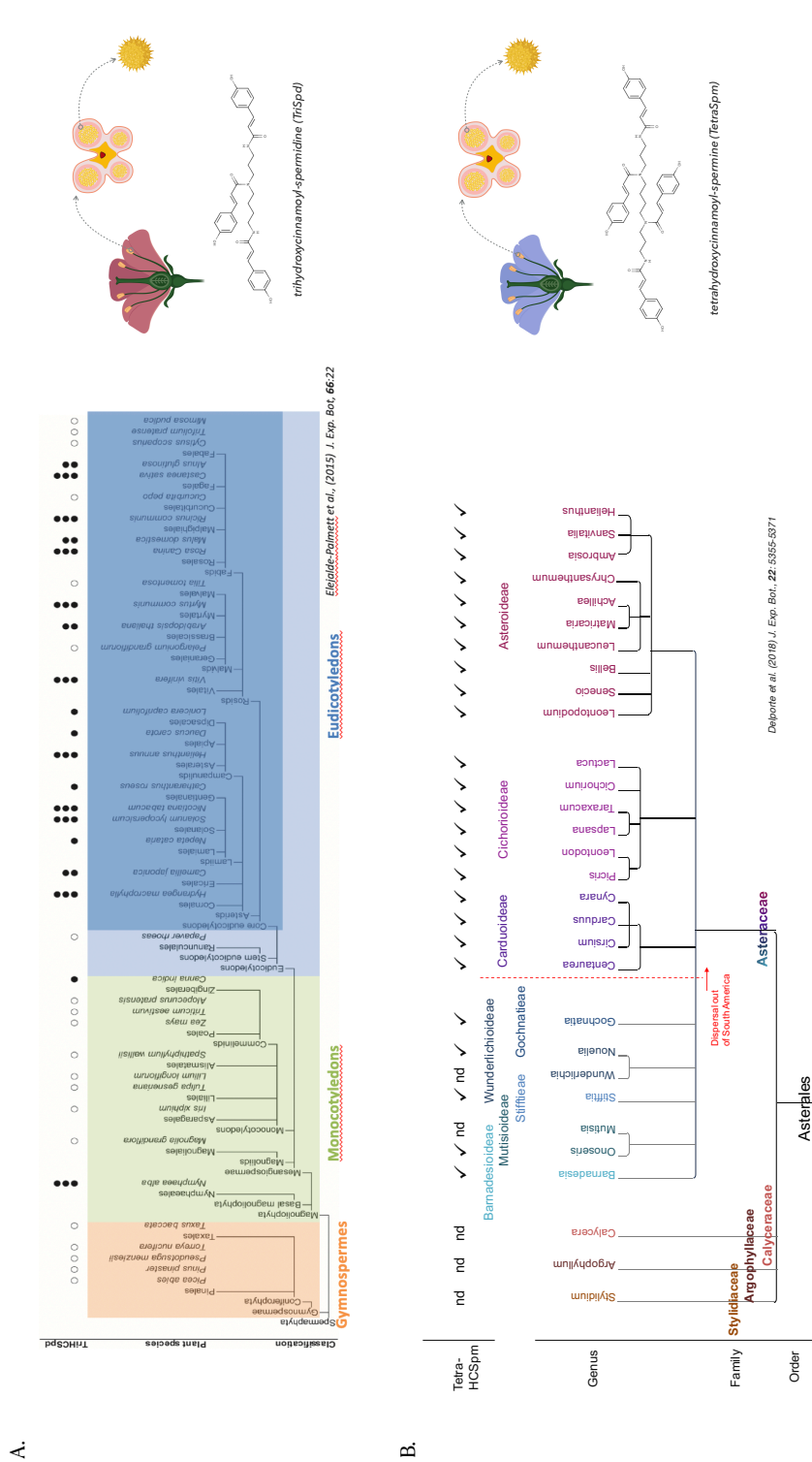


Figure 1.22 – Pollen exine markers in Eudicotyledons and in Asteraceae.
A. Trihydroxycinnamoyl-spermidine is considered a marker of the pollen exine of Eudicotyledons. Figure adapted from [Eliade-Palmett et al. 2015](#)

B. Tetrahydroxycinnamoyl-spermine is considered a marker of Asteraceae pollen exine in contrast with other Eudicotyledons. Figure adapted from [Delponte 2017](#)

1.4 Roles and functions of caffeoylquinic acids

Isochlorogenic acid, or dicaffeoylquinic acid, is a well studied potent specialized metabolite. Its chemical and physical characteristics make it a valuable plant natural product and highly beneficial to human health. Its role in plants however is not as well investigated. The presented work contributes to the characterization of its production in *C. intybus*.

1.4.1 Definition of caffeoylquinic acids

Caffeoylquinic acids are composed of a quinic acid moiety and of caffeic acid moieties. The four stereocenters of quinic acid (carbons 1, 3, 4, and 5) can be substituted by caffeic acid (Figure 1.23). A total of 15 combinations is possible, with four monoCQAs, six diCQAs, four triCQAs, and one tetraCQA. If we take a closer look on the properties of each component of the CQA molecule, we can gain insights as to their bioactivities. Indeed, quinic acid ((1S,3R,4S,5R)-1,3,4,5-tetrahydroxycyclohexane-1-carboxylic acid) polar surface area is 118 Å, whereas caffeic acid ((2E)-3-(3,4-dihydroxyphenyl)prop-2-enoic acid) is 77,8 Å making quinic acid much more polar (Kim et al. 2021). Another critical characteristic of caffeic acid is the presence of the five double bonds that enable it to stabilise free radicals by resonance. A higher degree of esterification of quinic acid with a caffeic acid moiety decreases the molecule's polarity and solubility and increases its lipophilicity. TetraCQA is in consequence a better scavenger of free radicals than triCQA, diCQA or monoCQA in that order. CQAs bioactivity is potentially influenced by their degree of esterification (Liang et al. 2019), making highly substituted CQA more desirable in industry and medicine but also more prone to alterations during extraction.

1.4.2 Identifying CQAs isomers and the difficulties that come with it

The major problem when it comes to CQAs is correctly identifying and extracting them. In the literature, there is a lot of misunderstanding over which CQA

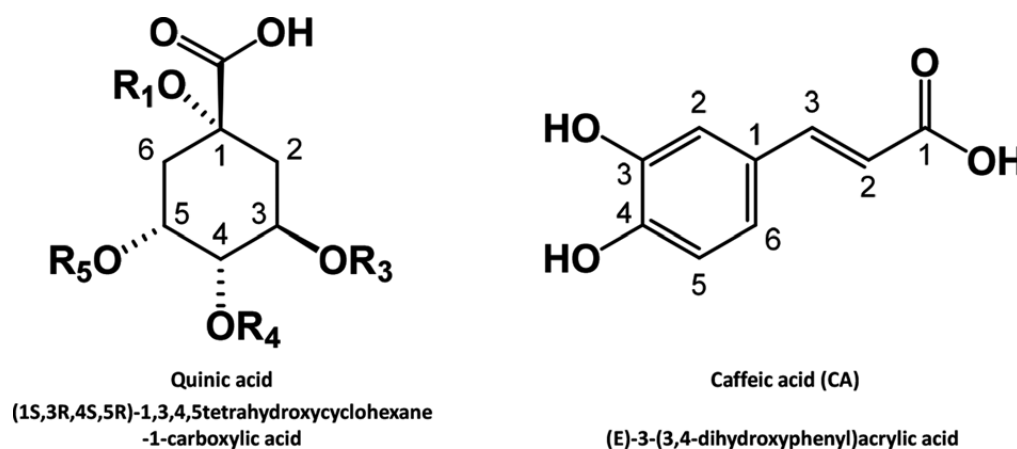


Figure 1.23 – Quinic and caffeic acids. Figure from [Alcázar Magaña et al. 2021](#).

isomers are detected. This is the result of a buildup of diverse problematics. The first one is the instability of CQAs. They are very prone to degradation, *cis* and *trans* isomerisation and acyl migration due to their sensitivity to UVs, heat and pH ([Xue et al. 2016](#); [Moglia, Lanteri, et al. 2014](#); [Deshpande et al. 2014](#)).

Xue et al. (2016) illustrated one of these features by exposing CQAs to light during 7 days resulting in significant *cis-trans* isomerisation into what could be assumed as more stable degradation products and isomers. Intramolecular acyl-migration can occur either *in vivo* (in the vacuole) as demonstrated by Moglia et al. (2016) or *in vitro* in heated aqueous solution or with the influence of the pH ([Xue et al. 2016](#); [Deshpande et al. 2014](#)). Another affect of heat is transformation. When heated up, 5-CQA ((1R,3R,4S,5R)-3-(((E)-3-(3,4-dihydroxyphenyl)acryloyl)oxy)-1,4,5-trihydroxycyclohexane-1-carboxylic acid) can transform into a mix of 5-CQA, 3-CQA and 4-CQA as well as the hydrolysis products quinic acid and caffeic acid ([Dawidowicz et al. 2016](#)). Extraction protocols of CQAs must be designed and carried out with great vigilance in regards of this physico-chemical features.

Another considerable issue is the heterogeneous nomenclature used to name mono-, di- and tri-CQAs stemming from the IUPAC ²⁹ rules for denominating molecules. A great deal of confusion arose in the literature between CQA isomers. Moreover, CQA isomers can have very similar physicochemical properties making them difficult to tell apart simply from bioactivity assays, especially in the absence of a commercial standard and given the previous points raised. Alcázar Magaña *et al.* (2021) give striking examples of the inadequacy of the IUPAC rules for CQAs. The main IUPAC nomenclature regarding a multi-substituted ring is to number in the direction that gives the lowest numbers for substituents. If we take the quinic acid molecule illustrated in Figure 1.23, this means that CQAs with substituents on positions 3 and 5 are bounded to be confused for each other in the literature. They give the case of 3-O-CQA & 5-O-CQA for which the IUPAC denominations are highly similar since for both the substituent position is considered to be 3 according to the lowest number rule. The only difference in the denomination is the stereochemistry of position 1 (S or R): 3-O-CQA is denominated (1 $\underline{\text{S}}$,3R,4S,5R)-1-(((E)-3-(3,4-dihydroxyphenyl)acryloyl)oxy)-3,4,5-trihydroxycyclohexane-1-carboxylic acid whereas 5-O-CQA is denominated (1 $\underline{\text{R}}$,3R,4S,5R)-3-(((E)-3-(3,4-dihydroxyphenyl)acryloyl)oxy)-1,4,5-trihydroxycyclohexane-1-carboxylic acid. A similar issue concerns 1,3- and 1,5-diCQA or 1,2,4-triCQA and 1,4,5-triCQA, often mistaken for each other in the literature due to inconsistent naming. To avoid further confusion and ambiguity, it is proposed to use the numbering of the quinic acid moiety for consistent naming across literature, which is also endorsed by the U.S. National Library of Medicine of the NIH ³⁰ (Kim *et al.* 2021).

The physicochemical properties of CQAs have to be taken into account when performing extraction of CQAs to prevent misidentification, alteration of the bioactive agents of a medicinal plant, heterogeneous results between batches or accessions of a same species.

²⁹ IUPAC: International Union of Pure and Applied Chemistry ³⁰ National Institutes of Health

1.4.3 Health benefits of caffeoylquinic acids

The abundance of CQAs in medicinal plants indicates that they may have health benefits for both animals and humans. Indeed, CQAs are highly valuable in agronomic or pharmaceutical applications. Their antioxidant activity is the most looked into, with treatment of Alzheimer or other inflammatory diseases in mind. It also possesses anti fungal and antibacterial activities (Bernard, Alves Dos Santos, et al. 2020). CQAs phenolic hydroxyl groups in a conjugated system make them electron rich molecules. This provides them with good scavenging properties and enables them to act as direct antioxidants.

Because of their antioxidant (Sroka et al. 2003; Bernard, Alves Dos Santos, et al. 2020; Nzekoue et al. 2020; Trendafilova et al. 2020), antibacterial (Bernard, Alves Dos Santos, et al. 2020; Hamed et al. 2020; Nzekoue et al. 2020), and antiviral properties (Ospanov et al. 2020; J.-Y. Park et al. 2012; P. Smith et al. 2016), CQAs have the potential to provide a wide range of health advantages. Beneficial effects in digestion (Gan et al. 2018), diabete-related symptoms (Imai et al. 2020) or in cancer cells inhibition (Kurata et al. 2007; Puanggraphant et al. 2011; Lodise et al. 2019; Murad et al. 2015). The neuroprotective activity of CQAs is being studied extensively due to the potential value of this research for public health (Sasaki et al. 2019; Nzekoue et al. 2020; Metwally et al. 2020), particularly in the case of Alzheimer disease (Nora E Gray et al. 2014; Anggreani et al. 2017; Matthews et al. 2020).

CQAs can act on a hub pathway of oxidative stress regulation by targeting the anti-inflammatory Keap1-Nerf2 pathway, conferring CQAs substantial interest in their use against age-related diseases (Brandes et al. 2020; Liang et al. 2019). Nerf2 is a hub regulator of cellular oxidative stress resistance and a common therapeutic target. The oral consumption of CGA³¹ in rats demonstrated a positive impact on Nerf2 signaling inducing ARE³² genes and thus the indirect production of glutathione and other antioxidant molecules. CQAs and particularly 1,5-diCQA and 3,5-diCQA, exhibit anti-inflammatory and great neu-

³¹ CGA: chlorogenic acid ³² ARE: Antioxidant Responsive Element

roprotective effects in Alzheimer disease notably by inhibiting the cell death of MCG5 and SH-SY5Y neuroblastomata cells (cancer cells developing from nerve cells) induced by β amyloids deposition and activation of the Nerf2 pathway and of mitochondrial genes responsible for electron transport for example (Nora E. Gray et al. 2015). Those neuroprotective effects of CQAs help reduce memory and cognitive deficits in the rat populations studied. Other molecular regulatory systems are influenced by CQA intake, such as Ca^{2+} homeostasis participating in the protection of neurons against glutarate exitotoxicity (Sunghwan Kim et al. 2012; Mikami et al. 2015).

CQAs antiviral effects are receiving renewed consideration in the current climate. Among 2878 compounds tested for TbCet1 inhibition (RNA triphosphatase essential to viral development), tetraCQA was identified as the most potent inhibitor. 3,5-diCQA also exhibited this activity but in a lesser manner by five-fold (P. Smith et al. 2016). Concerning the infamous SARS-Cov2³³, responsible for the ongoing COVID pandemic, it was demonstrated that catechols groups in CQAs may inhibit the papain-like cysteine protease (PL^{pro}) of the virus by interacting with its thiol nucleophile (Osmanov et al. 2020; J.-Y. Park et al. 2012).

Limitations of *in vivo* studies

The presented studies showcase *in vitro* and *in vivo* assays demonstrating the effects of multi-substituted CQAs either in their untransformed form as pure compounds or from intake of CQA rich foods. However, the bioavailability and thus bioactivity of CQAs greatly depends on the processes of absorption, distribution, metabolism and excretion (ADME). When orally supplemented with CGA, caffeic acid or quinic acid, rats showed very low absorption of the untransformed parent compound. Gonthier et al. (2003) found that CGA is hydrolysed in the large intestine by esterases from the gut microbia into caffeic acid and quinic acid that are then further metabolised into respectively *m-*

³³ SARS-Cov2: severe acute respiratory syndrome coronavirus 2

coumaric acid, derivatives of phenylpropionic, benzoic and in hippuric acids. These metabolites represent 57,4 % (mol/mol) of the CGA intake. Such findings raise the questions of the limitations in *in vivo* assays assessing multisubstituted CQAs bioactivity and of the identity of the bioactive compounds responsible for the beneficial effects seen in CQA supplemented dietary studies. Clifford and colleagues (2020) discuss the limitations of such investigations.

This emphasizes the need of research into the bioactivity of CQA breakdown products, without which we would be unable to fully comprehend the mechanisms underlying the health advantages found after CQA consumption. Caffeic acid as well as other degradation products of CQA have been shown to also have neuroprotective, anti-inflammatory and antioxydant effects but further characterization of these compounds bioactivities and bioavailability is needed, taking the focus off of CQAs (Alcázar Magaña et al. 2021).

1.5 Central role of acyltransferases in specialized metabolism

During my thesis, I focused on two metabolites of interest which are conjugates of hydroxycinnamic acids and polyamines or quinic acid respectively: TetraSpm and diCQA. They are both produced by the acylation reaction, which is widespread in the specialized metabolism of plants.

Acyltransferases are involved in the production of several metabolites accumulated in chicory, particularly hydroxycinnamic acids such as CTA ³⁴, diCTA ³⁵, CQA ³⁶ and diCQA ³⁷ (Figure 1.17). The compounds synthesized by acyltransfer on tartaric acid, CTA and diCTA, have been characterized *in vitro* in other accumulating species. CTA synthesis pathway was investigated in *Equisetum arvense* (*Equisetaceae*) revealing that *in vitro* a CTT ³⁸ was responsible for the transfer of a caffeoyl-CoA on tartaric acid and that diCTA was obtained through the action a CCT ³⁹ acylating a second caffeoylCoA moiety on the CTA molecule (H. Hohlfeld et al. 1995). In *Arachis glabrata* (*Fabaceae*), an HTT ⁴⁰ was proven to have a similar biosynthetic mechanism concerning CTA production *in vitro* (M. L. Sullivan 2014). More recently, another HTT was characterized in CTA production in *Echinacea purpurea* (L.) Moench, as well as a HQT in CQA production (Fu et al. 2021). HCT ⁴¹ and HQT ⁴² have also been characterized to be involved in the biosynthesis of CQA and diCQA as discussed in details in the introduction of Chapter 4.

1.5.1 Definition of acyltransferases

Acylation is the transfer of an activated acyl group on an acceptor molecule. It confers to molecules several properties such as solubility or stability which

³⁴ CTA: caftaric acid ³⁵ diCTA: chicoric acid ³⁶ CQA or CGA: chlorogenic acid ³⁷ diCQA: dichlorogenic acid ³⁸ CTT: hydroxycinnamoyl-CoA : meso-tartrate hydroxycinnamoyl transferase ³⁹ CCT: hydroxycinnamoyl-CoA : caffeoyl-meso-tartrate hydroxycinnamoyl transferase ⁴⁰ HTT: hydroxycinnamoyl-CoA : tartaric acid hydroxycinnamoyl transferase ⁴¹ HCT: hydroxycinnamoyl-CoA shikimate/quinate hydroxycinnamoyl transferase ⁴² HQT: hydroxycinnamoyl-CoA quinate hydroxycinnamoyl transferase

can enable them to achieve a storage compatible conformation, influence their transport or activity (Gomez et al. 2009). This reaction can be performed by several enzyme families in vascular plants, the main ones being SCPL⁴³ and BAHD⁴⁴ (Figure 1.24). SCPL are acyltransferases that use glucose esters as acyl donor to catalyze esterification or amidations (Milkowski et al. 2004). A newly discovered SCPL enzyme, EpCAS, was characterized to use caftaric and s as substrates to produce chicoric acid (Fu et al. 2021). The functions of SCPL are thus broader than previously thought.

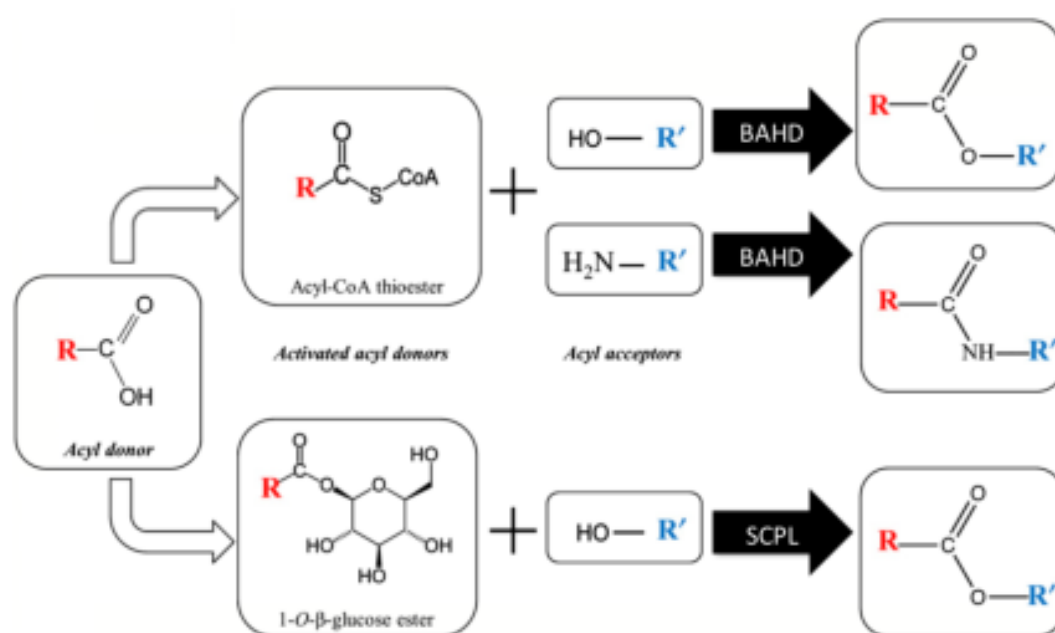


Figure 1.24 – BAHD and SCPL: two superfamilies of acyltransferases and their catalytic activities. Figure from Bonpart and colleagues (Bontpart et al. 2015).

BAHD use thioesters of Coenzyme A as activated donors for similar reactions (St-Pierre et al. 2000). BAHDs represent a huge specialized metabolism enzyme family, behind cytochromes P450, glycosyltransferases and peroxidases (St-Pierre et al. 2000). Their predicted number is between 50 and 120 copies in the

⁴³ SCPL: Serine CarboxyPeptidase-Like ⁴⁴ named after the initials of the first characterized enzymes of the family. BEAT: Benzyl alcohol O-acetyltransferase; AHCT: anthocyanin O-hydroxycinnamoyltransferase; HCBT: N-hydroxycinnamoyl/benzoyltransferase; DAT: deacetylvindoline 4-O-acetyltransferase

genomes of *Arabidopsis thaliana* (*Brassicaceae*), *Medicago truncatula* (*Fabaceae*), *Oriza sativa* (*Poaceae*), *Vitis vinifera* (*Vitaceae*), *Populus trichocarpa* (*Salicaceae*), *Cynara cardunculus* (*Asteraceae*) or *Pyrus bretschneideri* (*Rosaceae*) (D'Auria 2006 ; X.-H. Yu et al. 2009 ; Tuominen et al. 2011; Moglia, Acquadro, et al. 2016 ; C. Ma et al. 2017). This reflects the wide range of family in which they are encountered.

1.5.2 Features of BAHDs

From a genetic and biochemical perspective, a total of 75 BAHD genes have been characterized. Crystallography and site directed mutagenesis of these genes allowed to define common characteristics in this family.

These studies defined the characteristics of BAHD enzymes and provided sufficient knowledge to identify some of them by homology in other species. They possess two conserved motifs, HXXXD and DFGWG (Figure 1.25 A.). They are soluble monomeric proteins with a molecular mass comprised between 45 and 55 kDa and have a globular three dimensional structure constituted of two same size domains linked by a loop. The active site can be found in the space between the two main domains. The active site contains the motif HXXXD, conserved in all BAHDs (Figure 1.25 C.). The histidine residue of this motif has part a strong catalytic role. It could allow the deprotonation of the heteroatom of the acyl acceptor, hence enabling a nucleophile attack on the carbonyl of the acyl donor involved in the thiolester link, the release of the CoA group and the formation of the final conjugated metabolite (Figure 1.25 B.). The DFGWG motif is found towards the C-terminal part of the protein and plays a more structural role by stabilizing the three dimensional conformation of the protein. Its conservation is not ubiquitous among all identified BAHD (D'Auria 2006). BAHDs are involved in the synthesis of a wide array of specialized metabolites: phenylpropanoids, amides, volatile esters, terpenoids, alkaloids, anthocyanins, flavonoids, and acylsugars (D'Auria 2006; Tuominen et al. 2011). However their high diversity in functions impairs our ability to use their sequences to predict a BAHD enzyme function only by homology. This is due to their rapid evolutive history, which has resulted in sequence divergence, substrate

promiscuity, and functional divergence. Some BAHD who share only 40 to 50 % of sequence identity can perform acylation on the same substrates (ASAT⁴⁵ in *Solanaceae* all use sucrose/acetylated sucrose as substrates, Moghe, Leong, et al. 2017) whereas a single amino acid change can affect the substrate preferences of another BAHD (*Coleus blumei* HCT, Levsh et al. 2016). All these factors make the characterization of BAHD enzymes challenging.

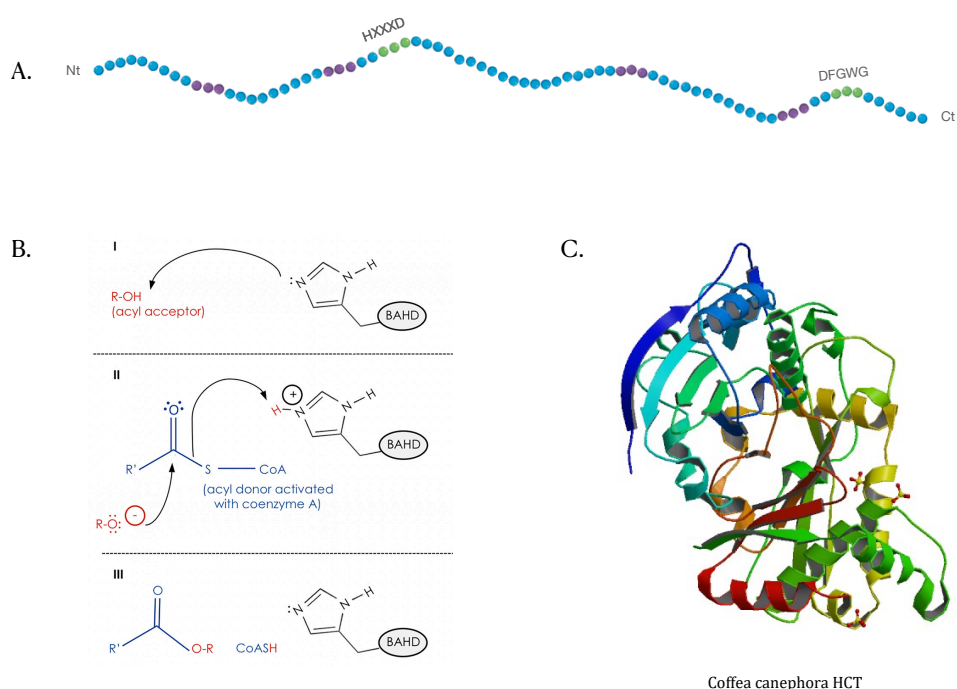


Figure 1.25 – Characteristics of BAHD enzymes.

A. Schematic representation of a BAHD protein with the conserved motifs HXXXD and DFGWG represented.

B. Simplified schematic of BAHDs acyl transfer activity, figure from (Haslam et al. 2017).

C. Structure of a BAHD characterized by crystallography, HCT of *Coffea canephora* (Lallemant et al. 2012).

1.5.3 Phylogenetic analysis of BAHDs

D'Auria and colleagues (2006) realized phylogenetic analysis using 46 biochemically and genetically characterized enzymes which highlighted the exist-

⁴⁵ 4-5 acylsugar acyltransferases

tence of five clades among the BAHD superfamily. Each clade appears to have distinct preferred substrates but these preferences are not always shared by all members of the clade and can also be common to members of other clades.

Clade I members act mainly on flavonoids, particularly anthocyanins.

Clade II encompasses principally CER2-like genes encoding which almost systematically lack the DFGWG motif, questioning the validity of these enzymes as BAHDs.

Clade III demonstrates a diverse array of substrates. It is divided into two subclades. Subclade IIIa BAHDs act in the synthesis of alkaloids such as vindoline, thebaine, cocaine. However other members can also catalyze the production of VOCs or hydroxycinnamoyl-spermidine. More than half of putative BAHDs identified in chicory belong to this clade (Delporte 2017). Subclade IIIb has only one characterized member involved in the synthesis of coniferylacetate (Dexter et al. 2007).

Clade IV was initially thought to be exclusive to monocotyledons with a member acting on agmatine as an acyl acceptor (Tuominen et al. 2011) but this was later contradicted by the identification of a clade member in *N. attenuata* (*Solanaceae*) with putrescine as an acyl acceptor (Onkokesung et al. 2012). Some studies classify it as adjacent to subclade Vb because of the similarities with the catalytic activities of SHTs present in this subclade.

Clade V has very diverse substrates including terpenoids, quinic acid, polyamines or alcohols with medium chain. The use of benzoyl-CoA as an acyl donor seems specific to this clade while hydroxycinnamoyl-CoA have been described as acyl acceptor in other clades. Subclade Va is composed of BAHDs catalyzing the production of VOCs including benzoate esters. It also includes SCT⁴⁶ and SDT⁴⁷ of *Arabidopsis thaliana* which are involved in the production of conjugates of the free polyamine spermidine (Luo, Fuell, et al. 2009). Subclade Vb members include SHTs as previously mentioned but also HCTs and HQTs. All members of this subclade are acyltransferases acting on hydroxycinnamic acids with various acyl acceptors. Acylation is performed on hydroxyl or amino groups.

⁴⁶ SCT: spermidine dicoumaroyl transferase ⁴⁷ SDT: spermidine disinapoyl transferase

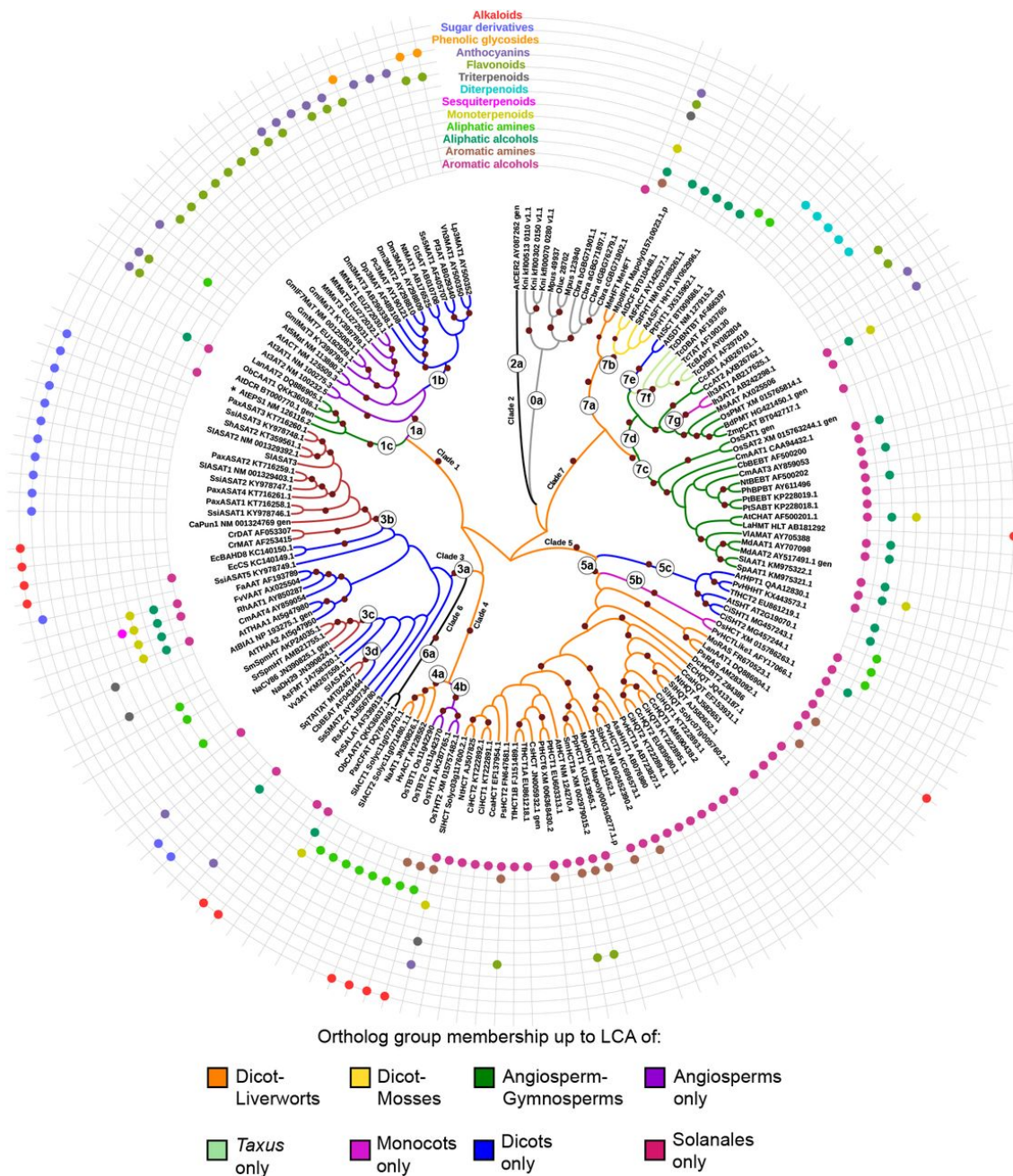


Figure 1.26 – Phylogenetic analysis of BAHD superfamily clades associated with substrates promiscuity by Kruse and colleagues (2020).

Legend is adapted from the original paper.

The tree was rooted using the algal enzyme clade (Clade 0a). Maroon circles on branches refer to clades with bootstrap values > 70. Clades were first defined based on deepest, high-confidence monophyletic clades.

The substrate promiscuity of BAHD complicates their characterization. *In vitro*, they are more promiscuous towards different substrates than *in planta*, where they are limited by the substrate availability. Heterologous expression can then be misleading in their characterization, and such studies must be considered these factors in mind (D’Auria 2006; Kruse et al. 2020).

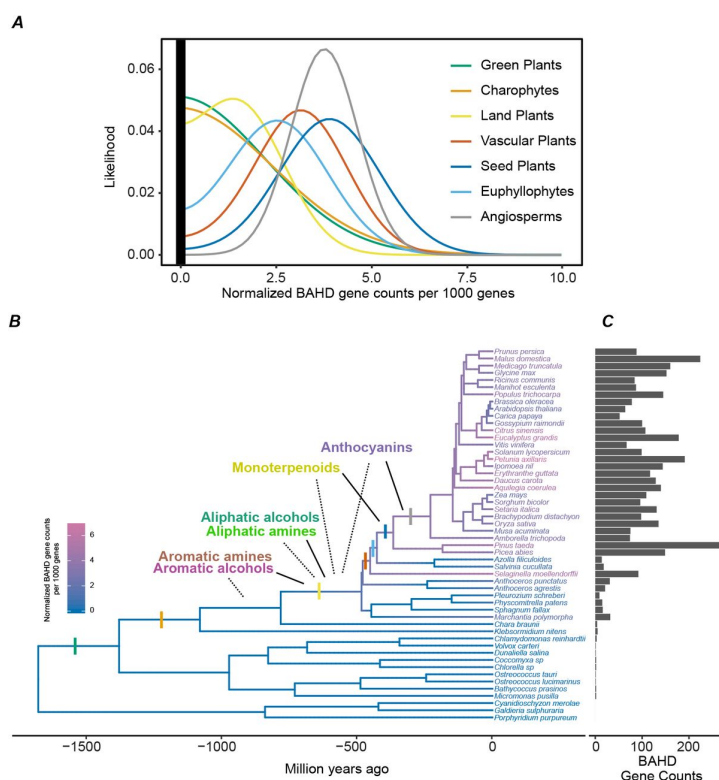


Figure 1.27 – Distribution of BAHD acyltransferases across plant species by Kruse and colleagues (2020).

Legend is adapted from the original paper.

A. Likelihood of the number of BAHDs per 1000 genes in the genome of different phylogenetic groups, as per the bound Brownian motion model.

B. Species tree of the analyzed species with sequenced genomes. One can note that the use of amines as acyl acceptors is established early in the evolution history of BAHDs.

Recently, Kruse and colleagues performed an in-depth study on BAHD enzymes promiscuity characteristics and used this superfamily as a model for fast evolving enzyme superfamily (Kruse et al. 2020). They redefined the clades established by D’Auria and colleagues (2006): instead of five clades, they divided the BAHDs into seven clades with clades I through IV remaining as previously

defined. The members of clade V were redistributed into three new clades (V to VII). Three subclades are particularly well conserved from angiosperms to liverworts: subclades IVa, Va and VIIa. Subclade IVa contains polyamine acyltransferases, subclade Va HCT/HQT enzymes and subclade VIIa alcohol acyltransferases (Figure 1.26). These phylogenetic results combined with the patterns in the enzymatic assays in substrates promiscuity they performed suggest that acylating aliphatic and aromatic alcohol and amine clades are the most ancestral and first to have been established in land plants (Figure 1.27).

1.5.4 Identified BAHDs in Chicory

The definition of conserved characteristics in BAHDs allowed for the identification of 81 putative BAHDs in chicory, but none belonged to clade II (Figure 1.28; [Delporte 2017](#)). Seven of the putative BAHDs belong to the Vb subclade, which encloses all of the BAHDs that catalyze the transfer of hydroxycinnamic acid on various acceptors. From those genes of interest, five were characterized by Guillaume Legrand ([2016](#)): 2 *HCTs* and 3 *HQTs*. The last two were annotated as SHTs⁴⁸ and named *CiSHT1* and *CiSHT2*. Their characterization was initiated by Marianne Delporte during her thesis ([2017](#)) and pursued during Guillaume Bernard' thesis ([2018](#)) as well as in the present work (Figure 1.28). However the work of Kruse and colleagues questions the validity of this Vb subclade. It is possible that this Subclade is actually be a combination of different clades some of them enclosing the characterized *HCTs* and *HQTs* and an other containing the SHTs.

⁴⁸ *SHT*: Spermidine Hydroxycinnamoyl Transferase

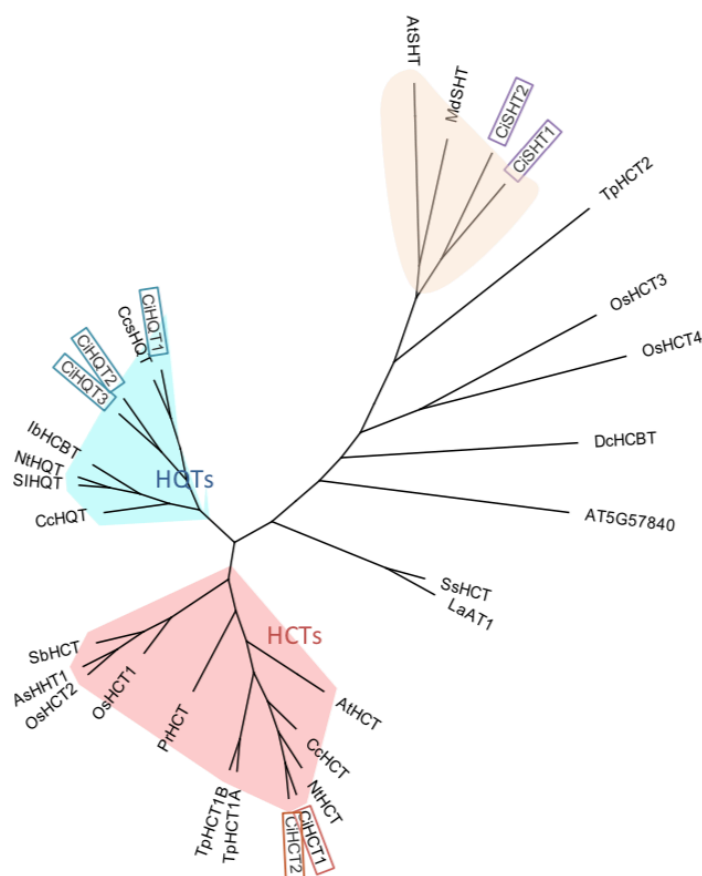


Figure 1.28 – Clade Vb of BAHD superfamily, figure from [Delporte 2017](#).

1.5.5 BAHDs are not the only option

BAHDs are good targets when attempting to find a gene encoding an acyltransferase due to their widespread presence. Nevertheless, possible acyltransferases present in other enzyme families should also be considered. Acylation was also described in other enzymes like GDSL-lipase like (more specifically GELP ⁴⁹; [Chepyshko et al. 2012](#)), as discussed in length in the introduction of Chapter 4 and in GNAT ⁵⁰ enzymes. The enzymes in the GNAT superfamily catalyze acylation from an acyl-CoA moiety to a variety of substrates (aminoglycosides,

⁴⁹ GELP: GDSL esterase/lipase protein ⁵⁰ GNAT: Gen5-related N-acetyltransferases

histones, arylalkylamines, decarboxylated amino acids, or polyamines) and are present in all domains of life (Vetting et al. 2005; Salah Ud-Din et al. 2016). Primarily studied in bacteria, they are also found in plants in which 17 members with diverse functions have been described (Roumani, Besseau, et al. 2021). The GNAT superfamily notably encompasses the DAT⁵¹ or SSAT⁵² and THT⁵³ families. DAT have polyamine acceptors (Pegg 2008; Lou et al. 2016) or hydroxycinnamoylation (Facchini et al. 2002). THTs are able to use tyramine as an acyl acceptor and have been mainly characterized in *Solanaceae* (H. Hohlfeld et al. 1995; J. Negrel and F. Javelle 1997; A. Schmidt et al. 1999; Farmer et al. 1999; Von Roepenack-Lahaye et al. 2003; K. Kang et al. 2009).

To this day, a comprehensive view of enzymatic families containing members with acylation abilities includes not only BAHD and SCPL, as previously thought (Bontpart et al. 2015), but also GELP and GNAT (Figure 1.29). Recently, an exhaustive review of N-hydroxycinnamoyl transferases was published, which gives a good overview of the various acyltransferases that exist and their versatility (Roumani, Besseau, et al. 2021).

⁵¹ DAT: diamine N-acyltransferase ⁵² SSAT: spermidine/spermine N1-acyltransferase

⁵³ THT: tyramine hydroxycinnamoyltransferase

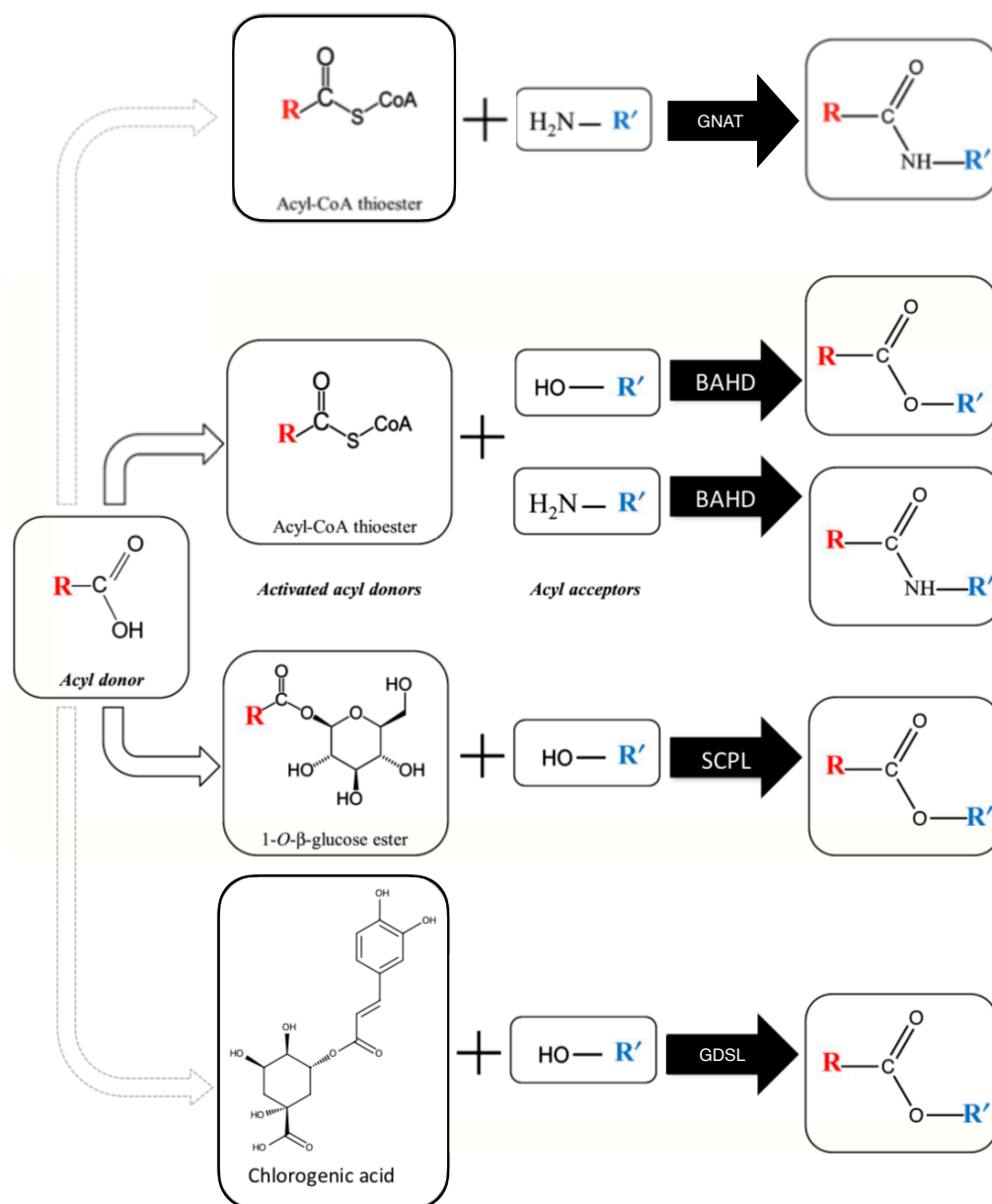


Figure 1.29 – Enzymes families in which acyltransferase activity was characterized, adapted from (Bontpart et al. 2015).

1.6 Objectives of this thesis

The main goals of my thesis were to contribute to the biochemical and physiological characterization of the enzymes involved in the biosynthesis of hydroxycinnamic acid conjugates of interest accumulated in chicory.

Ultimately, the goals of these researches are to be able to reconstruct such pathways in heterologous models in order to produce higher amounts of the molecules of interest and to understand their physiological roles through over-expression or mutagenesis. This would facilitate the study of the properties of those specialized metabolites *in vitro* and learn more about their roles for potential industrial applications. Moreover, unravelling their properties could shed light on their putative roles and functions *in planta*.

The first molecule I focused on is TeraSpm, found in the pollen coat of chicory. The goals were to unravel the mechanisms leading to its production and involving *CiSHT1* and *CiSHT2* as well as to deepen our understanding of the catalytic properties of SHTs and their evolutionary origin. Three aspects were explored by: (1) deciphering and validating this molecule's biosynthetic pathway *in vivo* (Chapter 2), (2) unraveling their evolutive history and relationship with the availability of free polyamine substrates and their peculiar activity (Chapters 2 and 3), and (3) identifying the catalytic sites required in their respective activities and substrate affinities (Chapter 3).

My second molecule of interest is isochlorogenic acid, accumulated in the roots of industrial chicory. The main objective was to characterize diCQA metabolism in chicory. These two objectives were addressed by: (1) studying the function of GDSL enzymes, our target genes for diCQA synthesis in chicory and (2) unraveling their evolutive history and their phylogenetic connection of GDSL with similar roles in other species (Chapter 4).

I used molecular biology, genetic engineering, biochemistry, and phylogenetic approaches to determine the properties and evolutive history of the enzymes involved in the biosynthesis of the natural products of interest.

Roles of CiSHT1 and CiSHT2 in the production of the fully substituted phenolamide tetracoumaroyl-spermine

2.1 Introduction

The known properties and biosynthesis of phenolamides have been reviewed in the introduction of this work, as well as their importance in the pollen grain (see section 1.3.1). Phenolamides constitute one of the classes of molecules accumulating in chicory with potential revalorization interest, but for which work remains to be done to unravel their full physiological properties, especially concerning TetraSpm. To that extent, the deciphering of its biosynthetic pathway within *C. intybus* is essential.

2.1.1 Putative activities of tetracoumaroyl-spermine

As previously mentioned, polyamines play an important role in several developmental steps of the pollen grain. Phenolamides could therefore be thought

of as a storage pathway to modulate the contents of free polyamines (Aloisi et al. 2016). However, this is not entirely consistent with studies conducted with *Arabidopsis*. The *Atsht* KO mutant, in which no trihydroxycinnamoyl-spermidine accumulates, exhibits phenotypic changes in the pollen grain structure, but with no effect on its fertility or resistance to UV-B (Figure 2.1; Grienenberger, Besseau, et al. 2009).

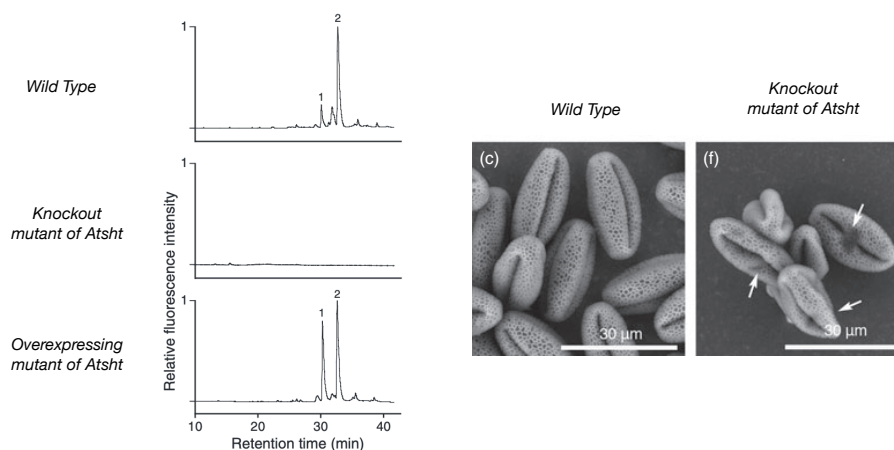


Figure 2.1 – Characterization of the pollen grain of *atsht* knockout (KO) mutant. Figures adapted from Grienenberger, Besseau, et al. 2009.

Right : Pollen wash from wild-type, *atsht* KO mutant and *atsht* KO mutant complemented with overexpressing *atsht* analyzed by HPLC.

Left : Comparison of wild-type and KO mutant pollen grains by scanning electron microscopy.

This experiment suggests that these compounds may play a structural role in the pollen grain. The content of free polyamines in the mutant pollen grain was unaffected, indicating the presence of an efficient homeostasis system, which is likely related to the importance of polyamines in pollen grain development (Fellenberg, Ziegler, et al. 2012). Several types of activities were discovered in *in vitro* assays of phenolamides extracted from pollen. Overall, their putative oxidative properties have been linked to antioxidative and antimicrobial properties, as well as a role in desiccation protection, germination, and stigate-pollen recognition (Ohta et al. 2007; Grienenberger, Besseau, et al. 2009; Elejalde-Palmett et al. 2015; Fellenberg and Vogt 2015; Delporte 2017; Vogt 2018; Kyselka et al. 2018; Huifang Zhang, R. Liu, et al. 2020). Recently antioxidative activity from

phenolamides was observed in pollen samples on bees (Huifang Zhang, R. Liu, et al. 2020).

The pollen of *Asteraceae* species is of particular interest. The reproductive organs of *Asteraceae* are well adapted for entomophilous pollination by specialist bees, allowing for simultaneous pollination of several flowers thanks to their organization in umbel inflorescences (Figure 2.2; Praz et al. 2008; Vanderplanck et al. 2017). It, however, restricts the range of insects able to pollinate *Asteraceae*.

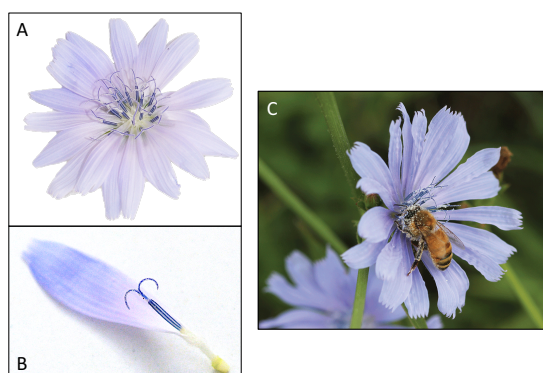


Figure 2.2 – *Asteraceae* reproductive organs specificities.

A and B: umbel and flower of *Cichorium intybus* (personal photos of Marianne Delporte)

C. bee visiting a *C. intybus* inflorescence (<http://flower.gainfx.us/flowers-and-bees/>)

Moreover, their pollen composition in polyamines and phenolamides is unique: spermine predominates over spermidine conversely to other Eudicotyledons. Phenolamides in the pollen of *Helianthus annuus* (*Asteraceae*) show antifungal properties, more specifically *p*-coumaroyl-putrescine hydrochloride, feruloyl-putrescine hydrochloride, N,N'-di(*p*-coumaroyl)-putrescine, di- and tri-*p*-coumaroyl-spermidines (Kyselka et al. 2018). It had also previously been demonstrated that specialist bees consuming *Asteraceae* pollen were more resistant to parasitism (Spear et al. 2016).

TetraSpm could thus be essential for *Asteraceae* pollen in various and non exclusive ways, but this natural compound may also be valued in human health. For instance it accumulates in the pollen during floral development, and especially ontogenesis, of a known medicinal *Asteraceae*, *M. chamomilla* (Eliasová et al.

2012). Its antioxidant activity is an obvious starting point (Ohta et al. 2007), but it has also been identified as a potent antagonist in two disease-related pathways. It is the first natural tachykinin NK1 receptor antagonist, a receptor involved in troubles such as depression, anxiety, insomnia but also in certain cancers (A. Yamamoto et al. 2002; S. Park et al. 2017). Its synthesized form was also reported as a strong inhibitor of HIV-1 protease (C.-M. Ma et al. 2001). Those putative health benefits enhance the importance of elucidating the biosynthetic pathways of such a potent molecule.

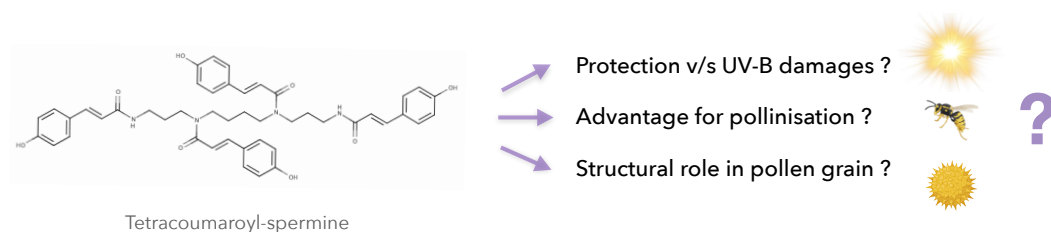


Figure 2.3 – Putative roles of tetracoumaroyl-spermine.

The conservation of tetrahydroxycinnamoyl-spermines in *Asteraceae*, as well as all of the potential functions *in planta* (Figure 2.3) and in health of fully-substituted phenolamides, demonstrates their importance for the plant and underlines the importance of elucidating all of their activities. This can only be accomplished by first deciphering their biosynthetic pathways in model species like *C. intybus*.

2.1.2 Characterized pathways of fully substituted phenolamides of aliphatic amines

The biosynthetic pathway of phenolamides is at a crossroad with polyamine and phenylpropanoid metabolisms (Bassard, Ullmann, et al. 2010) and its final step is performed by varied N-hydroxycinnamoyl transferases leading to their great diversity (Bassard, Ullmann, et al. 2010; Elejalde-Palmett et al. 2015; Figure 2.4).

Spermidine and spermine, backbones of the phenolamides of interest, are aliphatic polyamines with 3 or 4 amino groups, respectively. Their intracellular concentrations range from hundreds of micromolar to a few millimolar (Bassard, Ullmann, et al. 2010). When conjugated to HCA, they form phenolamides or HCAA. Their two biosynthetic pathways are conserved across the kingdoms of bacteria, animals and plants (Kusano et al. 2008). They originate from ornithine decarboxylated into putrescine by ODC¹, a polyamine with 2 amino groups, or from L-arginine (Figure 2.4). L-arginine can be either converted into agmatine by ADC² with is then hydrolyzed by AIH³ and CPA⁴ successively to produce putrescine. Another pathway is the reconversion of L-arginine into ornithine by ARG⁵. Once putrescine is produced, it can be converted into spermidine and spermine by SPDS⁶ and SPMS⁷ subsequently (Figure 2.4).

On the other hand, activated HCA derive from the amino acid Phenylalanine (Figure 2.4). Through the action of PAL⁸, Phenylalanine is converted into cinnamate, precursor of *p*-coumaric acid by C4H⁹. *p*-coumaric acid is the precursor of caffeic and ferulic acids through the successive actions of C3H¹⁰ and CCoAMT¹¹. The activated HCA can be obtained either *via* the action of 4CL¹² on their respective precursor, or by using *p*-coumaroyl-CoA as a precursor and the actions of C3H and CCoAMT (Figure 2.4).

¹ ODC: ornithine decarboxylase ² ADC: arginine decarboxylase ³ AIH: agmatine imidohydrolase ⁴ CPA: carbamoylputrescine amidohydrolase ⁵ ARG: arginase ⁶ SPDS: spermidine synthase ⁷ SPMS: spermine synthase ⁸ PAL: phenylalanine ammonia lyase
⁹ C4H: cinnamate 4-hydroxylase ¹⁰ C3H: cinnamate 3' hydroxylase ¹¹ CCoAMT: caffeoyl-CoA o-methyltransferase ¹² 4CL: 4-coumaroyl-coenzyme A ligase

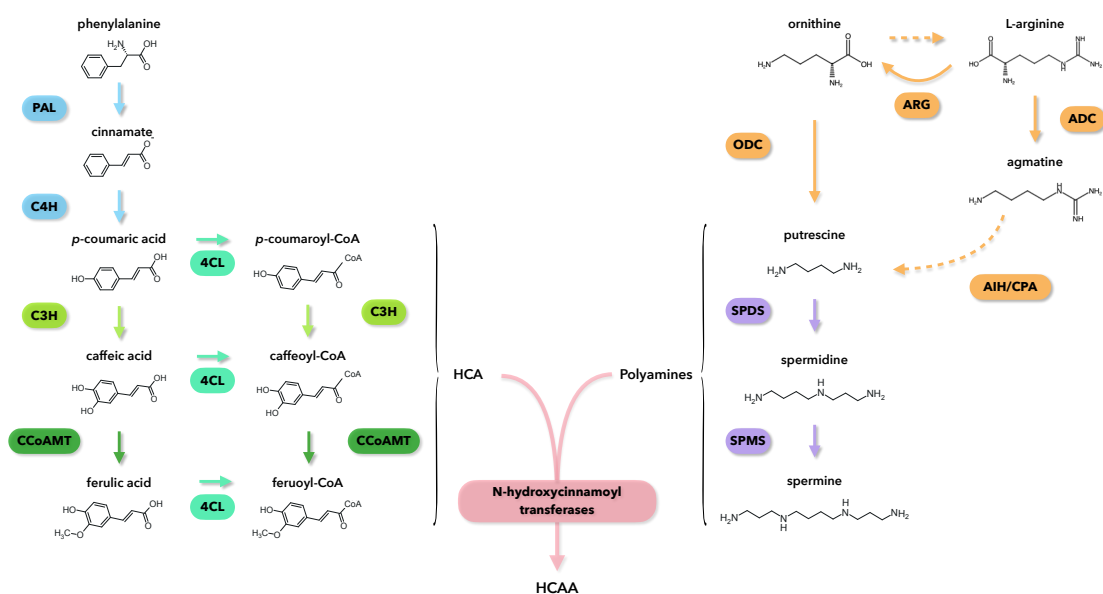


Figure 2.4 – The biosynthesis of HCAA is at the crossroads of HCA and polyamines synthesis.

Arrows represent synthesis and conversion. Arrows in dotted lines represent multiple steps of synthesis and conversion.

4CL: 4-coumaroyl-coenzyme A ligase; ADC: arginine decarboxylase; AIH: agmatine imino-hydrolase; ARG: arginase; C4H: cinnamate 4-hydroxylase; C3H: cinnamate 3' hydroxylase; CCoAMT: caffeoyl-CoA o-methyltransferase; CPA: carbamoylputrescine amidohydrolase; HCA: hydroxycinnamic acid; HCAA: hydroxycinnamic acid amides ODC: ornithine decarboxylase; PAL: phenylalanine ammonia lyase; SPDS: spermidine synthase; SPMS: spermine synthase.

These polyamines and HCA can be combined into HCAA of great variety thanks to the actions of diverse N-hydroxycinnamoyl transferases with diverse substrate preferences.

Phenolamide production pathways have some master regulators such as MYBs; known to also regulate HCTs expression. MYB8 was identified as a hydroxycinnamic acid transferases regulator in *N. attenuata* as well as in other species (Onkokesung et al. 2012 ; Jingying Liu et al. 2015). The phytohormones jasmonate and ethylene signaling pathways are also regulators of agmatine derivative contents through the positive regulator ORA59 of a ACT¹³ in *Arabidopsis thaliana* (J. Li et al. 2018).

¹³ ACT: agmatine coumaroyl transferase

In general, phenolamides can be found in all tissues either constitutively or in response to biotic or abiotic constraints (see section 1.3.2.a). However, fully substituted phenolamides of aliphatic amines (more particularly spermidine and spermine) are specifically expressed in the pollen coat. These compounds are synthesized in the tapetum through the action of SHTs, conjugating the activated hydroxycinnamic acid on the polyamine via N-acylation. This was first characterized in *A. thaliana* in which the production of the several derivatives involves also P450 cytochromes, AtSM1 (O-methyltransferase) that hydroxylates and methylates the phenolic moieties to produce from tricoumaroyl /caffeoyl /feruoyl-spermidine trihydroxyferuoyl-spermidine and dihydroxy-feruoyl-sinapoyl-spermidines (Figure 2.5) (Matsuno et al. 2009; Fellenberg, Milkowski, et al. 2008). This multistep biosynthesis of a variety of phenolamides derivatives seems to be specific to *Brassicaceae* in which the polyamines are substituted with a greater variety of hydroxycinnamic acids than in other families. In most species, the range of acyl donors for substitutions is much narrower and mainly comprise caffeoylCoA and coumaroylCoA (Elejalde-Palmett et al. 2015). This suggests that the acyl donor's nature may be less important than the number of substitutions in the physiological role of these compounds.

Whereas trihydroxycinnamoyl-spermidine is considered as a marker of most Eudicotyledons pollen coat (Elejalde-Palmett et al. 2015), tetracoumaroyl-spermine is a fully substituted HCAA unique to the pollen of *Asteraceae* (Delporte, Bernard, et al. 2018). To this day the biosynthetic pathway of this unusual pollen marker is investigated in only one model, *C. intybus*.

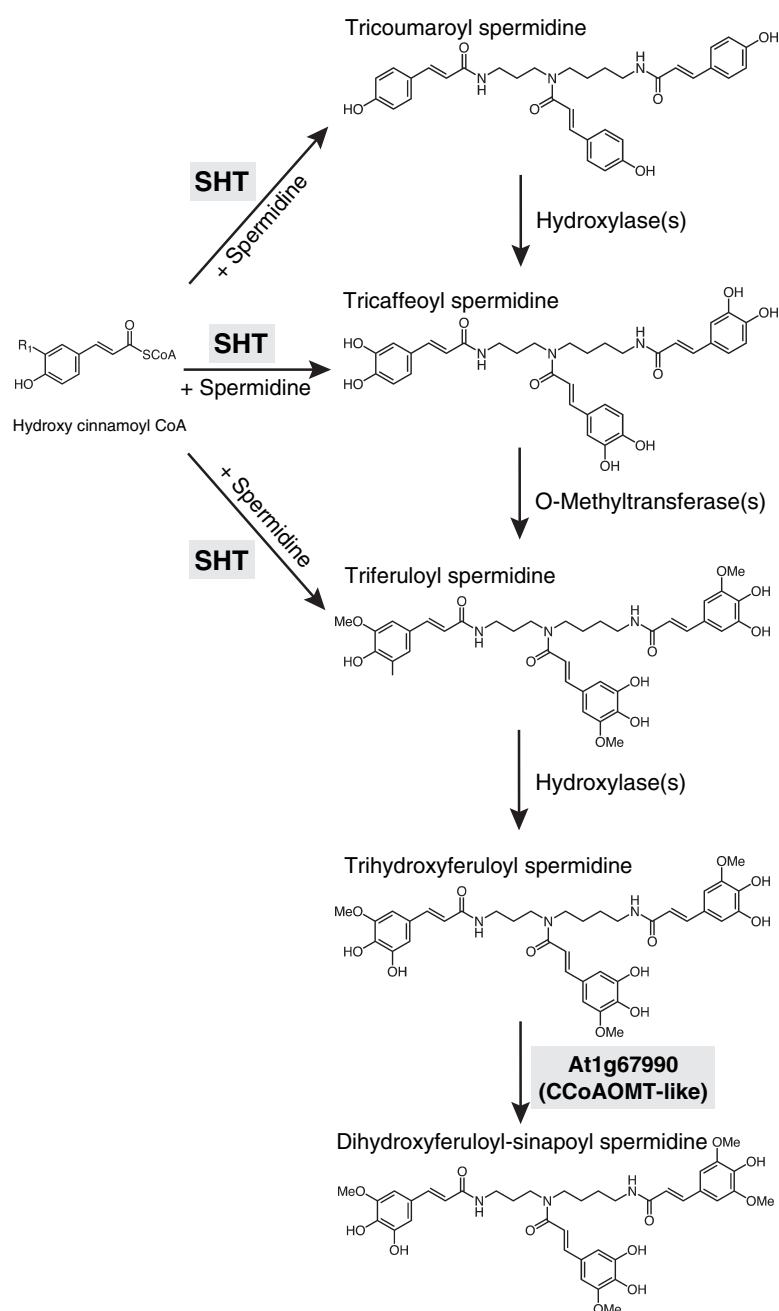


Figure 2.5 – Biosynthetic pathway of spermidine conjugates in *Arabidopsis thaliana* flowers. Figure and legend adapted from Grienenberger, Besseau, et al. 2009.

The three acyltransferase reactions catalyzed by spermidine hydroxycinnamoyl transferase (SHT) *in vitro* are indicated. At1g67990 is involved in the last O-methylation step. Other unknown methyltransferases may be involved in O-methylation of tricafeoyl spermidine.

2.1.3 Identification and characterization of target genes

Until our team's work, the identification and characterization of enzymes capable of catalyzing the acylation of secondary amino groups to produce fully substituted spermidines had only been done in two models: *A. thaliana* and *Malus domestica* in which SHTs were characterized for this reaction (Grienenberger, Besseau, et al. 2009; Elejalde-Palmett et al. 2015). The presence of this unusual fully substituted phenolamide with a spermine backbone in *C. intybus* pollen coat led the team to search by sequence homology of *AtSHT* homologues in chicory which could catalyze a similar acylation. Some N-acyltransferases were not considered as target genes in our pathway such as *SCT*¹⁴, *SDT*¹⁵ or *DH29*¹⁶ because their encoded proteins only acylated primary amine functions (Luo, Fuell, et al. 2009). Among the identified BAHD defined in chicory by our team, we identified two *CiSHTs* in *C. intybus* with high homology to *AtSHT* (53% and 57% for *CiSHT1* and *CiSHT2* respectively) (Delporte, Bernard, et al. 2018). We defined them as our target genes.

The presence of two copies of *SHTs* in chicory was quickly taken as a lead to explain the production of this unusual pollen marker. As previously mentioned, Tetraspm is accumulated in chicory floral buds as a major HCAA compound. The phenolic extraction of each floral part revealed it was highly accumulated in the androecium and the pollen coat (Delporte 2017). A correlation between the expression of the two *CiSHT* genes and the production of TetraSpm in the floral buds of chicory was evidenced (Figure 2.6 A., Delporte, Bernard, et al. 2018). However *in vitro* assays revealed that *CiSHT1* had only a very weak activity. This was confirmed in *in vivo* experiments in which the individual genes were overexpressed (Figure 2.6 B.). Overexpression of *CiSHT1* in either *atsht* KO *A. thaliana* floral buds or in chicory Hairy Roots demonstrated that no phenolamides accumulated in its presence, whereas overexpression of *CiSHT2* led to the production of intermediate substituted spermines and spermidines but not the fully substituted spermine (Figure 2.6 B.). The implication of *CiSHT1* and *CiSHT2* in TetraSpm production was not clearly established.

¹⁴ SCT: Spermidine DicoumaroylTransferase ¹⁵ SDT: Spermidine DisinapoylTransferase

¹⁶ DH29: spermidine acyltransferase

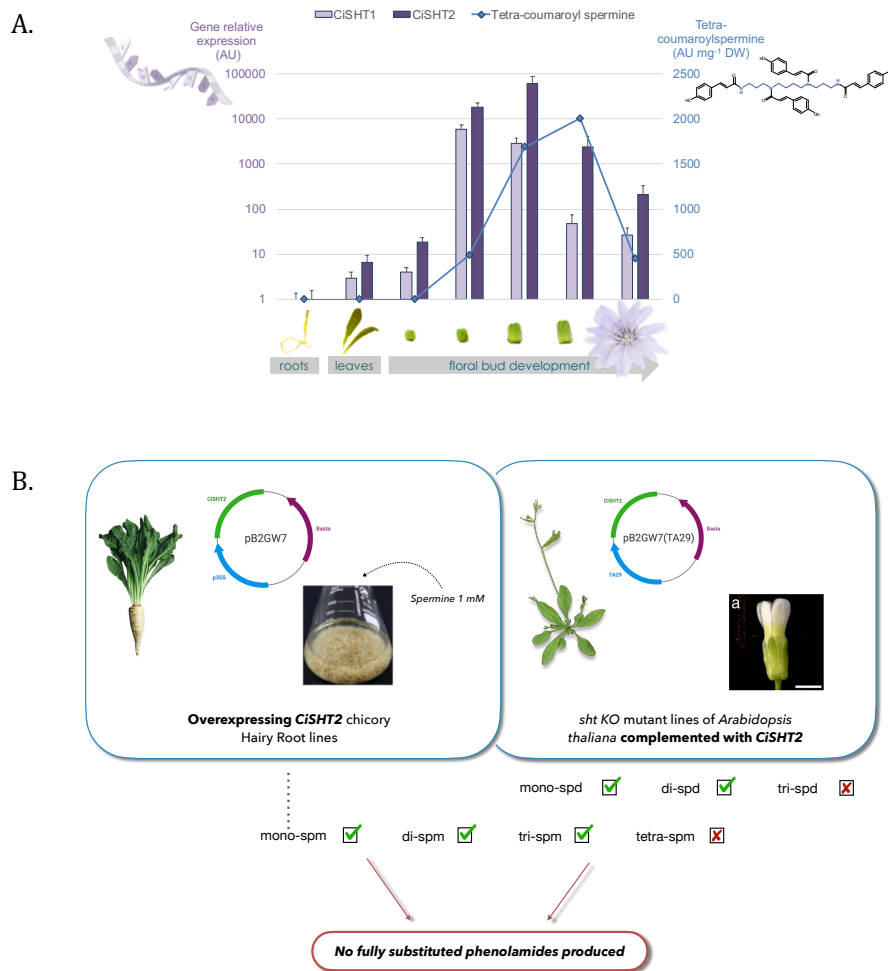


Figure 2.6 – Characterization of *CiSHT1* and *CiSHT2* in TetraSpm biosynthesis by Marianne Delporte and Guillaume Bernard.

A. Figure from Delporte, Bernard, et al. 2018. Correlation between the expression of *CiSHT1* and *CiSHT2* (qPCR) during the floral bud development with the accumulation of the metabolite of interest: TetraSpm.

B. Figure adapted from the results of Delporte, Bernard, et al. 2018. Overexpression of *CiSHT2* in two *in vivo* models and visual summary of the metabolic analysis.

2.1.4 Objectives of this Chapter

In order to investigate further the roles of *CiSHTs in planta* and unravel the molecular mechanisms leading to TetraSpm production, several strategies were used. They are presented in this Chapter under the form of an article recently submitted to *Plant Physiology* (november 2021) and additional elements.

Guillaume Bernard created hairy root KO-lines¹⁷ of *CiSHTs* (either single KO or double KO) using the CRISPR-Cas9 technology (see section A, Appendix A; [Bernard, Gagneul, et al. 2019](#)). The metabolic analysis of the regenerated KO plants provided new insights regarding the biosynthetic pathway of TetraSpm. *CiSHT1* was until then considered as non active in this pathway but these experiments unveil a higher importance of this gene in the pathway. They suggested that the successive actions of *CiSHT2* and *CiSHT1* led to TetraSpm production.

Therefore, to validate this hypothesis *in vivo*, both *CiSHTs* were overexpressed in two heterologous systems alone or combined (see section A). In both models used, *N. benthamiana* leaves and *Saccharomyces cerevisiae*, this hypothesis was confirmed.

The additional elements of this Chapter focus on the influence of the polyamine ratio on SHTs activity, already touched upon in the presented article. Indeed, the effect of polyamine availability on *CiSHTs* enzyme activity *in planta* is one of the main questions raised by our research. The starting point of this interrogation is the shift in the polyamine ratio between Eudicotyledons and *Asteraceae*. From an evolutionary point of view, one can wonder if the ratio shift between spermine and spermidine was the initiating event that allowed positive selection of the two duplicates of *SHTs* in *Asteraceae*. Therefore, activities of SHTs could be influenced by the polyamine available as acyl acceptors. The second hypothesis that can be contemplated is that SHT enzymes have an intrinsic substrate preference for acyl donors and acceptors. Accordingly, their activity would not be influenced by a modification of the available polyamine ratio. To test this hypothesis

¹⁷ KO: Knock-Out

a double complementation of *Atsht* KO mutants already complemented with *CiSHT1* was performed (Delporte 2017) to observe if the combined activity of both *CiSHTs* would lead to production of spermine or spermidine derivatives in this *in planta* system. The complementation of *Cishts* KO mutant chicory hairy root lines was also performed but the transformed lines have not yet been analyzed.

2.2 Four in a row: the dual action of two BAHD acyltransferases is required to promote tetra-hydroxycinnamoyl spermine accumulation in chicory flower buds

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Short title: Phenolamide synthesis in chicory pollen coat requires two SHTs

One sentence summary: Complete acylation of spermine in chicory flower buds requires the successive action of SHT2, which acylates the secondary amines of

free and monosubstituted spermine, and SHT1, which performs the two final acylation steps.

Author Contributions

C.R. and D.G. designed the experiments. G.B., J.B., M.D., R.M., A.B., A.W., J-X.F, D.M., S.Ber., S.Bas. performed the experiments and analyzed the data. G.B. and D.G. wrote the article and JB, M.D., A.B., S.Bes., R.M., A.Q., J.L.H. and C.R. contribute to writing and revised the manuscript. D.G. supervised the project.

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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 required the successive action of two enzymes, i.e. CiSHT1 and CiSHT2, two members of the BAHD enzyme family. Deletion of these genes in chicory using the 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* the synthesis and accumulation of the fully substituted spermine. Structural characterization of the main product of CiSHT2 using NMR revealed that CiSHT2 preferentially catalyzed N-acylation of secondary amines to form N⁵,N¹⁰-dicoumaroylspermine, whereas CiSHT1 would use this substrate to

synthesize tetracoumaroyl spermine. Interestingly, we showed that spermine availability may be a key determinant towards preferential accumulation of spermine derivatives over spermidine derivatives in chicory. Our results brought to light a neofunctionalization among the SHTs that was accompanied by a modification of free polyamine metabolism that has resulted in the accumulation of this new phenolamide in chicory and most probably in all *Asteraceae*. Finally, genetically engineered *Saccharomyces cerevisiae* was proven to be a promising host platform to produce these compounds.

Keywords: tetracoumaroyl spermine, *Cichorium intybus*, BAHD acyltransferases, metabolic diversification, SHTs.

2.2.1 Introduction

Plants accumulate a wide array of specialized metabolites. Historically defined as opposed to primary metabolites, these molecules have long been called secondary metabolites because they were often considered as evolutionary enigma or even waste products (Hartmann 2007). Primary metabolites, *i.e.* central metabolites, referred to molecules that are required for the survival of the host and which biosynthesis is fairly well conserved in all living organisms. Specialized metabolites 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 interactions (Pichersky and Lewinsohn 2011). Therefore, their synthesis 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 chemical diversity occurring in the biosphere (Pichersky and Lewinsohn 2011). Thus, terrestrialization of the green lineage is often associated with the emergence of these new molecules (Jing-Ke Weng 2014). They are involved in various ecological functions notably plant interaction with its environment, for example, pollinator attraction, symbiotic microorganism attraction, defense against pathogens and herbivores or environmental 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 random mutations (Chae et al. 2014). These genetic events occurring in the open reading frames or/and in the regulatory parts of the genes can promote the appearance of new biochemical paths leading to new metabolites associated with new functions. This evolutionary scenario is called neofunctionalization if one gene retains its ancestral function whereas the other evolves to promote a new function or subfunctionalization if both genes experience mutations and participate to a new function while contributing together to the maintenance of their ancestral function (Kroymann 2011; Jing-Ke Weng 2014; Moghe et al. 2015). Another model to explain appearance of new metabolic pathways is escape from adaptive conflict (Kroymann 2011; Moghe et al. 2015). In this model, a single gene evolved a new function at the expense of the ancestral function. This give rise to an adaptive conflict that is solved through gene duplication (Kroymann 2011). Most of the recruitments known to date are thought to be due to neofunctionalization (Moghe et al. 2015). In contrast to primary metabolism, in which selection results in enzymes with high efficiency and strict substrate specificity, enzymes from specialized metabolism have usually broad substrate specificity but low catalytic specificity, which favors the emergence of multiple new pathways during evolution (J.-K. Weng et al. 2012). This enzymatic promiscuity is thought to play a central role in evolution of specialized metabolic enzymes and generation of chemical diversity (J.-K. Weng et al. 2012). According to their chemical structures, specialized metabolites are ranked in three main classes: alkaloids, terpenoids (isoprenoids) and phenolics. These latter are widely distributed in the plant kingdom and they contribute to all aspects of plant life. Their basic structure is a six-carbon aromatic ring with one or more hydroxyl groups attached. The aromatic part of most phenolic compounds is synthesized by the phenylpropanoid pathway (Cheynier et al. 2013). The entry point of this path is the aromatic amino acid phenylalanine arising from the plastidic-located shikimate pathway. Deamination of phenylalanine by phenylalanine ammonia lyase (PAL) gives rise to cinnamic acid, which in turn can be converted into basic phenylpropanoid 105 backbones (C6-C3). All enzymes involved in this pathway have been known for years (Vanholme et al. 2010). These hydroxycinnamic acids are recruited to form majority of phenolic compounds including 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. For instance, caffeic acid can be combined to tartaric acid to form caftaric acid (caffeoyltartaric acid) as found in several members of the *Asteraceae* as in chicory, in perennial peanut and in horsetail (Hohlfeld et al. 1996; Sullivan 2014; Legrand et al. 2016). Caffeic acid can also be combined to quinic acid to form chlorogenic acid (caffeoylquinic acid) as found in tobacco, tomato or chicory (Niggeweg et al. 2004; Legrand et al. 2016) or isochlorogenic acid (dicaffeoylquinic acid) and tricaffeoyl quinic acid as in coffee, sweet potato or chicory (Kojima et al. 1985; Lallemand et al. 2012; Moglia et al. 2014; Legrand et al. 2016; Bernard, Alves Dos Santos, et al. 2020; Miguel et al. 2020). Many polyamines are conjugated with hydroxycinnamic acids to form phenolamides also termed phenylamides or hydroxycinnamic acid amides (Edreva et al. 2007; Roumani 2021). These polyamine-derived molecules are mostly known for their involvement in floral induction or as defense compounds (Bassard et al. 2010; Roumani 2021). Phenolic acids can be conjugated to either aromatic amines like tyramine, tryptamine, octopamine or anthranilate or aliphatic polyamines like putrescine, spermidine or spermine (Bassard et al. 2010; Roumani 2021). To date, all the enzymes involved in N-acylation of aliphatic polyamines belong to the superfamily of the BAHD acyltransferases (Roumani 2021). For instance, agmatine coumaroyl transferase (ACT) catalyzes acylation of agmatine in barley and *Arabidopsis* (Burhenne 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 disinapoyl transferase (SDT) and spermidine dicoumaroyltransferase (SCT) in *Arabidopsis* (Luo et al. 2009), by hydroxycinnamoyl-CoA:spermidine acyltransferase (DH29) in *N. attenuata* (Onkokesung et al. 2012) and by spermidine hydroxycinnamoyl transferase (SHT) in the tapetal cells of *Arabidopsis* and *Malus domestica* (Grienenberger et al. 2009; Elejalde-Palmett et al. 2015). We have previously identified and characterized two enzymes from chicory (*Cichorium intybus*), called 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, Noel, et al. 2006; Grienenberger et al. 2009; Elejalde-Palmett et al. 2015; Peng, Yang, et al. 2016; Delporte et al. 2018; Peng, Meyer, et al. 2019; 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 have evidenced that spermine-derived phenolamide 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, overexpression of *CiSHT2*, in chicory hairy roots that did not accumulate phenolamides naturally, or heterologous expression in *sht Arabidopsis* mutants devoided of fully substituted phenolamide accumulation in the pollen coat, led to similar inconsistency *i.e.* fully substituted phenolamides were either completely absent or were not the main phenolamides in chicory or *Arabidopsis*, respectively. 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. *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 evidences that *CiSHT2* acylates preferentially secondary amines using free or monoacylated polyamines as a substrate whereas *CiSHT1* catalyzes 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 investigations suggest that free polyamine avail-

ability may contribute to direct the flux toward either spermidine or spermine derivative production. Additionally, a bioconversion system using yeast as a chassis to produce spermine derivatives was successfully established but need further improvement for the efficient production of different isomers of acylated spermine.

2.2.2 Results

2.2.3 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 1356-bp and 1359-bp long, respectively and do not contain any intron (Delporte et al. 2018). The target sequences were selected to induce breaks at position 550 and 663 for *CiSHT1* and at position 235 and 351 for *CiSHT2* (Figure 2.7 A.). Three binary vectors were prepared but only two gave rise to mutant plants (pYLCRISPR-sgRNA1-sgRNA2-CiSHT2 was ineffective). The first, named pYLCRISPR-sgRNA1-sgRNA2-CiSHT1, was designed to generate a double mutation in *CiSHT1* and the second, termed pYLCRISPR-sgRNA1-sgRNA2-SHT1 / sgRNA1-sgRNA2-SHT2, to generate a double mutation in both genes, thus expressing;four sgRNAs (Figure 2.7 B. and C.). Through *Agrobacterium rhizogenes*-mediated transformation, 12 and 7 glufosinate-resistant hairy root lines were obtained with pYLCRISPR-sgRNA1-sgRNA2-CiSHT1 and pYLCRISPR-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-Curve (HRM) analysis. PCR products from plants with divergent melting-curves compared to the wild type, 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 (Figure 2.8). Three independent *sht1* mutant lines (8, 17 and 19; Figure 2.8 A.) arising from transformation with pYLCRISPR-sgRNA1-sgRNA2-CiSHT1,

three independent *sht2* mutant lines (21, 22 and 23; Figure 2.8 B.) and one *sht1/sht2* double mutant line (7; Figure 2.8 C.) arising from transformation with pYLCRISPR-sgRNA1-sgRNA2-SHT1/sgRNA1-sgRNA2-SHT2 were generated. Most of the mutations occurred at a position about 3 pb upstream the 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) (Figure 2.8 B.), which is consistent with what was shown in our previous study (Bernard, Gagneul, 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. 2016). The frame shifts induced by the genome editing introduced an early stop on mRNA translation in all the mutated hairy root lines (Figure 2.14).

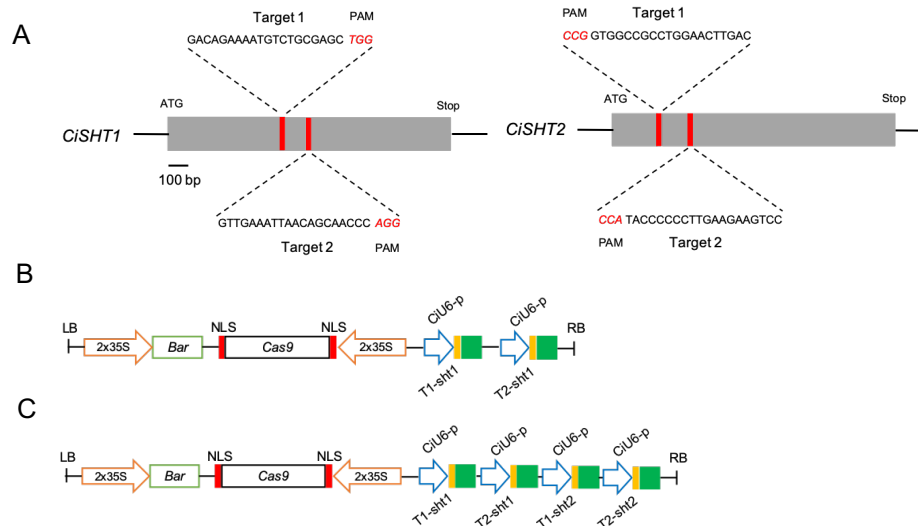


Figure 2.7 – Schematic diagram of *CiSHT1* and *CiSHT2* gene structures and of vectors used to generate chicory mutants.

A: Schematic diagram of *CiSHT1* and *CiSHT2* gene structures (grey rectangles) and target sequences (red lines). The translation initiation codon (ATG) and termination codon (stop) are shown. The target sequence is shown in capital letters, and the protospacer adjacent motif (PAM) sequence is marked in red. **B** and **C:** Schematic diagram of the pYLCRISPR-sgRNA1-sgRNA2-*CiSHT1* and pYLCRISPR-sgRNA1-sgRNA2-SHT1/sgRNA1-sgRNA2-SHT2 vectors, respectively. Bar: Phosphinothricin acetyltransferase. 35S: cauliflower mosaic virus 35S promoter. NLS: nuclear localization sequence. Tnos: gene terminator. CiU6-1P: *C. intybus* U6-1p promoter.

A

Plant line	Target 2	PAM	Mutation events
WT	TCACAATGTTGAAATTAACAGCAACCC	AGGTCGAGAAATTGCGAAACAAAGCTAATAAAAAG	
SHT1 (8) A1	TCACAATGTTGAAATTAACAGC - ACCC	AGGTCGAGAAATTGCGAAACAAAGCTAATAAAAAG	-1
SHT1 (8) A2	TCACAATGTTGAAATTAACAGC - ACCC	AGGTCGAGAAATTGCGAAACAAAGCTAATAAAAAG	-1
SHT1 (17) A1	TCACAATGTTGAAATTAACAGCAA	CCCAGGTCGAGAAATTGCGAAACAAAGCTAATAAAAAG	+1
SHT1 (17) A2	TCACAAT-(+46)-TTGAAATTAACAGCA	GAGGACCCAGGTCGAGAAATTGCGAAACAAAGCTA	+50, -1
SHT1 (19) A1	TCACAATGTTGAAATTAACAGCAA	TCCCAGGTCGAGAAATTGCGAAACAAAGCTAATAAAAAG	+1
SHT1 (19) A2	TCACAATGTTGAAATTAACAGCAA	TCCCAGGTCGAGAAATTGCGAAACAAAGCTAATAAAAAG	+1

B

Plant line	PAM	Target 1	PAM	Target 2	Mutation events T1/T2
WT	CGCCG	GTGGCCGCCTGGAAC TTGACTG...	CGCCA	TACCCCCCTTGAAGAAGTCCATTAT	
SHT2 (21) A1	CGCCG	GTGGCCGCCTGGAAC TTGACTG...	CGCCATAC	TCCCCCTTGAAGAAGTCCATTAT	wt / +1
SHT2 (21) A2	CGCCG	GTGG -- GCCTGGAAC TTGACTG...	CGCCATAC	----- GAAGAAGTCCATTAT	-2 / -7
SHT2 (22) A1	CGCCG	GTGGCCGCCTGGAAC TTGACTG...	CGCCATAC	- CCCCTTGAAGAAGTCCATTAT	wt / -1
SHT2 (22) A2	CGCCG	GTGGCCGCCTGGAAC TTGACTG...	CGCCATAC	CCCCCTTGAAGAAGTCCATTAT	wt / +1
SHT2 (23) A1	CGCCG	----- ACTTGTGACTG...	CGCCAT	----- AAGAAGTCCATTAT	-13 / -10
SHT2 (23) A2	CGCCG	GTG-----	-----	CCCCCTTGAAGAAGTCCATTAT	-139

C

Plant line	Target 2	PAM	Mutation events
SHT1 (WT)	ATGTTGAAATTAACAGCAACCC	AGGTC.	
SHT1/SHT2 (7) A1	ATGTTGAAATTAACAGCAAT	TCCCAGGTC	+1
SHT1/SHT2 (7) A2	ATGTTGAAATTAACAGCAAT	TCCCAGGTC.	+2

Plant line	PAM	Target 1	PAM	Target 2	Mutation events T1/T2
SHT2 (WT)	CGCCG	GTGGCCGCCTGGAAC TTGACTG...	CGCCA	TACCCCCCTTGAAGAAGTCCATTAT	
SHT1/SHT2 (7) A1	CGCCG	GTC --- GCCTGGAAC TTGACTG...	CGCCATAC	----- TTGAAGAAGTCCATTAT.	+1, -3/-5
SHT1/SHT2 (7) A2	CGCCG	GTG-----	-----	CCCCCTTGAAGAAGTCCATTAT	-139

Figure 2.8 – Genotypes of *sht1*, *sht2* and *sht1/sht2* mutants.

A: Mutation events induced on *CiSHT1* after transformation with pLYCRISPR-sgRNA1-sgRNA2-CiSHT1. **B:** Mutation events induced on *CiSHT2* after transformation with pLYCRISPR-sgRNA1-sgRNA2-SHT1/sgRNA1-sgRNA2-SHT2. **C:** Mutation events induced on *CiSHT1* and *CiSHT2* after transformation with pLYCRISPR-sgRNA1-sgRNA2-SHT1/sgRNA1-sgRNA2-SHT2. Target sequences are shown in blue letters, with PAM sequences in red. The changes in the number of nucleotides are shown on the right.

2.2.4 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* wild type strain (thereafter these 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 2.9). 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 2.9). In the flower buds of the *sht1* mutant lines, only partially substituted phenolamides *i.e.* dicoumaroyl spermine and tricoumaroyl spermine, were detected instead of tetracoumaroyl spermine and tricoumaroyl spermidine.

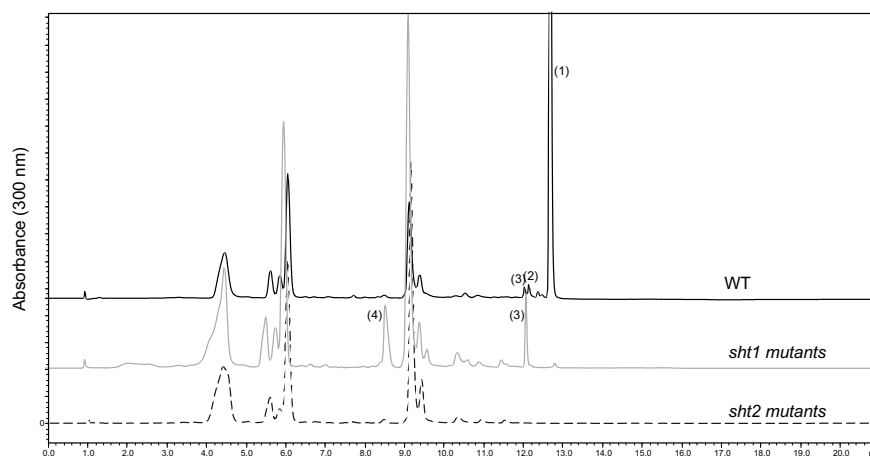


Figure 2.9 – Stacked HPLC chromatograms of extracts obtained from chicory flower buds at stage 13-15 isolated from WT (black line), *sht1* mutant (grey line) or *sht2* mutant (dotted line).

The identity of the numbered peaks was confirmed by mass spectrometry: (1) tetracoumaroyl spermine, (2) tricoumaroyl spermidine, (3) tricoumaroyl spermine, (4) dicoumaroyl 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 acylation steps whereas CiSHT1 would fulfil the acylations to produce the fully substituted phenolamides.

2.2.5 Transient expression of *CiSHT1* and *CiSHT2* in tobacco 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 *A. 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 tobacco leaves infiltrated with bacteria containing either the empty vector, pEAQ-HT and more interestingly with the pEAQ-HT-DEST1-SHT1 (35S-CiSHT1) vector (Figure 2.10).

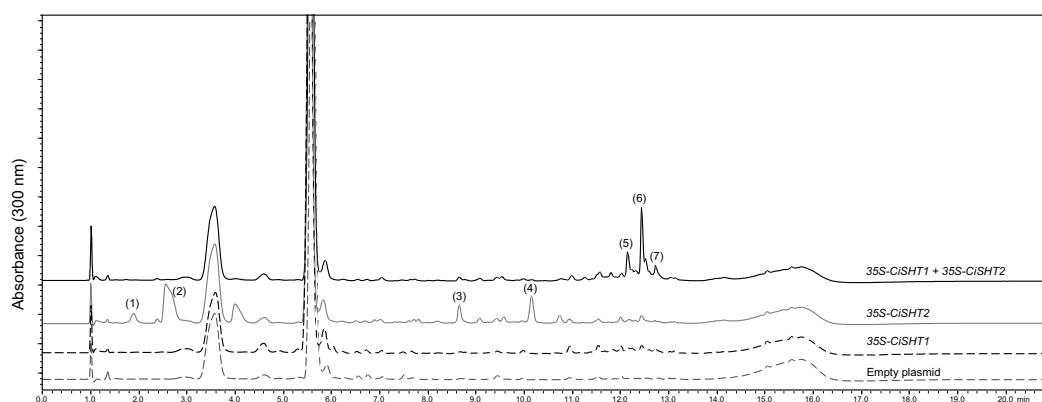


Figure 2.10 – Stacked HPLC chromatograms of extracts obtained from agroinfiltrated tobacco leaves.

Plants were infiltrated with either the empty plasmid (grey dotted line), *CiSHT1* (35S-*CiSHT1*; black dotted line), *CiSHT2* (35S-*CiSHT2*; grey line), or *CiSHT1* plus *CiSHT2* (35S-*CiSHT1*+35S-*CiSHT2*; black line). 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, (7) tetracoumaroyl spermine.

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 2.10). 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 tobacco plants were agroinfiltrated as already described and the metabolite profiles analyzed by hierarchical clustering (Figure 2.11 and Table 2.2). Three monocoumaroyl spermidine isomers, 2 dicoumaroyl spermidine isomers and 1 dicoumaroyl spermine were detected when tobacco leaves were agroinfiltrated with *CiSHT2*. Traces of tricoumaroyl spermidine were also detected. The main monocoumaroyl spermidine (MonoCSpd isomer 1) had a RT of 2.5 min in our chromatographic conditions (Figure 2.10) as also shown in our previous *in vitro* experiments (Delporte et al. 2018). It gave a $[M+H]^+$ ion at m/z 292.2013 as established by ESI-HRMS. Its molecular formula was determined to be $C_{16}H_{25}N_3O_2$ as expected for monocoumaroyl spermidine. ESI-MS-HRMS was performed to attempt to determine its precise structure. A previous study has shown that it was possible to distinguish the three regioisomers using this approach (Hu et al. 1996). Fragmentation pattern of each isomer was extensively examined by using either ^{15}N labelling or H/D exchange. Many fragments were common for all isomers but the relative intensity of the ions at m/z 218 and 221 were shown to be a diagnostic value. For N^1 -monocoumaroyl spermidine, the ion at m/z 221 was more intense than that at m/z 218 whereas an opposite trend was observed for N^{10} -monocoumaroyl spermidine. The ion at m/z 221 was slightly more intense than the ion at m/z 218 for N^5 -monocoumaroyl spermidine.

Contribution of the ion at m/z at 221 for N^5 -monocoumaroyl spermidine was attributed to an isomerization reaction responsible for the partial conversion of N^5 -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 behaviour towards fragmentation of the isomers (Figure 2.15).

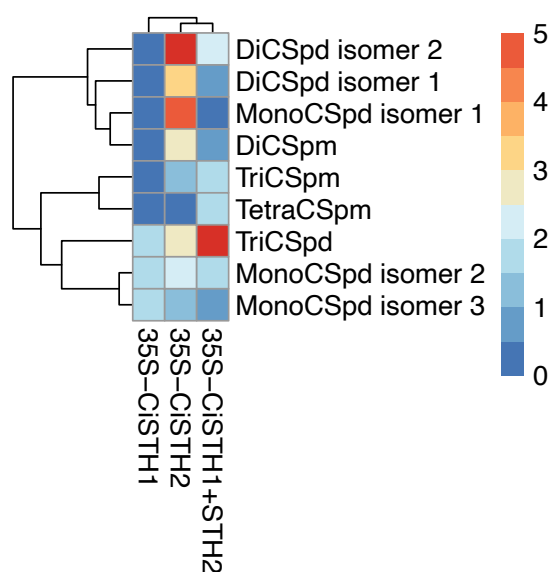


Figure 2.11 – Heatmap of spermine and spermidine derived phenolamides in *N. benthamiana* agroinfiltrated with *CiSHT1*, *CiSHT2* or *CiSHT1* plus *CiSHT2*.

Pick area means for each condition (eight plants) and for each metabolite are expressed per mg of dry material. A logarithmic neperien scale (\ln) was applied before the heatmap hierarchical clustering with the Ward's method. DiCSpd isomer: dicoumaroyl spermidine isomer, MonoCSpd: monocoumaroyl spermidine, DiCSpm: dicoumaroyl spermine, TriCSpm: tricoumaroyl spermine, TetraCSpm: tetracoumaroyl spermine, TriCSpd: tricoumaroyl spermidine.

A structure has been assigned to the three isomers and N^5 -monocoumaroyl spermidine was shown to be the main isomer accumulated in tobacco leaves (MonoCSpd isomer 1, Table 2.2). The identity of the two dicoumaroyl spermidine was not investigated further. Indeed it was already shown that their discrimination based on MS/MS data was not conclusive (Bigler et al. 1996). The main spermine derived products accumulated in tobacco leaves expressing

CiSHT2 (Figure 2.10, Figure 2.11 and Table 2.2) and accumulated in chicory *sht1* mutants (Figure 2.9) 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 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 $C_{28}H_{38}N_4O_4$ as expected for a dicoumaroyl spermine isomer (Figure 2.12 A.). In addition, NMR analysis revealed 1H and ^{13}C characteristic signals consistent with a spermine derivative substituted with coumaroyl groups (Table 2.1 and Figure 2.17). It should be pointed out that the number of 1H and ^{13}C 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^{14} positions or on N^5 and N^{10} positions. Long range 1H - ^{13}C correlations were investigated by 2D NMR using HMBC experiments to get more information about the connectivity of the coumaroyl groups on the spermine. $^3J_{HC}$ cross-peaks were observed between the protons H-4/H-11 (δ 3.57 ppm) and H-6/H-9 (δ 3.59 ppm) of the spermine and the equivalent quaternary carbons from coumaroyl groups (δ 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^5, N^{10} -dicoumaroyl spermine (Figure 2.12 C.). In the ESI-HRMS-MS spectrum, typical fragments were observed at m/z 147.0441, 204.1019, 275.1754 and 349.2600 confirming the identity of this compound (Figure 2.12 A. and B.). From the precursor ion at m/z 495.2709, a first loss of NH_3 (-17 Da) give 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 successive losses of NH_3 at one end and a propylamine chain at the other end (Figure 2.12 B.). To

conclude, the main spermine synthesized by CiSHT2 *in vitro* as well as *in vivo* was N⁵,N¹⁰-dicoumaroyl spermine. These data indicate that CiSHT2 efficiently catalyzes the acylation of secondary amines of spermine while CiSHT1 acylates primary amines of N⁵,N¹⁰-dicoumaroyl spermine.

Table 2.1 – ^1H and ^{13}C -NMR data of $\text{N}^5, \text{N}^{10}$ -dicoumaroyl spermine in methanol- d_4 .

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', α'
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', α' , CONH'
CONH'	169.9	-	-
1''	127.4	-	3'', 5'', α''
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)	4'', 6'', β''
α''	113.8	6.87 (d, J = 15.7)	1'', CONH''
β''	145.0	7.59 (d, J = 15.7)	2'', 6'', α'' , CONH''
CONH''	169.9	-	-

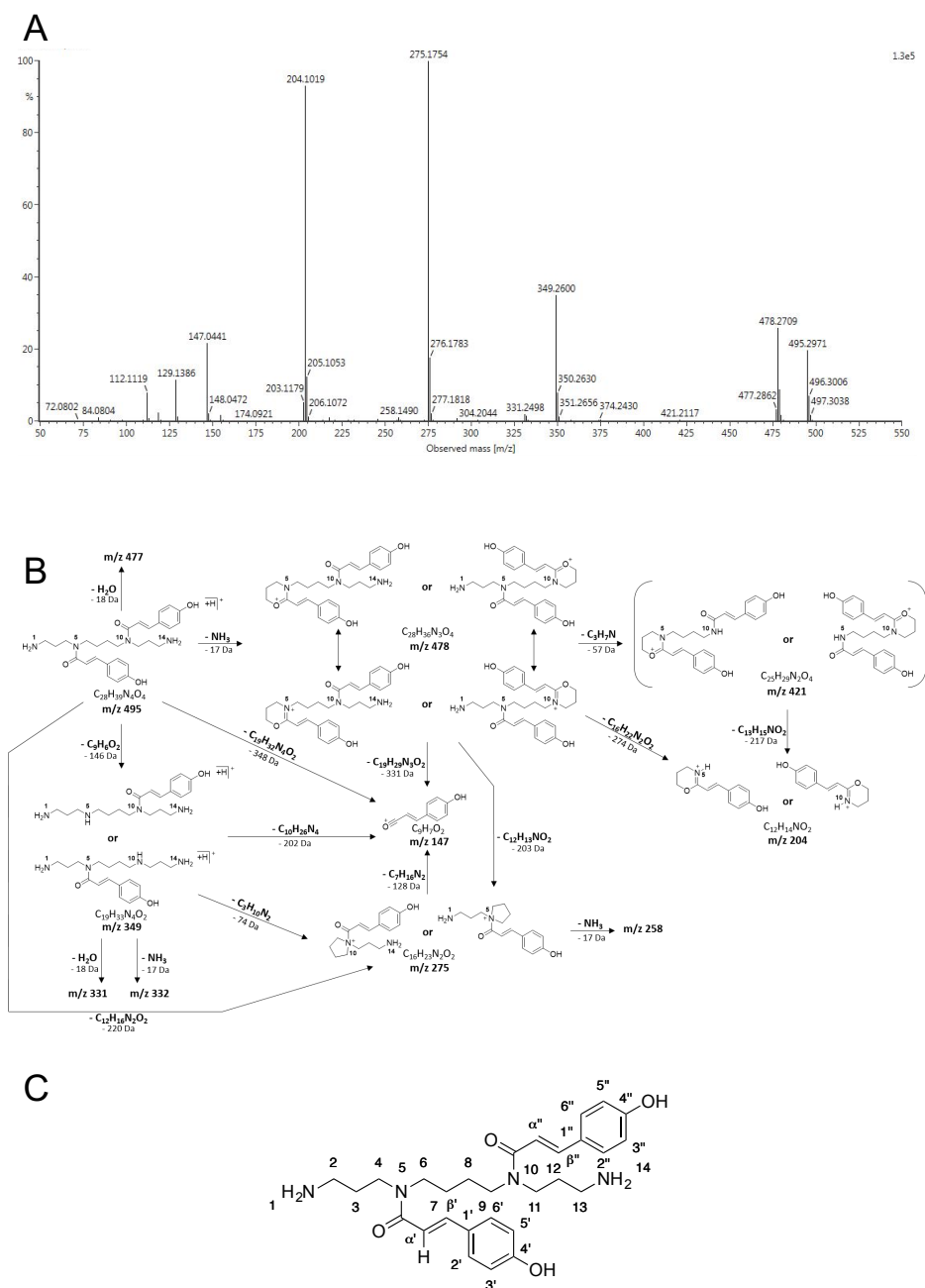


Figure 2.12 – ESI-MS-HRMS spectra of purified N^5,N^{10} -dicoumaroyl spermine. **A:** Fragmentation of purified N^5,N^{10} -dicoumaroyl spermine. The position of the coumaroyl moieties was deduced from RMN data (see Table 2.1 and Figure 2.17). **B:** Fragmentation scheme of N^5,N^{10} -dicoumaroyl spermine. **C:** Chemical structure of N^5,N^{10} -dicoumaroyl spermine. as deduced from NMR data.

2.2.6 Free polyamine availability controls the nature of the final product

While CiSHTs were shown *in vitro* to be able to catalyze spermidine and spermine acylation without apparent substrate preferences (Delporte et al. 2018), tobacco and *Arabidopsis* transformed with the chicory SHTs mainly accumulates spermidine derivatives whereas the main compound found in chicory flower buds is tetracoumaroyl 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 favour 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 tobacco leaves. In tobacco leaves, spermidine level ($1 \mu\text{mol} \cdot \text{g}^{-1} \text{DW}$) was higher than that of spermine ($0.12 \mu\text{mol} \cdot \text{g}^{-1} \text{DW}$). 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 $0.7 \mu\text{mol} \cdot \text{g}^{-1} \text{DW}$, 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.

2.2.7 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 48h, yeasts were pelleted, phenolic compounds extracted and analyzed by LC-UV (Figure 2.13). As expected,

no phenolamide could be detected when yeasts were transformed with *At4CL5* alone and in yeast coexpressing *At4CL5* and *CiSHT1* (not shown). In yeast transformed with *At4CL5* and *CiSHT2*, accumulations of monocoumaroyl spermidine and tricoumaroyl spermidine were evidenced but not that of spermine derivatives.

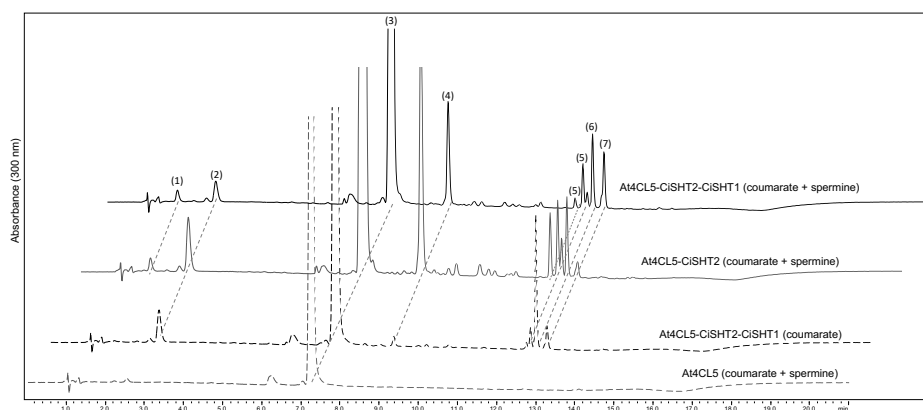


Figure 2.13 – Stacked HPLC chromatograms of extracts from transgenic yeast expressing different combination of genes.

Yeasts were transformed with *At4CL5* alone (dotted grey line), with *At4CL5*+*CiSHT2*+*CiSHT1* (dotted black line and black line) or with *At4CL5*+*CiSHT2* (grey line). The compounds added to the external medium are indicated in brackets. The identity of the numbered peaks was confirmed by mass spectrometry: (1) monocoumaroyl spermine, (2) monocoumaroyl spermidine, (3) coumarate, (4) dicoumaroyl spermine, (5) tricoumaroyl spermine, (6) tricoumaroyl spermidine, (7) tetracoumaroyl spermine.

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 *i.e.* 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 monocoumaroyl spermine, dicoumaroyl spermine and tricoumaroyl spermine occurred. In these conditions, yeasts accumulated also the spermidine derivatives but the main phenolamide was the N^5,N^{10} -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 when coumarate and spermine were added in the medium. In these conditions partially acylated spermine derivative accumulation decreased accordingly, and accumulation of spermidine derivatives was also effective. This heterologous system confirms our findings *i.e.* 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.

2.2.8 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 AtSHT and 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 (Grienenberger 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 need 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: CiSHT1 and CiSHT2 (Delporte et al. 2018). The biochemical and functional characterization of these two enzymes has evidenced 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 knock out *CiSHT1* and *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 CiSHT1 and CiSHT2 are required and sufficient to promote tetracoumaroyl spermine biosynthesis in chicory. The *sht2* mutants did not produce phenolamides whereas the *sht1* mutants only produced dicoumaroyl

and tricoumaroyl spermine but not the fully acylated spermine. These data indicate a role for CiSHT2 in the first acylation steps and for CiSHT1 in the last steps. The results obtained with these mutants are in accordance with the experiments done with chicory hairy roots overexpressing either *CiSHT1* and *CiSHT2* (Delporte et al. 2018). The requirement of both enzymes to synthesize the fully substituted phenolamides was further confirmed using tobacco and yeast heterologous systems. Thus, no phenolamides were produced when expressing only *CiSHT1* while *CiSHT2* expression only promoted partially substituted phenolamide 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 CiSHT2 is a dicoumaroyl spermine as determined by mass spectrometry. In order to decipher the reaction mechanism, this compound was purified and analyzed by NMR. It was identified as N⁵,N¹⁰-dicoumaroyl spermine.. These data suggest that CiSHT2 has a higher affinity for secondary amines of spermine and N⁵- or N¹⁰-monocoumaroyl spermine whereas CiSHT1 prefers primary amines of di- or tri-coumaroyl spermine. Taken together these results suggest that CiSHT2 would promote dicoumaroyl spermine and dicoumaroyl spermidine synthesis, whereas CiSHT1 would perform the last acylation steps toward fully substituted amine biosynthesis. More detailed *in vitro* biochemical experiments are necessary to assess these findings, but this task is hampered by the lack of commercial standards. A similar biochemical mechanism was suggested in *Nicotiana attenuate* (Onkokesung et al. 2012). An acyltransferase (DH29) was shown to catalyze the first acylation step in dicaffeoyl spermidine formation whereas a second enzyme named CV86 was suggested to act on monocaffeoyl spermidine synthesized by DH29 to promote diacylated spermidine synthesis. Position of caffeoyl moieties was not further investigated but both enzymes belong to the BAHD acyltransferase superfamily. Recently an enzyme able to acylate spermine has been identified in *Solanum richardii*, a wild relative of eggplant (Peng, Meyer, et al. 2019). This enzyme named SrSpmHT (Spermine hydroxycinnamoyl transferase) 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 N¹-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 emerge and evolved independently of SHT family (Roumani 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 amino 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 phenolamide 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 tobacco 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 406 that the ratio spermidine/spermine were 8 and 0.8 in tobacco 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 metabolic 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 et al. 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 anti-viral 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 derived compounds were the major products. Modification of polyamine metabolism towards spermine production may promote the synthesis of the spermine-derived phenolamides. In addition, the expected compounds were retained in the yeast intracellular medium and catalytic constraints, such as feedback inhibition, may not favor the synthesis of the fully acylated compounds. The engineering of a strain secreting the final products in the culture media should be considered. It was recently shown that yeast could be a suitable host platform for the production and excretion of spermidine-derived phenolamides (Perrin et al. 2021). Finally, the catalytic versatility of the enzymatic system characterized in this study may be used to synthesize related bioactive compounds such as the kukoamines widely studied, among other, that possess numerous pharmacological effects, such as antihypertensive, anti-inflammatory, anti-analgesic, anti-sepsis, autoimmune-enhancing and neuroprotective activities (Chantrapromma et al. 1981; Wang et al. 2016; Li et al. 2017). Yeast expressing SHTs from different species was already exploited to produce spermidine acylated with tridihydrocinnamic acids (Perrin et al. 2021). An optimized yeast strain could result in kukoamines production and maybe tetradihydrocaffeic spermine, a compound still never synthesized and biologically assessed.

In summary, we have shown, through *in vivo* experiments, that CiSHT1 and CiSHT2 are both required for the synthesis of tetracoumaroyl spermine in chicory flower buds. We proposed that appearance of this phenolamide is based on two evolutionary events in chicory *i.e.* modification of polyamine metabolism and gene duplication of an ancestral *sht* to give rise to neofunctionalization and appearance of a new metabolic pathway. In addition, we have generated two biological tools (chicory mutants and engineered yeasts) that could help to understand the *in planta* biological roles of phenolamides and facilitate their

valorization in human uses.

2.2.9 Material and Methods

Plant material

C. intybus (L.) line clone 17' (Florimond Desprez, Cappelle-en-Pévèle, France) as well as chicory hairy root lines overexpressing *CiSHT2* and plants regenerated from them (Delporte et al. 2018) were used in this study. Chicory and tobacco plants were grown in a greenhouse under a 16/8h light/dark cycle. Hairy roots and plantlets regenerated from them were grown in a growth chamber (16/8h light/dark cycle at 25/20°C).

Design of sgRNA, construction of CRISPR/Cas9 vectors and *A. rhizogenes* transformation

To introduce mutations in *CiSHT1* (GenBank accession MG457243.1) and *CiSHT2* (GenBank accession MG457244.1) coding sequences in order to knock-out the genes, two guides RNAs have been designed for each gene according to their location in the gene and their GC content using the software crispor.tefor.net (Haeussler et al. 2016) (Figures 2.18 and 2.19). The four targets were chemically synthesized by SIGMA-ALDRICH (Gillingham, UK). Primary vectors pKanCiU6-1p-sgRNA1-SHT1, pKanCiU6-1p-sgRNA2-SHT1, pKanCiU6-1p-sgRNA1-SHT2 and pKanCiU6-1p-sgRNA1-SHT2 containing the cassette “CiU6-1p-Guide-sgRNA scaffold were constructed as previously described (Bernard, Gagneul, et al. 2019). Binary vector pLYCRISPR-sgRNA1-sgRNA2-CiSHT1 harboring the two sgRNAs and the CAS9 was constructed as previously described (Bernard, Gagneul, et al. 2019). To construct binary vectors expressing four sgRNAs (two sgRNAs targeting SHT1 and two sgRNAs targeting SHT2), PCR using proofreading polymerase (PrimeStar HS DNA polymerase; TAKARA, Kasatu, Japan) with primary vectors as templates were run using the following primer pairs: GG1-F and GG1-R with pKanCiU6-1p-sgRNA1-SHT1, GG2-F and GG3-R with pKanCiU6-1p-sgRNA2-SHT1, GG3-F and GG4-R with pKanCiU6-1p-sgRNA1-SHT2, GG4-F and GG2-R with pKanCiU6-1p-sgRNA2-SHT2. The primer sequences are listed in Table 2.3.

The BsaI restriction site and the 4 nucleotides recombination sites for Golden Gate restriction/ligation method was added in the 5' extremity of each primer. Then PCR products were purified with NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel, Düren, Germany) and were assembled with Golden Gate restriction/ligation method in pYLCRISPR/Cas9P35S-B as described by Ma and Liu (2016) to generate pYLCRISPR-sgRNA1-sgRNA2-SHT1/sgRNA1-sgRNA2-SHT2 and pYLCRISPR-sgRNA1-sgRNA2-CiSHT1. The pYLCRISPR-sgRNA1-sgRNA2-CiSHT2 was also generated but was not effective to generate mutants. The *A. rhizogenes* strain 15834 (kindly provided by Marc Buée, INRAE, Nancy, France) was independently transformed by electroporation with the binary vectors as described elsewhere (Nagel et al. 1990).

Hairy root culture and selection and regeneration of plants

Hairy root induction was performed on leaves of clone 17' using *A. rhizogenes* strains (wild-type or transformed with either pYLCRISPR-sgRNA1-sgRNA2-CiSHT1 or pYLCRISPR-sgRNA1-sgRNA2-SHT1/sgRNA1-sgRNA2-SHT2) as described previously (Bernard, Gagneul, et al. 2019) and the infected leaves were placed on Murashige and Skoog Hairy Root (MSHR) solid medium (macro and micro-elements, Fe-Edta, vitamins (Murashige et al. 1962), added with $30 \text{ g} \cdot \text{L}^{-1}$ sucrose, $6 \text{ g} \cdot \text{L}^{-1}$ agar, pH 5.6) in Petri dishes, and grown at $25^\circ\text{C} \pm 2^\circ\text{C}$ under 16 h light ($37.6 \pm 10.1 \mu\text{mol} \cdot \text{m}^{-2}\text{s}^{-1}$) and at $20^\circ\text{C} \pm 2^\circ\text{C}$ under 8 h dark cycles. Individual putative transgenic hairy roots of approximately 3 cm long, emerging from transformed leaf explants transformed were excised and immediately transferred on Petri dishes with MSHR/2 solid medium supplemented with $30 \text{ g} \cdot \text{L}^{-1}$ sucrose, $6 \text{ g} \cdot \text{L}^{-1}$ agar and added with $300 \text{ mg} \cdot \text{L}^{-1}$ ampicillin to remove *Agrobacteria* and $10 \text{ mg} \cdot \text{L}^{-1}$ glufosinate-ammonium to select transformants. Regeneration of shoots from hairy root cultures was spontaneous on MSHR/2 medium. The regenerated shoots were excised from the hairy roots and grown separately for 6 weeks on H10 solid medium for root development (Bernard, Gagneul, et al. 2019). After this time, the well-developed plantlets were then transferred to the greenhouse and grown until flowering. These plants do not need vernalization for flowering (Bogdanovic et al. 2014) which occurs as early

as 1 week after regeneration. Flower buds were harvested at developmental stage 13-15 when the phenolamide contents were shown to be the highest (Delporte et al. 2018). Samples were immediately immersed in liquid nitrogen and store at -80°C until use.

Genomic DNA extraction and high-resolution melting (HRM) temperature analysis

Genomic DNA from hairy roots was extracted using the NucleoSpin Plant II kit (Macherey-Nagel, Düren, Germany) according to the manufacturer instructions. PCR was performed using Type-it HRMTM PCR (QIAGEN) according to the manufacturer instructions. Twenty ng genomic DNA were used as template for PCR. The primers used for the detection of gene editing events are listed in Table 2.3 and were designed using Primer3 and Netprimer softwares. The PCR products were analyzed by HRM using Rotor-Gene 6000 cycler. Data were analyzed with Rotor Gene Q Serie software. Edited DNA fragments were amplified with primer pairs flanking the target regions (Table 2.3). PCR products were analyzed by Sanger sequencing method and the chromatograms were analyzed with CodonCodeAligner software (Xing et al. 2014). Primer positions are indicated in figures 2.18 and 2.19.

Transient expression in *N. benthamiana*

Previously reported entry vectors pDONR207_SHT1 and pDONR207_SHT2 were used to generate expression vector for transient expression in tobacco (Delporte et al. 2018). The open reading frames of *CiSHT1* and *CiSHT2* were introduced in pEAQ-HT-DEST1 vector by LR recombination to generate pEAQ-HT-DEST1-SHT1 and pEAQ-HT-DEST1-SHT2 (Sainsbury et al. 2009). The empty pEAQ-HT vector was used as a negative control. These three vectors were independently introduced in *A. tumefaciens* GV2260 by electroporation. *Agrobacteria* were grown and prepared for agroinfiltration as previously described (Legrand et al. 2016). For transformation, *agrobacteria* strains were prepared alone or mixed to reach a final OD600 of 0.8 (equal concentration of each strain). Tobacco was then infiltrated with strains harboring pEAQ-HT as a

control, with pEAQ-HT-DEST1-SHT1 alone, with pEAQ-HT-DEST1-SHT2 alone or with a mixture of strains containing pEAQ-HT-DEST1-SHT1 or pEAQ-HT-DEST1-SHT2. Four to six-week-old plants per combination were used. After 4 days, the infiltrated leaves were collected, immediately frozen in liquid nitrogen and stored at -80°C until use.

Expression in *S. cerevisiae*

The vectors pDRf1-4CL5-GW, pDRf1-4CL5 and pRS423-FjTAL were provided by Dominique Loqué (Eudes, Baidoo, et al. 2011; Eudes, Mouille, et al. 2016). pRS423-GW was generated by BP reaction with pRS423-FjTAL and pDONR221. pDONR-SHT1 and pDONR-SHT2 were used to generate pDRf1-4CL5-SHT2, pDRf1-4CL5-SHT1 and pRS423-SHT1 by LR recombination with pDRf1-4CL5-GW or pRS423-GW. The *S. cerevisiae* pad1 knockout (*MATa his3Δ1 leu2D0 met15Δ0 ura3Δ0 Δ pad1*, ATCC 4005833) were transformed using the freeze-thaw method (Winzeler et al. 1999; Gietz et al. 2007). Yeasts were selected on solid medium containing yeast nitrogen base (YNB) without amino acids supplemented with 2% glucose and 1X dropout-uracil, 1X dropout-histidine or 1X dropout-uracil-histidine. Overnight cultures from 4 independent single colonies of recombinant yeast harbouring pDRf1-4CL5, pDRf1-4CL5-SHT2, pDRf1-4CL5-SHT1 or pDRf1-4CL5-SHT2 plus pRS423-SHT1 constructs were mixed and grown in 1X YNB medium supplemented with 2% glucose and the required yeast synthetic drop-out medium supplement. These cultures were used to inoculated 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 tobacco, 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 a 0.45- μ m filters. For tobacco used for the statistical analysis, eight 4 to 6 weeks-old-plants per combination were used. Hundred mg of dry leaves were extracted for 10 min at 60°C under agitation (2000 rpm) four times with 0.4 mL of hexane and finally four times with 0.4 mL of a mixture of water/methanol (1:1). The polar extract was diluted (1/10) before ESI-MS-HRMS analysis. For metabolite measurement of yeast, supernatant and pellet were separately collected by centrifugation (20000 g, 5 min, 4°C). The supernatants were passed through a 0.45- μ m filter prior to HPLC-UV analysis. The pellets were ground with glass beads in a mixture of methanol/water/acetic acid (75/23/2, v/v/v). Homogenates were clarified by centrifugation (14 000 g, 4 °C, 10 min) and passed through a 0.45- μ m filter before HPLC-UV analysis.

Metabolite analyses by HPLC-DAD

Metabolite analysis was carried out using Prominence HPLC system (Shimadzu) consisting of a quaternary pump (LC-20AD) and a UV-visible diode-array detector (DAD; SPD-20A). Analyses were performed on a 100 \times 4.6 mm Kinetex 2.6 μ m PFP 100 Å column (Phenomenex, <http://www.phenomenex.com/>). The chromatographic separation was performed using water (solvent A) and acetonitrile (solvent B), both acidified with 0.1% ortho-phosphoric acid. The solvents were delivered at a flow rate of 1.1 mL \cdot min⁻¹ and the oven temperature was set at 45°C. The HPLC conditions were as follow: start at 5% solvent B, 6 min gradient to 20% solvent B followed by 1 min isocratic 20% solvent B, and 1 min gradient to 25% solvent B were applied before returning to 5% solvent B and 7 min re-equilibration. Ten μ L of the extracts were injected on the column.

Extraction and analysis of free polyamines

Thirty milligrams of powdered samples were thoroughly mixed with 400 μ L of 1 mmol \cdot L⁻¹ HCl supplemented with 10 μ mol \cdot L⁻¹ heptanediamine, as an internal standard, on a magnetic stirring plate (2000 rpm) for 1 h at 4 °C. The homogenates were then centrifuged for 15 min at 10 000 g at 4 °C, and the supernatants collected. The pellets were further extracted twice with 400 μ L

of $1 \text{ mmol} \cdot \text{L}^{-1}$ HCl supplemented with $10 \text{ } \mu\text{mol} \cdot \text{L}^{-1}$ heptanediamine. After short stirring, the homogenates were centrifuged for 15 min at 10 000 g at 4°C . The combined supernatants were used as the crude extracts for characterization and determination of free amines and polyamines and stored frozen at -20°C before chromatographic analyses. HPLC and fluorescence spectrophotometry were used to separate and quantify amines prepared as their dansyl derivatives as already described (Smith et al. 1985) with some modifications as follows. Aliquots ($200 \text{ } \mu\text{L}$) of the supernatant were added to $200 \text{ } \mu\text{L}$ of saturated sodium carbonate and $600 \text{ } \mu\text{L}$ of dansyl chloride in acetone ($7.5 \text{ mg} \cdot \text{mL}^{-1}$) in a 5 mL tapered reaction vial. After a brief vortexing, the mixture was incubated in darkness at room temperature for 16 h. Excess dansyl chloride was converted to dansylproline by 30 min incubation after adding $300 \text{ } \mu\text{L}$ ($150 \text{ mg} \cdot \text{mL}^{-1}$) of proline. Dansylated amines were extracted in 1 mL ethyl acetate. The organic phase was collected then evaporated to dryness, and the residue was dissolved in methanol and stored in glass vials at -20°C . Standards were processed in the same way, and two to 50 nmol (per assay) were dansylated for each standard alone or in combination. Free amines were quantified after yield correction with the internal standard and calibration with external standards. The HPLC column was packed with reverse phase Spherisorb ODS2 C18 (particle size $5 \text{ } \mu\text{m}$; $4.6 \times 250 \text{ mm}$, Waters, Milford, USA). The mobile phase consisted of a solution of $17.5 \text{ mmol} \cdot \text{L}^{-1}$ potassium acetate (pH 7.17) as eluent A and acetonitrile as eluent B. The solvent gradient was as follows: 0 min, 70% A; 2 min, 58% A; 24 min, 58% A; 25 min, 55% A; 32 min, 55% A; 52 min, 45% A; 53 min, 35% A; 64 min, 35% A; 70 min, 20% A; 75 min, 100% B; and 80 min, 70% A. The flow rate of the mobile phase was $1.5 \text{ mL} \cdot \text{min}^{-1}$. For fluorescence detection of dansyl amines, an excitation wavelength of 366 nm was used with an emission wavelength of 490 nm. Identities of peaks of amines were confirmed by spiking the sample with known amounts of authentic standards (Sigma, St. Louis, Missouri, USA). The HPLC design consisted of a thermoelectron pump (SpectraSystem P1000 XR, Thermo Fisher, San Jose, California, USA) and (Spectra- Series AS100) autosampler with a $20 \text{ } \mu\text{L}$ injection loop, and detection through an FP-2020 Plus fluorometer (Jasco, Inc., Easton, Maryland, 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 g 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 2h at 4 °C in the dark under agitation. Homogenate was then clarified by centrifugation (5 min at 6000 rpm) and the supernatant was passed through a 0.45- μ m 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 reach a final volume of 2 ml. This concentrated fraction was further purified using a purify flash 4250250 (Interchim). Purification was performed on a 250x21 mm Interchrom 2mm 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 \cdot min⁻¹. The column was equilibrated with 20% solvent B. The separation conditions were as followed: 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.

LC-ESI-HRMS and NMR analysis

Electrospray high resolution mass spectrometry (ESI-HRMS) analysis, was performed according to the procedure described in our previous work (Delporte et al. 2018). For nuclear magnetic resonance (NMR) analysis, pure compound was dissolved in 0.75 mL of solvent (methanol- d_4 or DMSO- d_6). Six hundred μL 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, Wissembourg, France) equipped with a z-gradient inverse probe head (TXI, 5-mm tube). The TOPSPIN (V3.2, Bruker) software was used. The 1D proton spectra were acquired using 32 scans of 128 K data points, using spectral widths of 8403 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 12019 Hz in both dimensions. For the TOCSY, a mixing time of 100 ms were employed. The NUS based 2D HSQC spectra were acquired using 16 scans per 8 K increments that were collected into 4K data points, using spectral widths of 12019 Hz in F2 and 26412 Hz in F1. The NUS based 2D HMBC spectra were acquired using 64 scans per 8 K increments that were collected into 4K data points, using spectral widths of 12019 Hz in F2 and 37732 Hz in F1. The number of NUS sampling points was 256 complex points (3.125% sampling density of 8 K points)(Le Guennec et al. 2015). All non-zero filled obtained spectra were manually phased and baseline-corrected, calibrated with solvent signal (^1H (3.31) and ^{13}C (49.0) for methanol- d_4)(Fulmer et al. 2010).

Statistical analysis

For heatmap construction, pick area for each quantification ion were evaluated and expressed per mg of dry materials. A logarithmic neperien scale (\ln) was applied to the means for each condition (eight plants) before the heatmap hierarchical clustering with the Ward's method. The heatmap was visualized (using the pheatmap-package, Rsoftware (v. 4.0.3, company Foundation for Statistical Computing, Vienna, Austria)).

Acknowledgments

Authors would like to thank Professor George Lomonossov (John Innes Center) for providing the pEAQ vectors (pEAQ-HT and pEAQ-DEST1). This work has been performed using infrastructure and technical support of the Plateforme Serre, cultures et terrains expérimentaux - Université de Lille for the greenhouse.

2.2.10 Supplementary data

Plant line		
SHT1 (WT)	... NERECEQETAVTMLKLTATQVEKLRNKANKSQESEMSR...	451 aa
SHT1 (8) A1	NERECEQETAVTMLKLTAPRSRNCETKLIKVRRVR*	252 aa
SHT1 (17) A1	NERECEQETAVTMLKLTATPGREIAKQS*	245 aa
SHT1 (17) A	NERECEQETAVTMLKLTAIPLFWKPWWHILYYSF*	251 aa
SHT1 (19) A1	NERECEQETAVTMLKLTAIIPGREIAKQS*	245 aa
Plant line		
SHT2 (WT)	...LSSIAGGRLELDCNADGVQFIEAYADMKLVLDLDDFMQSPIFHHLIPSVDYRHTPLEEVPLLLQATKFVCGGW...	452 aa
SHT2 (21) A1	...LSSIAGGRLELDCNADGVQFIEAYADMKLVLDLDDFMQSPIFHHLIPSVDYRHTPP*	128 aa
SHT2 (21) A2	...LSSIAGGRLELDCNADGVQFIEAYADMKLVLDLDDFMQSPIFHHLIPSVDYRHTKKSHYCFCKLQNLQVEVGL*	145 aa
SHT2 (22) A1	...LSSIAGGRLELDCNADGVQFIEAYADMKLVLDLDDFMQSPIFHHLIPSVDYRHTPLKSHYCFCKLQNLQVEVGL*	147 aa
SHT2 (22) A2	...LSSIAGGRLELDCNADGVQFIEAYADMKLVLDLDDFMQSPIFHHLIPSVDYRHTPP*	128 aa
SHT2 (23) A1	...LSSIADLTAMRMVFSSSKLTPT*	95 aa
SHT2 (23) A2	...LSSIAGAPGT*	84 aa
Plant line		
SHT1 (WT)	...ECEQETAVTMLKLTATQVEKLRNKANKSQESEMSRGFTRYEVVTA...	451 aa
SHT1/SHT2 (7) A1	...ECEQETAVTMLKLTAIIPGREIAKQS*	245 aa
SHT1/SHT2 (7) A2	...ECEQETAVTMLKLTAIIPRSRNCETKLIKVRRVR*	253 aa
SHT2 (WT)	...LSSIAGGRLELDCNADGVQFIEAYADMKLVLDLDDFMQSPIFHHLIPS...	452 aa
SHT1/SHT2 (7) A2	...LSSIAGAPLKKSHYCFCKLQNLQVEVGL*	101 aa
SHT1/SHT2 (7) A1	...LSSIAGAPGT*	84 aa

Figure 2.14 – Predicted amino acid sequences of SHT1 and SHT2 of mutants used in this study.

Both alleles are shown (A1 and A2). The amino acids that differ from the wild type sequences are in grey. (*) depicts stop codons. The number of amino acids of the predicted proteins is shown on the right. AA : amino acids.

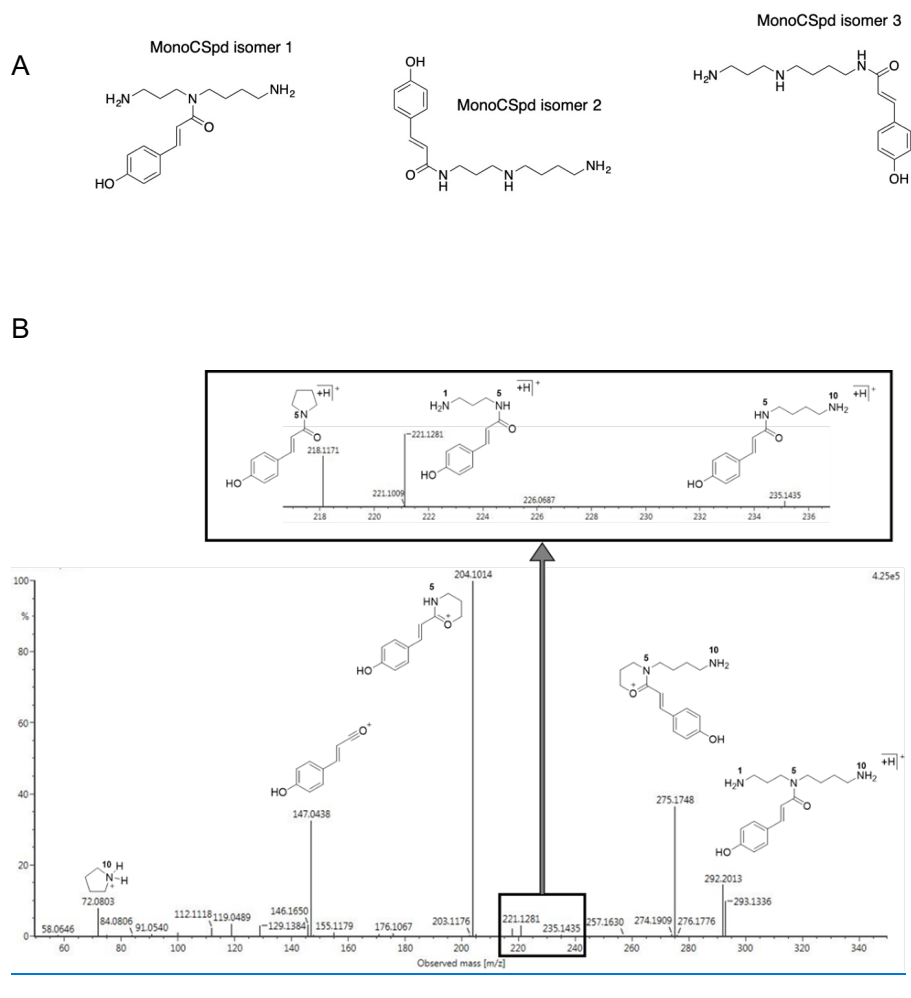
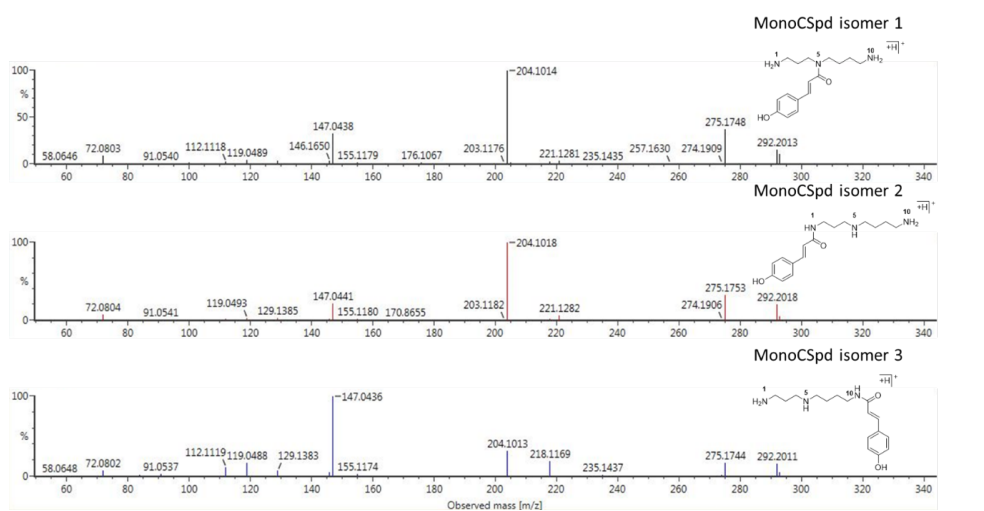


Figure 2.15 – Chemical structures of monocoumaroyl spermidine isomers as determined by mass spectrometry Part 1.

A: Chemical structures of the three isomers as determined by mass spectrometry. **B:** ESI-MS/HRMS spectrum and structural annotation of the fragment ions of the main monocoumaroyl spermidine (MonoCSpd isomer 1).

C



D

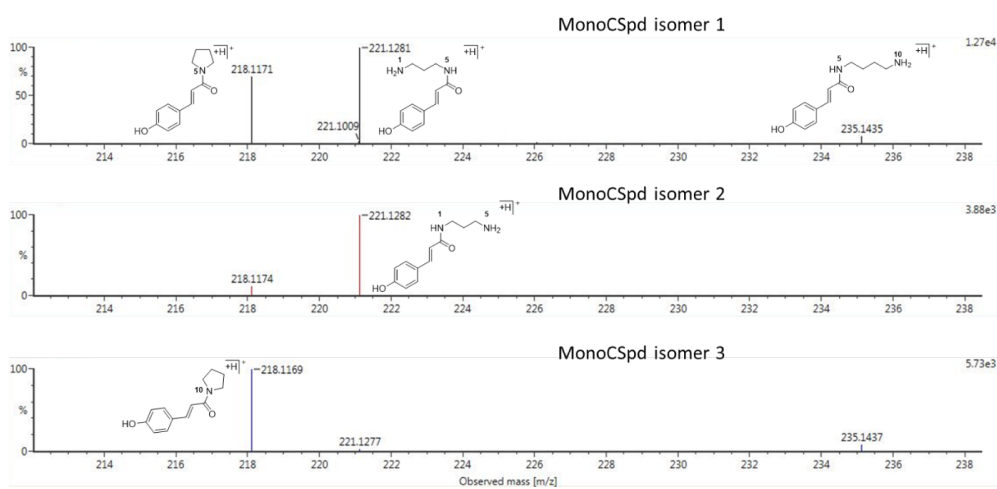


Figure 2.16 – Chemical structures of monocoumaroyl spermidine isomers as determined by mass spectrometry Part 2.

C: Stacked ESI-MS/HRMS spectra of the three isomers. **D:** Stacked ESI-MS/HRMS spectra of the three isomers expanded over the mass range 213-238 Da in order to illustrate the different intensity ratios of the ions at m/z 218 and 221 existing when comparing isomers.

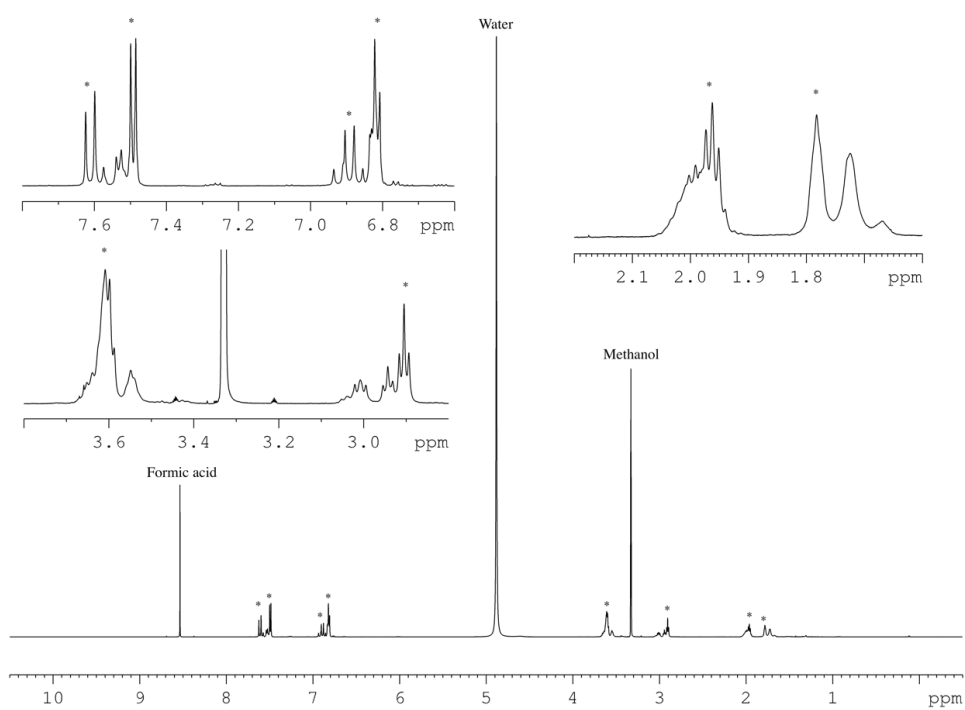


Figure 2.17 – ^1H NMR spectra of $\text{N}^5, \text{N}^{10}$ -dicoumaroyl spermine. Signals of the major compound are marked with *.

CiSHT1

ATCAAGGTTTCCAACAAGACTTCTTCCATGGTTAAACCAGCTGAACCAACATGGTCCGGTAAGCTAGCCTTATCAGAACT
 TGATCAAACCTGGTGCACAACCTCATGTTCCAACCATTTACTTCTATGCACAATCCCCTGAGGACTGGGTCACTGTTCTAC
 ATACCCTAAAAACTTCCCTAAGTTGTACCCTAGTTCATTTCTATCCATTGGCTGGCCGGCTGTCTTCTATTGTCTGGAGGC
 CGTTTGGAGCTTGATTGCAATGCGGCCGGCGTGCATTTACAGAAGCTTACGCTGACAAGAACTTACTGATCTGGACAG
 TCTGTTACCATCGCCCATGTATCACCAACTTATTCCTTCAATAGATTATCAAAGTACACTTCTTGAAGAAATCCCATGT
 TAGTTTGC**CAAGTCACAAGGTTCA**TTTGTGGAGGGTTAGCCTTGGTGTAACATATCGCATGTTGTGTGCTGACGGACAA
 AGTGCGCTTCATTTTGTGACCGAATGGGGCTCGGATTT**CA**CGAGGTGAGTTATTAGAGTTACCACCTTATCTT**GACAGAAA**
 ATG**TC**TCGGAG**CTGG**GGATCCATCAAGGGCAAGCTCGAGGTTTGAACATGCAGAGTTCGATCCCTCCACCAGTCCTTTTAG
 ATCAATCTGGCAATGAAAGAGAATGTGAGCAGGAGACCGCGGTCACAAT**GTTGAAATTAACAGCAACCCAGG**TCGAGAAA
 TTGCGAAACAAAGCTAATAAAAAGTCAGGAGAGTGAGATGAGCCGTGGATTTACACGGTATGAGGTTGTA**ACTGCACACAT**
 ATGGAGAAGTGCATGCAAAGCGGTAATCACAACCAGAGCAGCCA**ACTGCCTTGGCTATTTGCAT**TGATATTCGAAGTA
 AGATGACGCCACCATTGCCAAGAAAGTACTTTGGAACCGAATTGTCAATGCGATTGCCATCGGCTGCTCCGGTGAGATA
 ATTTCAAACCTTTAGGATACGTTTCGGGTAAACTAAGGGAGGTGATTGAAAAGGTTGACGATGGCTACGTGCACTCGGT
 GTTGGACTTCTGAAGAGTCAACAAGATTTATCACAGTTTCGAGAGTTGCAACCAACAAGTAACAGTGATGAGCATTTT
 ACGGCAATCCAAATCTTGGGGTGATAAGTTGGTTAAACTACCAATCTATGGTGCCGATTTTGGATGGGGAAAGGAGATT
 CATATGGGCCCCGGAACACATGATTCGGATGGT**GAT**CTTTT**GAT**CTTCA**TGG**CATAGATGGAGATGGATCGTTGATAGT
 TGCATTATGTTTACAGTCGAGGCATATGGAAGATTTCCAGAAGGTGTTCTACCAAGATATTGATATGGAATGT**TGA**

Figure 2.18 – *CiSHT1* DNA sequence, sgRNA target sites and the primer sites for sequencing and HRM analysis.

Blue nucleotides indicate the target sites, bold red nucleotides indicate the PAM sequences. Nucleotides highlighted in blue indicate start and stop codons. Black arrows represent primers used for sequencing. Green arrows represent primers for HRM analysis of target 1. Blue arrows represent primers used for HRM analysis of target 2.

CiSHT2

ATCAGAGTACCGTGAAATCTTCTTCCATGATCAAACCAGCCGAACCAACATGGTCTGGTAGGCTCCCACTATCCGAACT
 AGACCAAACAGGAATGAACACACATGTTTCAACCATATATTTCTACACAAAACCCCTCTCAGGACTGGGACACTGTCTCTGC
Seq-SHT2-F
 AAACCTTAAAAGCTTCCCTCACCACCACACTTGTTCATTTCTATCCTTTAGCCAGCCGGCTATCTTCCATCG**CCG**GTGGC
HRM-T1-SHT2-F
CGCCTGGAACTTGACTGCAATGCGGATGGTGTTCAGTTCATCGAAGCTTACGCCGACATGAAACTAGTTGACTTAGATGA
HRM-T1-SHT2-R *HRM-T2-SHT2-F*
 TTTTCATGCAATCTCCAATATTTACCACCTTATTCCTTCCGTAGATTATCG**CCA**TACCCCCCTTGAAGAAGTCCATTAT
 TGCTTTTGCAAGCTACAAAATTTGTGTGTGGAGTTGGTCTTTAGGGTTTTGTATATCACATGTTGTGTGCGACGGTCAA
HRM-T2-SHT2-R
 AGCGCACTTCATTTTCTGTCTGAATGGGCTAGAGCTTGTGCTGGTGAGCCGCTGGATTCACCACCTCATATCGATAGGAA
Seq-SHT2-R
 ATGTTTGCACGCTGGTGAGCCACCGACCACCACATGTTCAAGCATCGAACAACATGTACAGTTTCGTCTCCACCGATCC
 TTTTAGGTCAATCAAGCAATGAAAATGAACGTACCCAGGAAACCACGTAGCAATGCTGAAACTAACAGAACTCTAGTGC
 GAAAAACTGCGAAATAAAGCTAATAAAAAGCCGAAAACCTGAAACGGACCGTGGTTTTACACGGTATGAGGTTGTAACATGC
 ACATGCATGGCGAACAGCATGCAAAGCTCGTAATCTAGAACCAGAGCAGCCAACTGCAGTAGGCATATGTATTGATGTTTC
 GAACCAGAATGAAGCCGCGTTGCCGAAAAGTACTTTGAAAACGCCATTATAGACGTGATTGCCACCGGACTTCCGGT
 GAGTTACTTTTCGAGACATTAGGGTATGTTTCGACTAAGATAAGGAACCGCATCGAGAAGGTTAATGATGAGTATGTGAA
 TTCAGTGATTGACTTCTTAAAAATCAAGAAGATTTATCAAAGTTCCAAGATTGCAACGATTTGGGGACGATGAAGGGC
 CGTTTTACGGGAATCCGAATCTGGGAGTGGTAAGCTGGTTAACATTGCCGATGTACGGTGTGATTTGGGTGGGTAAG
 GAGGAATTTACAGGTCAGGAACCGATGATGCTGATGATGGAGACTTTTGTATCTACGTGGGGAAGAGGGAGGTGGATC
 TCTGGTTGTAGCATTTGTTTGCAGGTGAGACACATGGAGGATTTCAAAGGGTGTCTATCAAAGCATTGAAGAT**TGA**

Figure 2.19 – *CiSHT2* DNA sequence, sgRNA target sites and the primer sites for sequencing and HRM analysis.

Blue nucleotides indicate the target sites, bold red nucleotides indicate the PAM sequences. Nucleotides highlighted in blue indicate start and stop codons, respectively. Black arrows represent primers used for sequencing. Green arrows represent primers for HRM analysis of target 1. Blue arrows represent primers used for HRM analysis of target 2.

Table 2.2 – Relative quantification of phenolamides in *N. benthamiana* agroinfiltrated with *CiSHT1*, *CiSHT2* or *CiSHT1+CiSHT2*.

Plants were infiltrated with either the empty plasmid (Control), *CiSHT1* (35S-*CiSHT1*), *CiSHT2* (35S-*CiSHT2*), or *CiSHT1* plus *CiSHT2* (35S-*CiSHT1*+35S-*CiSHT2*). Pick areas for each ion quantification are expressed per mg of dry weight (DW). RT: Retention time. See legends of Figure 2.11 for details of compound names.

Name	Elemental composition	Quantification ion trace	Control	Relative quantity ($Area \cdot mg^{-1} DW$)			
				35S- <i>CiSTH1</i>	35S- <i>CiSTH2</i>	35S- <i>CiSTH1+STH2</i>	
Mono <i>CiSpd</i> isomer 1	$C_{16}H_{25}N_3O_2$	292.2013	0.027	0.037	101.800	1.325	
Mono <i>CiSpd</i> isomer 2	$C_{16}H_{25}N_3O_2$	292.2013	0.044	5.870	9.041	5.521	
Mono <i>CiSpd</i> isomer 3	$C_{16}H_{25}N_3O_2$	292.2013	0.042	6.456	4.367	2.762	
Di <i>CiSpm</i>	$C_{28}H_{38}N_4O_4$	495.2971	0.026	0.041	20.140	1.967	
Di <i>CiSpd</i> isomer 1	$C_{25}H_{31}N_3O_4$	438.2394	0.100	0.381	21.822	1.816	
Di <i>CiSpd</i> isomer 2	$C_{25}H_{31}N_3O_4$	438.2394	0.125	0.210	242.257	9.040	
Tri <i>CiSpm</i>	$C_{37}H_{44}N_4O_6$	641.3352	0.146	0.120	3.052	6.841	
Tri <i>CiSpd</i>	$C_{34}H_{37}N_3O_6$	584.2749	0.364	5.928	13.289	324.356	
Tetra <i>CiSpm</i>	$C_{46}H_{50}N_4O_8$	787.3723	0.326	0.221	0.277	6.525	

Table 2.3 – Sequence of primers used in this study.

Primer sequence 5' to 3'		Application	Target	Name
ATCGACAGAAAATGTCTGCGAGC	Fwd	Target 1	CiSHT1	T1-SHT1-F
AAACGCTCGCAGACATTTTCTGT	Rev	Target 1	CiSHT1	T1-SHT1-R
ATCGTTGAAATTAACAGCAACCC	Fwd	Target 2	CiSHT1	T2-SHT1-F
AAACGGGTTGCTGTTAATTTCAA	Rev	Target 2	CiSHT1	T2-SHT1-R
ATCGTCAAGTTCAGGCGGCCAC	Fwd	Target 1	CiSHT2	T1-SHT2-F
AAACGTGGCCGCTGGAACCTGA	Rev	Target 1	CiSHT2	T1-SHT2-R
ATCGGACTTCTTCAAGGGGGTA	Fwd	Target 2	CiSHT2	T2-SHT2-F
AAACTACCCCCTTGAAGAAGTC	Rev	Target 2	CiSHT2	T2-SHT2-R
ATATATGGTCTCACTCGAAAAGAACCAACCTGTTTTCATAGC	Fwd	Golden Gate		GG1-F
ATTATTGGTCTCAACCTAAAAAAGCACCGACTCGGTG	Rev	Golden Gate		GG1-R
ATATATGGTCTCAAGGTAAGAACCAACCTGTTTTCATAGC	Fwd	Golden Gate		GG2-F
ATTATTGGTCTCACAGA-AAAAAAGCACCGACTCGGTG	Rev	Golden Gate		GG3-R
ATATATGGTCTCATCTGAAAAGAACCAACCTGTTTTCATAGC	Fwd	Golden Gate		GG3-F
ATTATTGGTCTCAGACTAAAAAAGCACCGACTCGGTG	Rev	Golden Gate		GG4-R
ATATATGGTCTCAAGTCAAAGAACCAACCTGTTTTCATAGC	Fwd	Golden Gate		GG4-F
ATTATTGGTCTCAACCGAAAAAAGCACCGACTCGGTG	Rev	Golden Gate		GG2-R
CGAATGGGCTCGGATTTTAC	Fwd	HRM-Target 1	CiSHT1	HRM-T1-SHT1-F
TCGACCTGGGTTGCTGTTAA	Rev	HRM-Target 1	CiSHT1	HRM-T1-SHT1-R
CGATCCTCCACCAGTCCTTT	Fwd	HRM-Target 2	CiSHT1	HRM-T2-SHT1-R
AATCCACGGTCTATCTCACT	Rev	HRM-Target 2	CiSHT1	HRM-T2-SHT1-R
CCTCACCACCACACTTGTTT	Fwd	HRM-Target 1	CiSHT2	HRM-T1-SHT2-F
ACTGAACACCATCCGCATTG	Rev	HRM-Target 1	CiSHT2	HRM-T1-SHT2-R
GCTTACGCCGACATGAAACT	Fwd	HRM-Target 2	CiSHT2	HRM-T2-SHT2-F
AGACCAACCTCCACACACAA	Rev	HRM-Target 2	CiSHT2	HRM-T2-SHT2-R
AAGTCACAAGGTTTCAATTTGTGGAG	Fwd	Sequencing	CiSHT1	Seq-SHT1-F
TATCAATGCAAATAGCCAAGGCAG	Rev	Sequencing	CiSHT1	Seq-SHT1-R
AGACCAAACAGGAATGAACAC	Fwd	Sequencing	CiSHT2	Seq-SHT2-F
ATCGATATGAGGTGGTGAATCC	Rev	Sequencing	CiSHT2	Seq-SHT2-R

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2.3 Additional elements: Complementation of *Atsht*-KO mutant lines of *A. thaliana* with *CiSHT1* and *CiSHT2* of *C. intybus*.

2.3.1 Introduction

As showcased in the introduction and article constituting the core of this chapter, catalytic activities and, in particular, substrate affinities of SHTs are only partially understood. In this section, we focused on the influence of acyl acceptor availability on activities of CiSHTs. This work aims to elucidate *SHTs* evolutive history and its relationship with the evolutive shift in polyamine ratio in *Asteraceae* pollen.

Indeed, spermidine appears to be the ancestral acyl acceptor for fully substituted phenolamides, which are markers of the pollen coat of Eudicotyledons. If CiSHTs have a higher affinity for spermidine than for spermine, this could indicate that they have retained their ancestral function and affinities. Thus, the *SHT* gene duplication in Chicory, and by extension in *Asteraceae*, would have occurred prior to the polyamine ratio shift in this family. If, on the other hand, their substrate preference for spermine remains even when fed higher levels of spermidine, this could indicate that their activity is due to an intrinsic characteristic. The *SHT* gene duplication in *Asteraceae* may have occurred after the polyamine ratio shift and was positively selected for its relevance to substrate availability in its environment.

To that purpose, the double complementation of *Atsht* KO lines of *A. thaliana* with the *CiSHTs* genes, and the complementation of double-*Cishts* KO lines of *C. intybus* with the *AtSHT* gene (Figure 2.20) were performed.

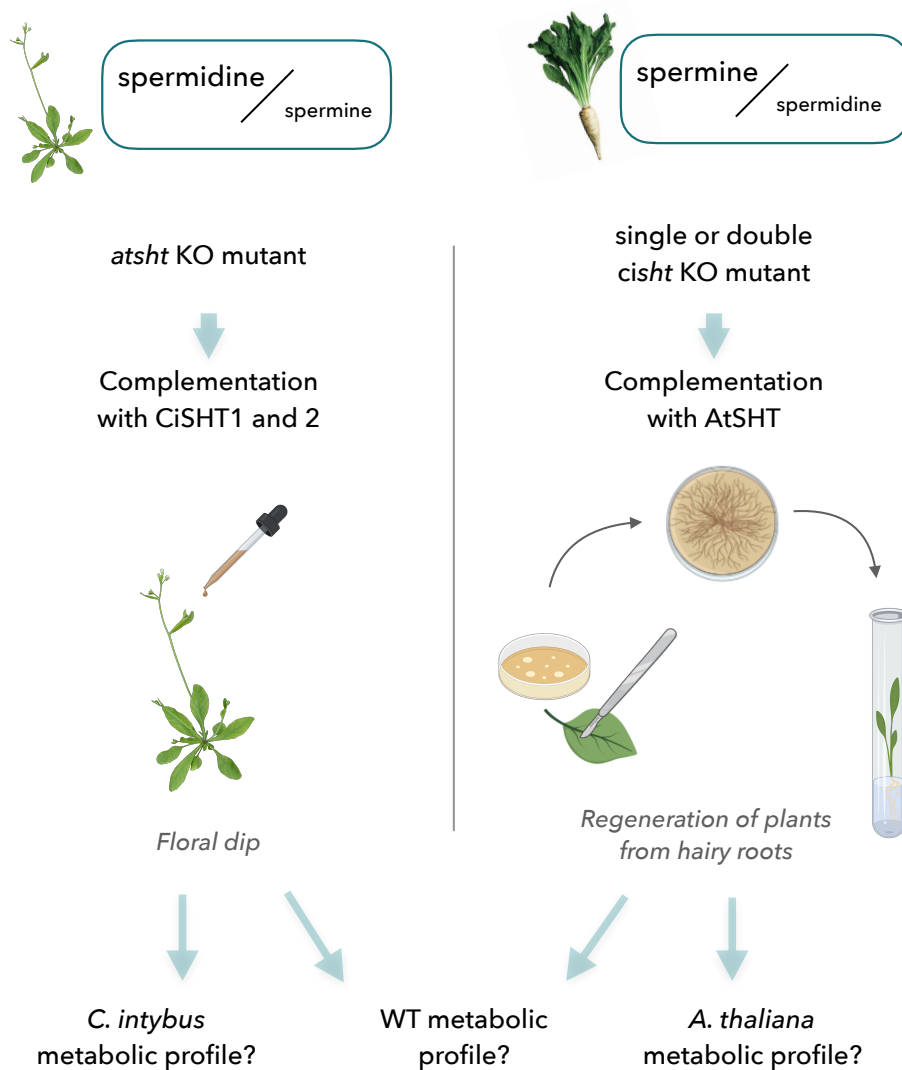


Figure 2.20 – Diagram of the methodology approach used for the study of the influence of the polyamine ratio on SHT activity *in planta*

Complementation of KO (Knock Out) mutants in both species is done by expressing *SHT(s)* of a species with a different polyamine ratio in their anthers using a TA29 promoter. Two methods of stable transformation are represented: floral dip for *A. thaliana* and modified Hairy Roots for *C. intybus*. The analysis of the floral buds metabolic profiles of the complemented lineages will provide insights as to the influence *in planta* of the polyamine ratio on the studied SHT enzymes.

2.3.2 Material and Methods

Plant material

N. benthamiana plants were cultivated as described in section 2.2.9 on page 107.

Lines of *A. thaliana* *Atsht* KO mutant complemented with *CiSHT1* were complemented with *CiSHT2* as well (Figure 2.20). Those lines were created during Marianne Deplore thesis (Delporte, Bernard, et al. 2018) using a modified pB2GW7(TA29) vector (Karimi et al. 2002) conferring a Basta resistance to the transformed individuals. Stable homozygous F2 generation individuals were used for the transformation using the floral dip method (Clough et al. 1998; Figure 2.22 A.). The floral buds of the F2 double complemented generation were collected in liquid nitrogen for further analysis.

Double *Cisht* KO chicory hairy root lines (Bernard 2018) were used for the complementation experiments. They are described in the presented article.

Vectors construction for specific overexpression in the tapetum of *Cisht1*

The hygromycine resistance conferred by the vecteur pH2GW7 (Karimi et al. 2002) allows for the selection of double mutants. This vector was modified to replace promoter 35S by the promoter TA29, a strong and specific promoter of tapetum cells (Grienenberger, Besseau, et al. 2009; Koltunow et al. 1990) using In-Fusion cloning technique (Clontech) with the restriction enzymes *SacI* and *SpeI*. Promoter TA29 was cloned from *N. Tabacum* gDNA using primers 5'- ATGTATGATAATTCGAGCTCCTTTTTGGTTAGCGAATGCA-3' and 5'- TTGTGATATCACTAGTTAGCTAATTTCTTTAAGTAAAAAC-3'. Donor plasmid pDONR207_SHT2 (described in Delporte, Bernard, et al. 2018) was used to clone *CiSHT2* gene in the expression vector pH2GW7(TA29) with the Gateway method. In parallel, donor plasmid pDONR207_AtSHT (described in Delporte 2017) was used to clone *AtSHT* in the expression vector pH2GW7(TA29) with the Gateway method.

Transformation of *A. thaliana* lines for overexpression of *Cisht2*

A. tumefaciens were transformed as described in section 2.2.9 with pH2GW7(TA29)_CiSHT2. The chosen lines of *atsht* KO *A. thaliana* overexpressing *CiSHT1* were transformed using the floral dip method as described in Clough and Bent (1998) (Figure 2.22 A.). The resulting seeds were selected on MS media containing $50\text{mg} \cdot \text{L}^{-1}$ at each generation. 6 homozygotes lineages of F2 generation (Figure 2.22 A.) were obtained and analyzed in HPLC (as described in section 2.2.9) using at least 6 pooled individuals.

Transformation of *C. Intybus* hairy root lines for overexpression of *Atsht*

Creation of *cisht* KO lines of chicory complemented with pH2GW7(TA29)_*Atsht* was performed using the method described in section 2.2.9.

2.3.2.a Metabolite extraction and HPLC analysis

Metabolite extraction and HPLC analysis were performed as described in section 2.2.9.

2.3.3 Results

Complementation of KO mutants of *Atsht* and *Cishts* with heterologous enzymes allows to test the activities of CiSHTs in an environment with an inverted spermine/spermidine ratio (Figure 2.20). The metabolic profile of the double KO mutant *Cishts* overexpressing *AtSHT*, and reciprocally of the two *CiSHTs* in the *Atsht* KO mutant, would provide valuable insight into the impact of free polyamine availability on enzymatic activity. Preliminary findings for the double complementation of *Atsht* KO mutant are presented here.

► **Phenolamide analysis in WT Arabidopsis, in Arabidopsis *sht* mutant and in Arabidopsis *sht* mutant overexpressing CiSHT2**

Compounds	m/z ES(-)	Relative quantity (%)		
		WT	sht	sht/CiSHT2
Feruloyl spermidine	320 [M-H]-	4.2	nd	92.5
Diferuloyl spermidine	496 [M-H]-	14.2	nd	2.4
Trihydroxyferuloyl spermidine	720 [M-H]-	13.9	nd	nd
Dihydroxyferuloylsinapoyl spermidine	734 [M-H]-	100	nd	3.8
Diferuloyl spermine	553 [M-H]-	nd	nd	9
Triferuloyl spermine	729 [M-H]-	nd	nd	11

For relative quantification, phenolamide signals were normalized to the dihydroxyferuloyl sinapoyl spermidine signal.
Nd: not detected.



Figure 2.21 – Mass spectrometry analysis of the metabolic profile of *Atsht* KO mutant lines complemented with *CiSHT2* floral buds. Figure from [Delporte 2017](#)

During her thesis, Marianne Delporte overexpressed *CiSHT1* or *CiSHT2* in the *atsht* KO mutant however the double complementation was not performed. Given the successive actions of CiSHTs in the production of TetraSpm, double complementation appears to be necessary. Nonetheless, the mass-spectrometry analysis of the *atsht* KO lines overexpressing *Cisht2* demonstrated that CiSHT2 alone only partially complements the phenolamide deficiency in the pollen coat of the *atsht* mutant (Figure 2.21). This metabolic profile could be compared to that of a double-complemented line to learn more about the impact of polyamine availability on SHT enzymes (Figure 2.20).

atsht KO lines of *A. thaliana* already complemented with *Cisht1* were complemented with *Cisht2* as well. The floral buds of the F2 generation were analyzed

by HPLC (Figure 2.22).

Three lines used were designated as C1F5, C1G5 and C1H2. At least two complemented filial lineages each were selected until F2 generation, designated as C1F5C2, C1G5C2 and C1H2C2 (Figure 2.22 A.).

The metabolic profiles reveal two things : the wild-type metabolic composition of *A. thaliana* floral bud is not re-established by the double *Cishts* complementation (Figure 2.22 B., C. and D.). Some of the major peaks 2 and 3 of the WT (at 11.5 and 12.5 min respectively) seem to be present in only in small amounts in the C1G5C2 and C1F5C2 double complemented lines (Figure 2.22 B. and C.). However, in the double-complemented lines, we can observe that peak 1 (at 10.2 min) present in the Col-0 WT is recovered compared to the *atsht* KO mutant and single complemented lines. This peak 1 is recovered in all lines but some demonstrate higher expression: both C1H2C2 lines and the C1G5C2 2-1A line (Figure 2.22 B. and D.). The corresponding molecule seems to be a spermidine derivative since it was already present in the WT which does not accumulate spermine derivatives.

In conclusion, some HCAA derivatives of the WT can be recovered in the presence of the two CiSHTs, but the chicory enzymes have a preferential action on different derivatives than AtSHT. Further analysis with mass spectrometry will reveal the identity of the produced metabolites and shed light on the roles of CiSHTs *in planta* as well as the influence of polyamine availability on their activity. The putative spermidine derivative identity of the compound most accumulated in the double-complemented mutants C1H2C2 1A, C1H2C2 5D and C1G5C2 2-1A, whilst different than the derivatives produced by AtSHT, CYP98A9/A8, CYP98A8 and AtSM1 (Figure 2.5 on page 78; [Grienenberger, Besseau, et al. 2009](#)), shows that the highest contents of spermidine in this model might influenced the *in planta* activity of CiSHTs.

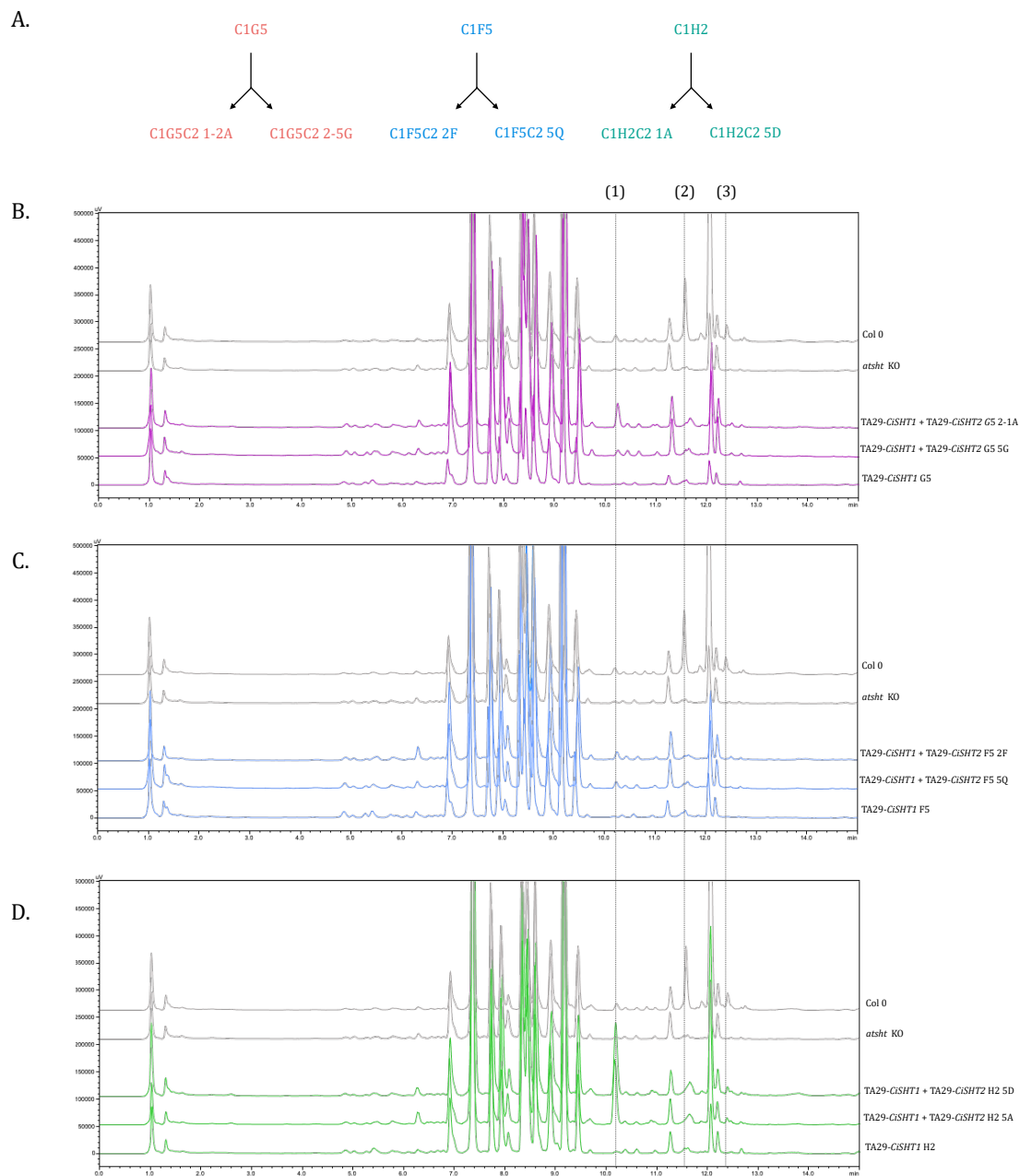


Figure 2.22 – Complementation of *Atsh1* KO *Arabidopsis* with both *CiSHTs*

A. Lines used in this work.

B. Stack of HPLC chromatograms of complemented C1G5 *Arabidopsis* lines with *CiSHT2* and controls.

C. Stack of HPLC chromatograms of complemented C1F5 *Arabidopsis* lines with *CiSHT2* and controls.

D. Stack of HPLC chromatograms of complemented C1H2 *Arabidopsis* lines with *CiSHT2* and controls.

2.4 Discussion

The substrate affinities and types of N-acylation that each CiSHT enzyme can perform were determined. We were able to map out the biosynthetic pathway of TetraSpm in chicory based on these findings (Figure 2.23).

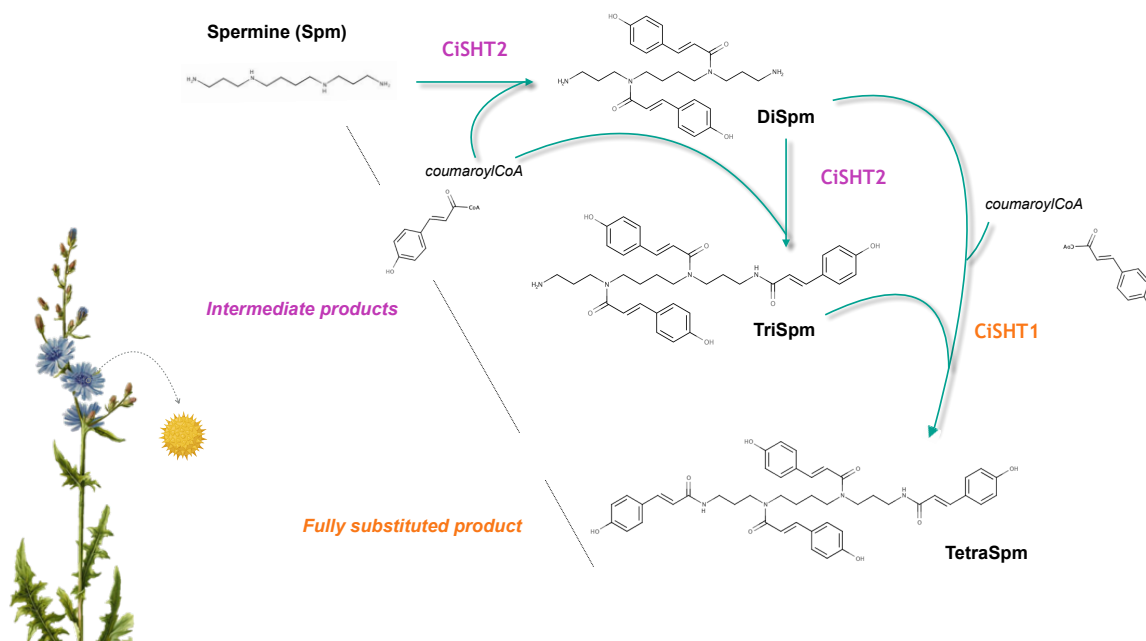


Figure 2.23 – Proposed Tetracoumaroyl-spermine biosynthetic pathway in *Cichorium intybus*

mono-, di- and tri- substituted spermine are designated as intermediate products in this biosynthetic pathway.

We addressed the influence of polyamine ratios available *in planta* on enzyme activity and substrate preferences. We also demonstrated that substrate preferences *in vitro* can differ from those *in planta*, which was highlighted for AtSHT as well in Chapter 3.

The events leading to the catalytic activity of SHTs in *Asteraceae* and their affinity for spermine have not yet been elucidated. In order to determine if the higher availability of spermine in *Asteraceae* pollen coat is at the origin of the activities exhibited by CiSHTs *in planta*, complementation of KO mutants

experiments were realized. Complementation of KO mutants of *Atsht* and *Cishts* with heterologous enzymes is another aspect of this research (Figure 2.20). The metabolic profile of the double KO mutant *Cishts* overexpressing *AtSHT*, and reciprocally of the two *CiSHTs* in the *Atsht* KO mutant, would provide valuable insight into the impact of free polyamine availability on enzymatic activity. The preliminary results presented here seem to confirm this hypothesis.

The various metabolic profiles observed in the different lines obtained could be due to the transformation method. Each transformation event is distinct, and T-DNA from *A. tumefaciens* can be inserted in a different part of the host cell's genome each time (Gelvin 2021). While this is not particularly an issue for transient expression, when it comes to stable transformation, the localization of the inserted T-DNA in the genome has more consequences in the gene expression. The localization of the *CiSHTs* genes in some transformation events may have been more favorable to their proper overexpression in *Arabidopsis* tapetum crating lines with more remarkable metabolite profiles.

The complementation of *Cishts* double KO hairy root lines with pH2GW7(TA29)_AtSHT has been initiated but the specific timing of flowering of the regenerated *C. intybus* plants did not allow further analysis of the transformed lines. Their metabolic profiles will be of great interest and allow a proper comparison of the effects of spermidine over spermine ratio on the studied SHT enzymes.

These results could indicate that that conserved the ancestral affinity towards spermidine and therefore that the duplication of the SHT gene in the *Asteraceae* family occurred before a polyamine ratio shift event. However, no evidence of a causal relationship has been found regarding the correlation between free polyamine ratio and SHT activity observed in *C. intybus* and *A. thaliana*.

The work of Peng and colleagues also supports this hypothesis (H. Peng, Meyer, et al. 2019). In *Solanum richardii*, derivatives of spermine (di- or tri- substituted by caffeoyl moiety) have been identified when they are absent from other related *Solanum* species (Meyer et al. 2015). It appears that a shift in polyamine ratio occurred within this *S. richardii* compared related to *Solanum* species, similarly to

the *Asteraceae* family but to a substantially smaller scale. Moreover, in contrast to what was thought to be a SHT from its domesticated relative *S. melongena*, which uses spermidine as a substrate, Peng and colleagues demonstrated that the SHT from *S. richardii* uses spermine as a substrate. This SrSpmHT's catalytic activity is thus associated with the higher spermine accumulation in this species' fruits, leaves, and cotyledons. However, these *SpmHTs* have been later on characterized as *SCT*¹⁸ or *SDT*¹⁹ through phylogenetic analysis. Like *SCT* and *SDT*, they are able to acylate primary amine functions but not secondary amine functions like SHTs of *A. thaliana*, *M. domestica* or *C. intybus*. The association between the polyamine ratio shift between these *Solanum* species and the activity and substrate preferences of these *SCT/SDT* enzymes remains analogous to the phenomenon observed within SHTs.

A screening of polyamine contents (particularly spermidine and spermine) in *Asteraceae* as well as close species could help pinpoint if and when an event that changed the polyamine ratios in *Asteraceae* compared to other Eudicotyledons families occurred. In addition, these data could help define the relationship between the subfunctionalization in *SHTs* and this polyamine ratio shift. A molecular engineering approach upstream of the pathway to study the metabolism of polyamines in the species of interest is also proposed. For instance, creating *C. intybus* and *A. thaliana* mutants of *SPMS*²⁰ and / or *SPDS*²¹ in order to obtain individuals with an inversion or modification of the polyamine ratio in the anthers. Overexpression of one of the genes could be envisioned using the promoter TA29 to modulate polyamine availability *in situ* (Koltunow et al. 1990).

The deciphering of these pathways allows for the reconstruction of the entire pathway in yeast, as described in the preceding article. Similar to Perrin and colleagues, we could improve our system by using a different yeast strain and the most efficient enzymes for our pathway (Perrin et al. 2021). They were able to produce a total of 19 different HCAA derivatives, including some new to nature by evaluating the efficiency of different *SHT* orthologues and *4CL*²²

¹⁸ *SCT*: Spermidine Dicoumaroyl Transferase ¹⁹ *SDT*: Spermidine Disinapoyl Transferase
²⁰ spermine synthase ²¹ spermidine synthase ²² 4-coumarate:coenzyme A ligase

isoforms separately and in duo. This work led to the metabolic engineering of a sustainable biosynthetic pathway in a modified *S. cerevisiae* strain WT303 without providing external precursors (hydroxycinnamic acids or polyamines) by choosing the most optimal *TAL*²³ and including necessary THCSpd²⁴ modifier enzymes such as *CYP98A8* as seen in the *Brassicaceae* biosynthetic pathway (Figure 2.5 on page 78). This high-quality work allows for efficient yields of metabolite of interest production, flexibility in the type of derivatives formed and is cost efficient due to the absence of external precursors input.

The research presented in this chapter raised questions not only about CiSHTs' evolutive history, but also about the origin of their distinct substrate affinities, despite the fact that their sequences are so similar. According to both Delporte and colleagues (Delporte 2017) and Kruse and colleagues (Kruse et al. 2020), *SHTs*, *HCTs* and *HQTs* belong to the same BAHD clade, Clade V. However, They place the three families of enzymes in different subclades: *SHTs*, *HCTs* and *HQTs* all belong to subclade Vb according to Delporte and colleagues whereas the updated BAHD phylogenetic tree of Kruse and colleagues places them in different subclades (Va for *HCTs* and *HQTs*; Vc for *SHTs*). Their relationship to one another remain unchanged in both phylogenetic analysis: *HCTs* and *HQTs* respectively cluster together, when *SHTs* cluster together as well as with *TpHCT2*. Moreover, *HCTs/HQTs* and *SHTs* share the same substrate affinities for aliphatic amines, an ancestral activity of the BAHD superfamily (Kruse et al. 2020). However, *HCTs* and *HQTs* are present in Dicotyledons and Liveworts whereas the subclade Vc is only found in Dicotyledons. The emergence of *SHTs* following a duplication and then sub- or neo-functionalization of *HCTs* is thus a possibility. They would have acquired the catalytic ability to acylate secondary amine groups during these events.

In the following chapter, these topics will be further addressed with a focus on the origin of the duplication of the SHT gene in *C. intybus* and the origins of their novel acquired substrate affinities and catalytic properties.

²³ tyrosine ammonia-lyase ²⁴ trihydroxycinnamoyl-spermidine

Molecular engineering of SHT

Introduction

In chapter 2, we have demonstrated that CiSHT2 and CiSHT1 act in a sequential manner and that they are able to use either spermine or spermidine as aliphatic polyamine substrates. In this chapter, molecular features of these two very similar proteins that are responsible for these catalytic differences were investigated. The structural difference between chicory SHTs and other Eudicotyledons SHTs were also searched in an attempt to decipher why their substrate promiscuity is different.

CiSHTs do differ from other Eudicotyledons SHTs in several ways. The presence of two *SHT* copies is the most obvious. TetraSpm two-step production process is also unusual. However, attempting to determine the enzymatic properties of each CiSHT *in vitro* is not feasible. Because CiSHTs are interdependent in this pathway, traditional enzymatic characterization methods are not viable. Furthermore, the lack of standards for the intermediate products of the pathway (*i. e.* monocoumaroyl-spermine , dicoumaroyl-spermine , and tricoumaroyl-spermine) renders *in vitro* assays impossible. Thus, in order to determine the elements that distinguish *CiSHTs* from other *SHTs*, typical characteristics of *SHTs* had to be determined first. Model *SHTs* were to be used to lay the groundwork

before moving on to further characterization of *CiSHTs*.

A novel BAHD, termed *spmHT*, with a clear preference for spermine as acyl acceptor was identified and characterized in *Solanum richardii* (*Solanaceae*) (H. Peng, Meyer, et al. 2019). This protein is present in the fruits and flowers of this non domesticated variety of eggplant. In contrast to the domesticated cultivars studied, *S. richardii* the ratio between spermine and spermidine is in favor of spermine. In this study, three points should be highlighted. The first is that this *SpmHT* is more closely related to *NaDH29* phylogenetically (subclade IIIc of BAHDs, Kruse et al. 2020) than to SHTs (subclade Vc of BAHDs, Kruse et al. 2020) and it can only acylate primary amines of spermine. The second is that it lacks the FYGN motif, which is present in all SHT able to promote acylation on secondary amine functions, but this does not affect its preference for spermine as a substrate (Delporte 2017). The last aspect is that *spmHT* seems to be silenced in other eggplant cultivars. Thus, the authors suggest that the expression of *SHT* is exclusive to these cultivars. There is no evidence of a fully substituted spermine, which is consistent with Marianne Delporte's earlier work (see introduction of chapter 2). As a result, it was not a good model for our study.

The acylation activity of *CiSHT2*, on the other hand, is very similar to that of *AtSHT* characterized by Grienberger (2009, Figure 3.1). However, no *TetraSpm* was found in *A. thaliana* pollen coat. We established in chapter 2 that the ratio between spermidine and spermine are inverted between *C. intybus* and *A. thaliana*. The *in vitro* enzymatic assays with recombinant *CiSHT* proteins purified from BL21 *E. coli* shows that they are able to use spermidine as a substrate (Delporte, Bernard, et al. 2018). So, the first hypothesis to that peculiar activity and metabolite presence in *Asteraceae* is due to the accumulation of more spermine than spermidine in *C. intybus*. Thus, in both *A. thaliana* and *C. intybus*, the polyamine backbone of the fully substituted phenolamide marker of their pollen coat coincides with the polyamine in favor in the ratio of spermidine to spermine accumulating in their reproductive organs.

TpHCT2 (*Trifolium pratense* hydroxycinnamoyl-CoA:shikimic acid/quinic acid hydroxycinnamoyltransferase 2) is a BAHD enzyme which protein has a high sequence homology with those of SHT. It belongs to the same subclade as SHTs (Vc) and not with other HCT/HQT (Va) (Kruse et al. 2020). Despite this phylogenetic promiscuity, TpHCT2 exhibits a different activity than SHTs. It catalyzes the production of phasic acid through the acyltransfer of caffeoylCoA onto a malate residue (M. Sullivan 2009; Figure 3.1). CoumaroylCoA can also be used as an acyldonor. An alignment with known SHT such as AtSHT (*Arabidopsis thaliana* Spermidine Hydroxycinnamoyl Transferase) or SHT1s and SHT2s of *Asteraceae*, all catalyzing amidations, reveals that instead of the consensus motif and putative catalytic site of SHTs FYGN, the phenylalanine and tyrosine residues are inverted in TpHCT2 (YFGN) (Figure 3.1). It was hypothesized that this modification in the consensus motif of SHT could have given rise to a different activity in TpHCT2. TpHCT2 and AtSHT were thus chosen for this study because they both perform one step reactions, contrary to CiSHTs, and their enzymatic properties have been characterized previously (Grienenberger, Besseau, et al. 2009; M. Sullivan 2009).

In large and fast evolving superfamilies of enzymes involved in the synthesis of specialized metabolites such as BAHDs, unraveling the origin of a change in substrate preferences and promiscuity is a challenge. Here we ponder on a smaller scale what Kruse and colleagues recently questioned using the BAHD superfamily as a model (2020): does this substrate diversity originates from ancestral substrate promiscuity or did it emerge after the duplication and neofunctionalization of the SHT in *Asteraceae* (Delporte 2017)? Does this novel activity and thus unusual natural product occur as a result of a specialization of SHTs in *Asteraceae* or because of a difference in substrate availability? Which event is at the origin of the other: what is the evolutive history of CiSHTs? And what makes them different from SHTs of other Eudicotyledons?

To answer those questions, a more extensive *in silico* search was performed on the protein sequences of CiSHTs and of SHTs of *Asteraceae* to identify putative active sites as well as conserved amino acid common to each kind of SHT or

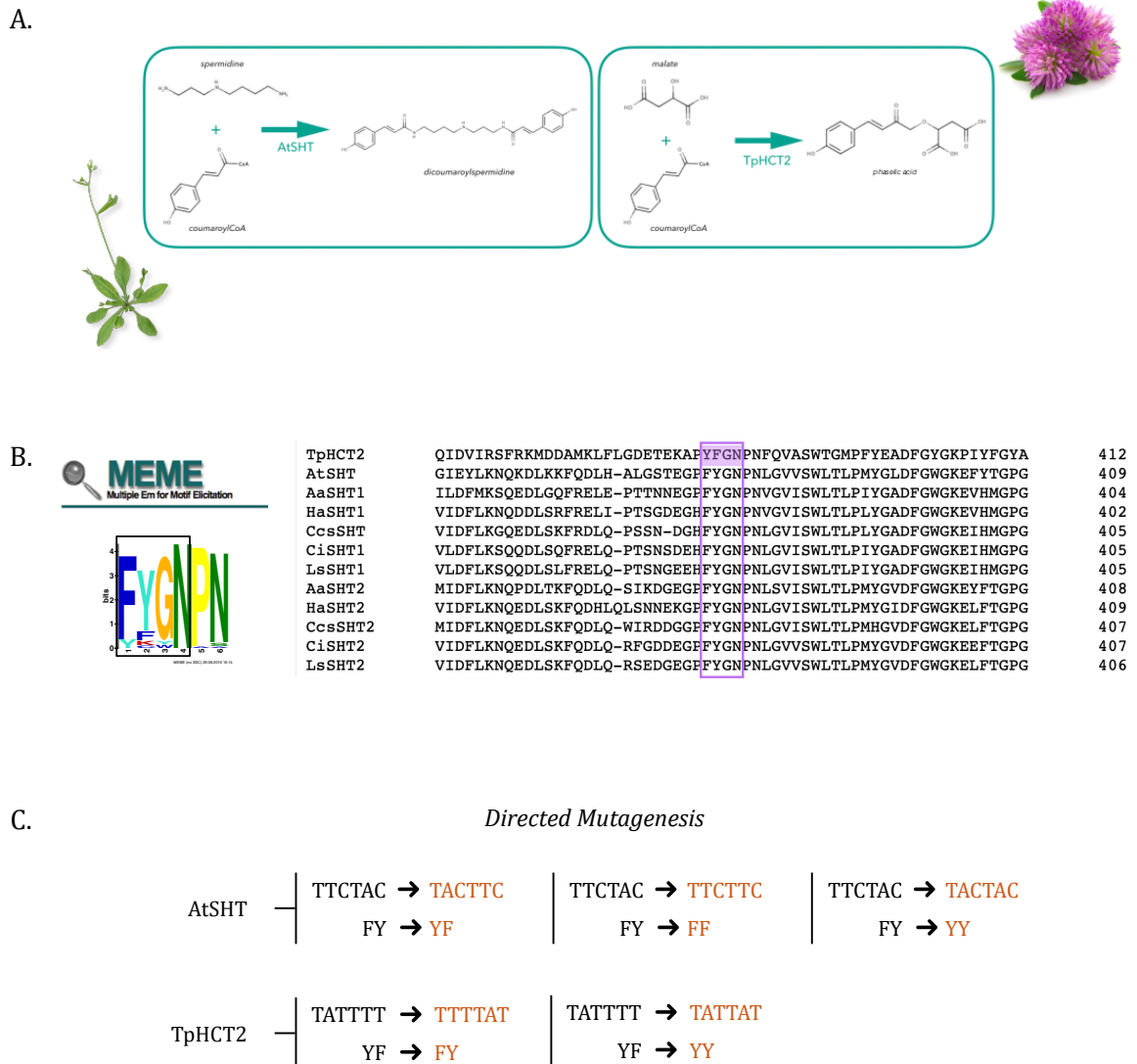


Figure 3.1 – Characteristics of the proteins of interest and mutation strategy
A. Main activities of AtSHT (Grienenberger, Besseau, et al. 2009) and TpHCT2 (M. Sullivan 2009).
B. Identification of the FYGN consensus motif in SHTs and alignment of SHTs with TpHCT2 highlighting the inversion of two amino acid residues in its sequence (overlaid in purple)
C. Nucleotide and protein sequences of the performed directed mutagenesis on the consensus motif

amino acid conserved in SHT2s as well as AtSHT (section 3.2.2).

Firstly, a motif of interest in the BAHD enzymes AtSHT (*A. thaliana*) and TpHCT2 (*T. pratense*) was studied (Grienenberger, Besseau, et al. 2009; M. Sullivan 2009). AtSHT and TpHCT2 both group with CiSHT1 and CiSHT2 in the phylogenetic tree of the BAHD superfamily in subclade Vc, rendering their study even more relevant to gain understanding regarding their evolutive history (Kruse et al. 2020).

AtSHT was first assayed in the presence of variety of common acyl acceptors (section 3.2.1). The main goal was to determine if this enzyme could use spermine as a substrate. The measurement of the activity of TpHCT2 towards the same range of acyl acceptor was also conducted. Site-directed mutagenesis was performed on the above-mentioned amino acid motif in both proteins and the activities of the mutated proteins produced in two different heterologous systems were examined (Figure 3.2).

In parallel of the site-directed mutagenesis on AtSHT and TpHCT2, search for potential essential amino acids responsible for the modification in substrate promiscuity of CiSHTs was conducted. To this end, chimeras of CiSHT1 and CiSHT2 were generated to observe if one or several part(s) of the proteins would lead to an inversion of activity hence giving us a better idea as to where a putative essential amino acid for substrate affinity would be located (Figure 3.2). These studies will contribute to answering the questions raised in this introduction.

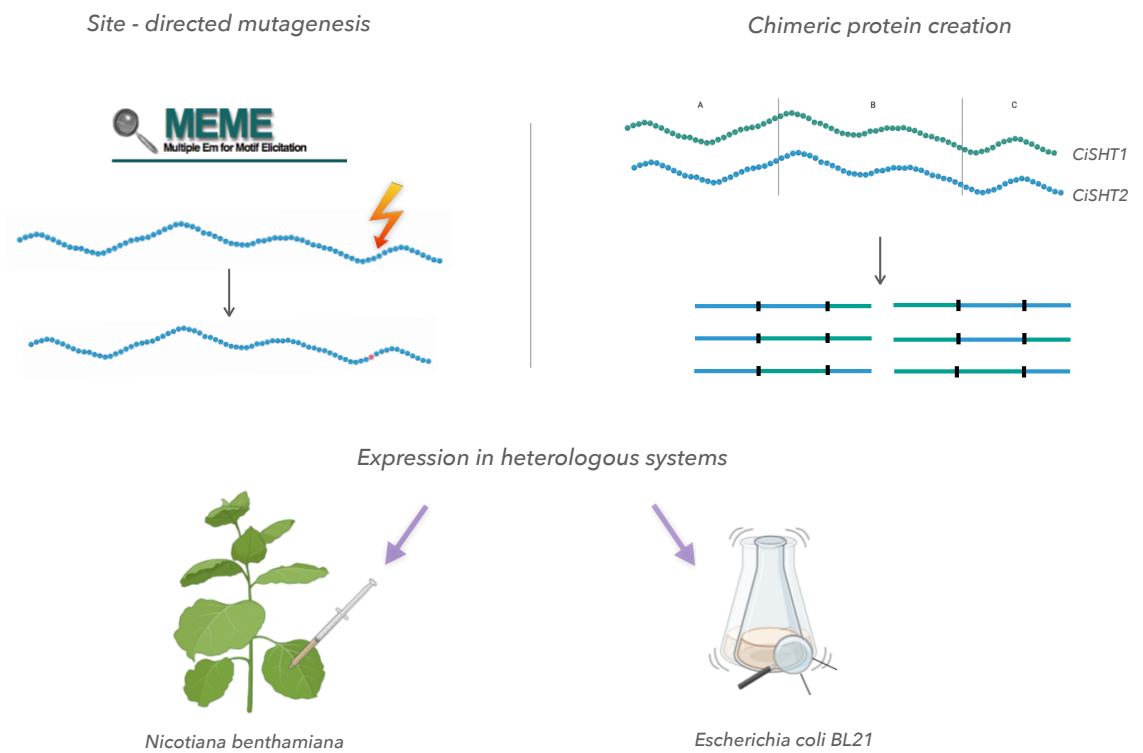


Figure 3.2 – Strategies for SHT-like structural analysis

3.1 Material and Methods

3.1.1 Plant Material

N. benthamiana for transient expression

N. benthamiana seeds were sown on soil and cultivated in culture rooms under a 16 h/8 h day/night photoperiod with artificial light ($70 \mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$) at 26 °C. Five to six-week-old plants were used for agroinfiltration experiments, as described in [Waadt et al. 2008](#).

3.1.2 Methods

Computational analysis

Conserved motif determination was conducted with the MEME suite ([Bailey et al. 2009](#)). The prediction of putative N-terminal signal peptide (SP) was performed using TargetP 2.0 (<http://www.cbs.dtu.dk/services/TargetP/>). To predict putative amino acid sites for post-translational modifications such as glycosylation, the ModPred tool was used (<http://montana.informatics.indiana.edu/ModPred/>). The alignments and phylogenetic analyses were performed using the MEGA X software (<https://megasoftware.net>; [S. Kumar et al. 2018](#)). Alignments were done using the MUSCLE program. To determine the parameters of the phylogenetic studies, a model was generated and gave the following parameters: Maximum Likelihood Tree with the heuristic method was Nearest-Neighbor-Interchange (NNI). Phylogenetic trees were reconstructed using this algorithm with 1000 replications of bootstrap. Three dimensional modelling was performed for the CiSHTs proteins. Alignment of the sequences of native and chimera CiSHTs with cleaned up sequence of homologue *CcHCT* (PDB reference 4G0B; [Lallemand et al. 2012](#)) were performed with ExpasyClustal ([Madeira et al. 2019](#)). Homology models done with modeller 9.25 ([Sali et al. 1993](#); [Fiser et al. 2000](#)) using the model-multiply.py and loop.py codes for each protein sequences. Results were analyzed and images generated with PyMOL version 2.x ([Schrödinger 2021](#)).

Site-directed mutagenesis by overlap extension using polymerase chain reaction

Generation of site-directed mutated proteins was done using two consecutive PCRs using proofreading polymerase (PrimeSTAR HS DNA polymerase, Takara). DNA sequences were amplified from *C. intybus* cDNA using the external oligo primers binding at the 5' or 3' extremities of the cDNA sequence respectively, as well as primers that hybridize in the site of interest containing one or two mismatching nucleotides (see Figure 3.1) in order to introduce the desired mutations and overlapping sequences flanking the mutation site. The obtained DNA fragments were purified according to the manufacturer's instruction (Macherey-Nagel PCR purification kit). The purified PCR fragments were amplified in a second PCR using the external oligo primers binding either at the starting ATG codon or the stop codon and containing the gateway adaptors for subsequent cloning in donor vectors. The entry clones (pDONR207_AtSHT, pDONR207_AtSHT*YF, pDONR207_AtSHT*YY, pDONR207_AtSHT*FF and pDONR207_TpHCT2, pDONR207_TpHCT2*FY, pDONR207_TpHCT2*YY) were obtained through BP recombination of the PCR products with pDONR207 (Invitrogen) and sequenced. For further expression analysis, the open reading frames were introduced into pEAQ-HT_DEST1 expression vector (kindly provided by Professor George Lomonosof, John Innes Center) by LR recombination for transient expression in *N. benthamiana* (pEAQ-HT-DEST1_AtSHT, pEAQ-HT-DEST1_AtSHT*YF, pEAQ-HT-DEST1_AtSHT*YY, pEAQ-HT-DEST1_AtSHT*FF and pEAQ-HT-DEST1_TpHCT2, pEAQ-HT-DEST1_TpHCT2*FY, pEAQ-HT-DEST1_TpHCT2*YY) or in pDEST17 (pDEST17_AtSHT, pDEST17_TpHCT2 and pDEST17_TpHCT2*FY) for expression in *E. coli* BL21-CodonPlus-(DE3)-RIL cells. Primers are listed in Table 3.1.

Chimera construction by overlap extension using polymerase chain reaction

Generation of chimeric proteins was done using a similar protocol as for the site-directed mutagenesis experiment (3.1.2) using two consecutive PCRs using proofreading polymerase (PrimeSTAR HS DNA polymerase, Takara).

DNA sequences were amplified using the external oligo primers as well as

primers that hybridize in the site of interest overlapping on the flanking sequences of the site of cutoff between two sections of the genes of interest (see Figure 3.9). The obtained DNA fragments were purified according to the manufacturer's instruction (Macherey-Nagel PCR purification kit). The purified PCR fragments were amplified in a second PCR using the external oligo primers containing the gateway adaptors for subsequent cloning in donor vectors. The entry clones (pDONR207_CHIM_I to VI) were obtained through BP recombination of the PCR products with pDONR207 (Invitrogen) and sequenced. For further expression analysis, the open reading frames were introduced respectively into pEAQ-HT_DEST2 expression vector (Invitrogen) that fused a N-terminal 6xHisTag to the protein of interest by LR recombination for transient expression in *N. benthamiana* (pEAQ-HT_DEST2_CHIM_I to VI). Previously reported donor vectors pDONR207-SHT1 and pDONR207-SHT2 (Delporte 2017) were used to generate expression vector as well (pEAQ-HT_DEST2_CiSHT1 or 2). The primers are listed in Table 3.1.

Expression, purification and *in vitro* assays of recombinant proteins in BL21 *Escherishia coli*

Expression and purification of the recombinant proteins

Recombinant proteins with an N-terminal 6XHis-tag pDEST17_AtSHT, pDEST17_TpHCT2 and pDEST17_TpHCT2*FY were expressed in *E. coli* BL21-CodonPlus-(DE3)-RIL cells. Following induction with 1 mM isopropyl β -D-1-thiogalactopyranoside (IPTG), cells were harvested by centrifugation (10 000 g, 4 °C, 30 min) and pellets stored at -80°C. Cell pellets were resuspended in the extraction buffer (50 mM NaH₂PO₄, 500 mM NaCl, 20 mM imidazole pH 7.4) added with 1 mg · mL⁻¹ lysozyme and the suspensions were incubated on ice for 30 min. After sonication on ice, samples were centrifuged (5 000 g, 4 °C, 30 min) and the supernatants were loaded onto 1 ml HisTrap HP columns (GE Healthcare) and processed according to the manufacturer's procedures. Protein concentrations of the extracts were determined using Bio-Rad Protein Assay using bovine serum albumin as a standard. SDS-PAGE and immunoblot analysis was conducted as described elsewhere (Sambrook et al. 1989). The following antibody combi-

nation were used for immunodetection: penta-His antibody (Qiagen)/alkaline phosphatase (AP)-conjugate anti- mouse IgG (Promega). To estimate molecular masses, the Precision Plus Protein All blue Standards ladder (Bio-Rad) was used.

***In vitro* assays**

The standard *in vitro* assay was performed in a total volume of 50 μL containing 400 μM acyl donor (hydroxycinnamoyl-CoA) and 2 mM acyl acceptor freshly prepared in 50 mM phosphate buffer (Figures 3.5, 3.4, 3.6). Hydroxycinnamoyl-CoA were prepared enzymatically as described in (Legrand et al. 2016). After incubation at 28°C from 0h to 24h, the reactions were stopped by adding 15 μL acetonitrile and 1 μL 12 N HCl. pH optima were established according to the native proteins preferential pH previously determined: pH 9 for AtSHT and pH 6.5 for TpHCT2 (Grienenberger, Besseau, et al. 2009; M. Sullivan 2009). Reaction mixtures were then centrifuged at 14 000g for 5min and filtered through a 0.45 μm filter before HPLC analysis.

Transient expression of recombinant SHT proteins in *N. benthamiana*

Vectors pEAQ-HT-DEST1_AtSHT, pEAQ-HT-DEST1_AtSHT*YF, pEAQ-HT-DEST1_AtSHT*YY, pEAQ-HT-DEST1_AtSHT*FF and pEAQ-HT-DEST1_TpHCT2, pEAQ-HT-DEST1_TpHCT2*FY, pEAQ-HT-DEST1_TpHCT2*YY as well as the empty vector pEAQ-HT were introduced by electroporation in *Agrobacterium tumefaciens* strain GV2260. The same procedure was done for vectors pEAQ-HT-DEST2_CHIM_I to VI, pEAQ-HT-DEST2_CiSHT1 or 2 as well as the empty vector pEAQ-HT.

All strains were selected on Yeast Extract Beef (YEB) medium (10 $\text{g} \cdot \text{L}^{-1}$ beef extract, 5 $\text{g} \cdot \text{L}^{-1}$ yeast extract, 10 $\text{g} \cdot \text{L}^{-1}$ peptone, 15 $\text{g} \cdot \text{L}^{-1}$ sucrose, 0.5 $\text{g} \cdot \text{L}^{-1}$, MgSO_4 , 20 $\text{g} \cdot \text{L}^{-1}$ at pH 7.2) supplemented with 100 $\mu\text{g} \cdot \text{mL}^{-1}$ kanamycin. The recombinant bacteria strains were grown overnight in YEB medium supplemented with 100 $\mu\text{g} \cdot \text{mL}^{-1}$ kanamycin at 28 °C with shaking at 200 rpm until OD600=0.6. The cultures were then pelleted via centrifugation (10 min, 3000 g) and resuspended in infiltration buffer (10 mM MgCl_2 , 10 mM MES, pH 5.7, 100 μM acetosyringone) to OD600=0.6. Each construct was used to infiltrate the

abaxial air space of the two young leaves of 5-6 weeks old *N. benthamiana* plants using a needleless syringe (Waadt et al. 2008). After four days, the infiltrated leaves were collected and immediately frozen in liquid nitrogen. The leaves destined to metabolic extraction were freeze-dried before extraction.

Phenolic compounds extraction and HPLC analysis

The lyophilized plant material was powdered and 25 mg was resuspended in 1 mL of a methanol/water/acetic acid mixture (75/23/2, v/v/v). After a 1 h incubation in the dark under agitation at 4 °C, the homogenates were clarified by centrifugation (14 000 g, 4 °C, 10 min) and a second extraction with 1 mL of solvent was done. The pooled supernatants were passed through a 0.45 μm filter. Metabolites were analyzed by HPLC/DAD. The HPLC system was a Prominence HPLC system (Shimadzu, Marne la Vallée, France) consisting of a quaternary pump (LC-20AD) and a UV-visible diode array detector (SPD-20A). Analyses were performed on a 100 mm \times 4.6 mm Kinetex 2.6 μm PFP 100 Å column (Phenomenex). The chromatographic separation was performed using water (solvent A) and acetonitrile (solvent B) both acidified with 0.1 % ortho-phosphoric acid. The solvents were delivered at a flow rate of 1.1 $\text{mL} \cdot \text{min}^{-1}$ and the oven temperature was set at 45°C. The HPLC conditions for metabolic contents analysis were as follow: start at 10 % solvent B, 7 min gradient to 30 % solvent B followed by 3 min gradient to 70 % and 1.5 min of isocratic 70 % solvent B. Then, 1 min gradient to return to 10 % solvent B and 8.5 min of isocratic re-equilibration at 10 % solvent B.

Annexes

Table 3.1 – Primers used in the structural analysis of SHTs

Code	Sequence	Target cDNA	Usage	Fwd or Rev
DG460	888gACAAGTTTGTACAAAAAAGCAGGCTccaccATGGTTACCAATTAATAAAATTCCTTAC	TpHCT2	Plant overexpression	Fwd
DG461	888gACAAGTTTGTACAAAAAAGCAGGCTccaccATGGCTCCCAATACCTTTAGAAAAATCTTA	ASHT	Plant overexpression	Fwd
DG391	888gACCACCTTTGTACAAGAAAGCTGGGTCTAGATATCTTCATAAAAGTGTTCCTTG	ASHT	Plant overexpression	Rev
DG389	888gACCACCTTTGTACAAGAAAGCTGGGTcTCATATATCTCATAGAAAGTAC	TpHCT2	Plant overexpression	Rev
DG484	AGCAGGGAAGGTCCATACACGGAAACCCAAATCCT	ASHT*YY	FYGN site directed mutagenesis	Fwd
DG485	AAGATTTGGTTTCCGTAGTATGGACCTTCCCGTGT	ASHT*YY	FYGN site directed mutagenesis	Rev
DG486	AGCAGGGAAGGTCCATTCGGAAACCCCAATCCT	ASHT*FF	FYGN site directed mutagenesis	Fwd
DG487	AAGATTTGGTTTCCGAAGAATGGACCTCCCGTGT	ASHT*FF	FYGN site directed mutagenesis	Rev
DG488	ACTGAAAAAGCACCATATATGGAAATCCAAATTTT	TpHCT2*YY	FYGN site directed mutagenesis	Fwd
DG489	AAAATTTGGATTTCCATAATATGGTCTTTTTCAGT	TpHCT2*YY	FYGN site directed mutagenesis	Rev
DG490	ACTGAAAAAGCACCAATTTTGGAAATCCAAATTTT	TpHCT2*FF	FYGN site directed mutagenesis	Fwd
DG491	AAAATTTGGATTTCCAAAAAATGGTCTTTTTCAGT	TpHCT2*FF	FYGN site directed mutagenesis	Rev
DG492	AGCAGGGAAGGTCCATACCTCCGGAAACCCCAATCCT	ASHT	FYGN site directed mutagenesis	Fwd
DG493	AAGATTTGGTTTCCGAAGTATGGACCTCCCGTGT	ASHT	FYGN site directed mutagenesis	Rev
DG494	ACTGAAAAAGCACCAATTTATGGAAATCCAAATTTT	TpHCT2	FYGN site directed mutagenesis	Fwd
DG495	AAAATTTGGATTTCCATAAATGGTCTTTTTCAGT	TpHCT2	FYGN site directed mutagenesis	Rev
DG125	888gACAAGTTTGTACAAAAAAGCAGGCTccATGAAGGTTTCCAAACAAGACTTCT	CiSHT1	Plant overexpression	Fwd
DG126	888gACCACCTTTGTACAAGAAAGCTGGGTcCAACATCCATATCAATATCTTGGT	CiSHT1	Plant overexpression	Rev
DG285	888gACAAGTTTGTACAAAAAAGCAGGCTccATGAGAGTCACCGTGAATCTTC	CiSHT2	Plant overexpression	Fwd
DG286	888gACCACCTTTGTACAAGAAAGCTGGGTcCAATCTTCAATGCTTTGATAGAAC	CiSHT2	Plant overexpression	Rev
DG338	GCTCTAGCCCAATCAGACAGAAATGAAGCCACTTTGTCC	CiSHT2B-CiSHT1A	Chimera construction	Rev
DG339	CTGTCTGAATGGCTAGAGC	CiSHT2	Chimera construction	Fwd
DG340	CAGATTCGGATTCCTCCGTAAAA	CiSHT2	Chimera construction	Rev
DG341	TTTTACGGGAATCCGAATCTGGGGGTGATAAGTTGGTTAACA	CiSHT2B-CiSHT1C	Chimera construction	Fwd
DG342	CCGAGCCCAATCGGTCCAAAAATGAAAGTGGCTTTGACC	CiSHT1B-CiSHT2A	Chimera construction	Rev
DG343	GTGACCGAATGGGCTCGGA	CiSHT1	Chimera construction	Fwd
DG344	AAGATTTGGATTTCCGTAAAAAATG	CiSHT1	Chimera construction	Rev
DG345	CATTTTACGGCAATCCAAATCTTGGAGTGGTAAGCTGGTTAACAT	CiSHT1B-CiSHT2C	Chimera construction	Fwd

3.2 Results

3.2.1 Directed mutagenesis on a conserved motif of SHTs

As illustrated in the introduction to this chapter, TpHCT2 and AtSHT were used to study the conserved motif of SHT proteins, FYGN. The activities of the native proteins had to be assessed before performing site-directed mutagenesis on this site to provide a control condition. TpHCT2 and AtSHT were expressed in a BL21 *E. coli* system and purified (Figure 3.3).

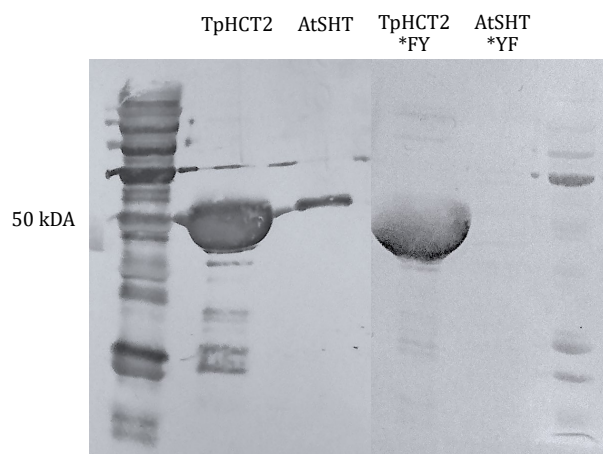


Figure 3.3 – Immunoblot analysis confirming the expression of the recombinant proteins produced in BL21 *E. coli*. The proteins were detected with an anti-His antibody. All proteins were successfully purified except for AtSHT*YF as can be seen on the Western-Blot membrane. The figure is composed of different membranes.

The activities *in vitro* of both purified enzymes were assayed using different acyl acceptors and at two pHs. AtSHT preferential pH is 9 whereas the preferential pH of TpHCT2 is 6.5. All assays were performed at both pH and following a 24h kinetic. To see if TpHCT2 could synthesize polyamine derivatives, we assayed its activity toward spermine, spermidine as well as other common acyl acceptors. We employed coumaroylCoA in all assays for an easier comparison with assays with CiSHTs since they preferentially use this acyldonor *in vivo*. The

controls for each assay were done by omitting the acyl acceptor.

At its optimal pH (6.5), TpHCT2 demonstrates the ability to only use malate, its preferential acyl acceptor, to produce coumaroylmalic acid (Figure 3.4). TpHCT2 can not utilize polyamines, quinate nor shikimate in those conditions. However, at pH 9 its flexibility for acyl acceptors increases since it can utilize more substrates with a clear preference for spermidine and malate. Derivatives of the polyamines (respectively undetermined derivatives of spermine and monocoumaroyl-spermidines) are produced and higher contents of coumaroyl-malate (Figure 3.4).

AtSHT displays an activity only at its optimal pH (9) and only towards spermine and spermidine (Figure 3.5). Major phenolamides produced are respectively tricoumaroyl-spermine and dicoumaroyl-spermidine. No other acyl acceptors were used by the enzyme (Figure 3.5).

TpHCT2 has more versatility and promiscuity when it comes to acyl acceptors and pH conditions. It does, however, produces derivatives with fewer substitutions than AtSHT.

A directed mutagenesis experiment was done to introduce the FYGN motif in TpHCT2 and the YFGN motif in AtSHT to test the hypothesis that the inversion between the phenylalanine and tyrosine residues in the consensus motif is responsible for such divergent abilities to utilize substrates (Figure 3.1 C.). Mutated proteins were named TpHCT2*FY and AtSHT*YF. Both native and mutated proteins were heterologously expressed in *N. benthamiana* (not shown). The mutations however resulted in a complete inactivation of the proteins. The proteins were then mutated on only one of the residues. Only mutated proteins TpHCT2*YY, AtSHT*FF and AtSHT*YY were successfully cloned. After expression in *N. benthamiana*, a similar result was observed. No activity was detected for any of the mutated proteins (not shown). Although the altered proteins did not show the expected changes in substrate affinities, the data nonetheless highlighted the importance of this consensus motif in both AtSHT and TpHCT2.

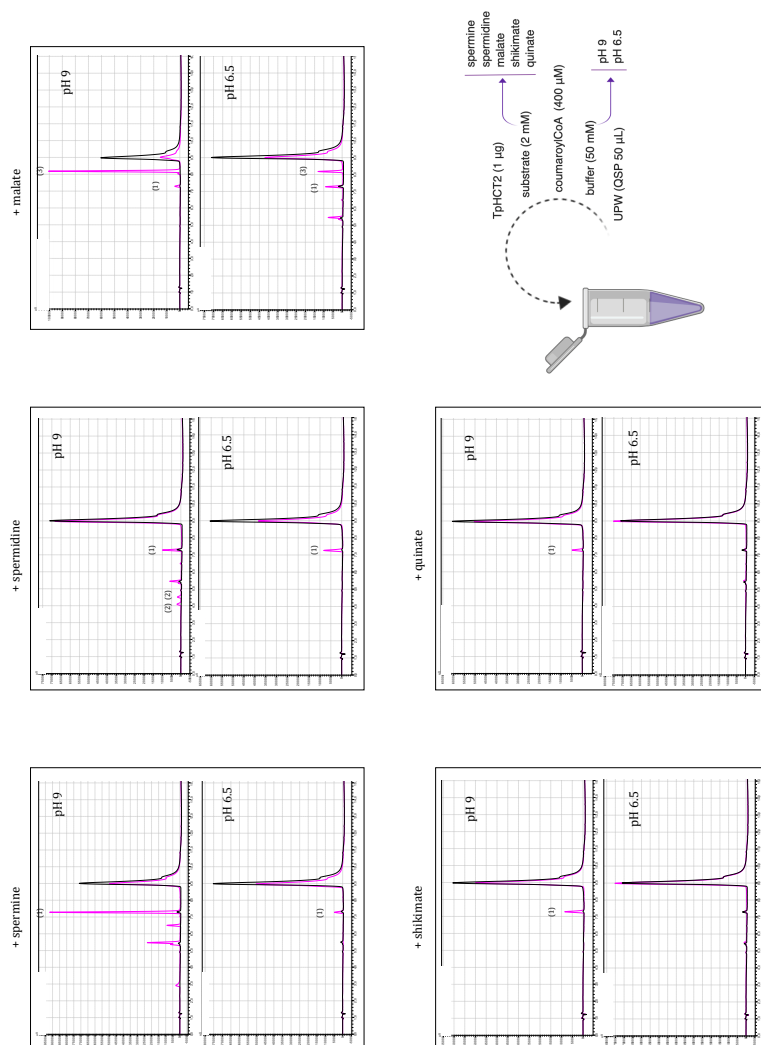


Figure 3.4 – *In vitro* assays of TpHCT2 activity towards a variety of acyl acceptors at different pH. Assays were incubated 3 h at 28 °C in obscurity and analyzed in HPLC. Controls performed by omitting the acyl acceptor in the mix are represented in black. (1) coumaroyl-glycerol (2) monocoumaroyl-spermidine (3) coumaroyl-malate

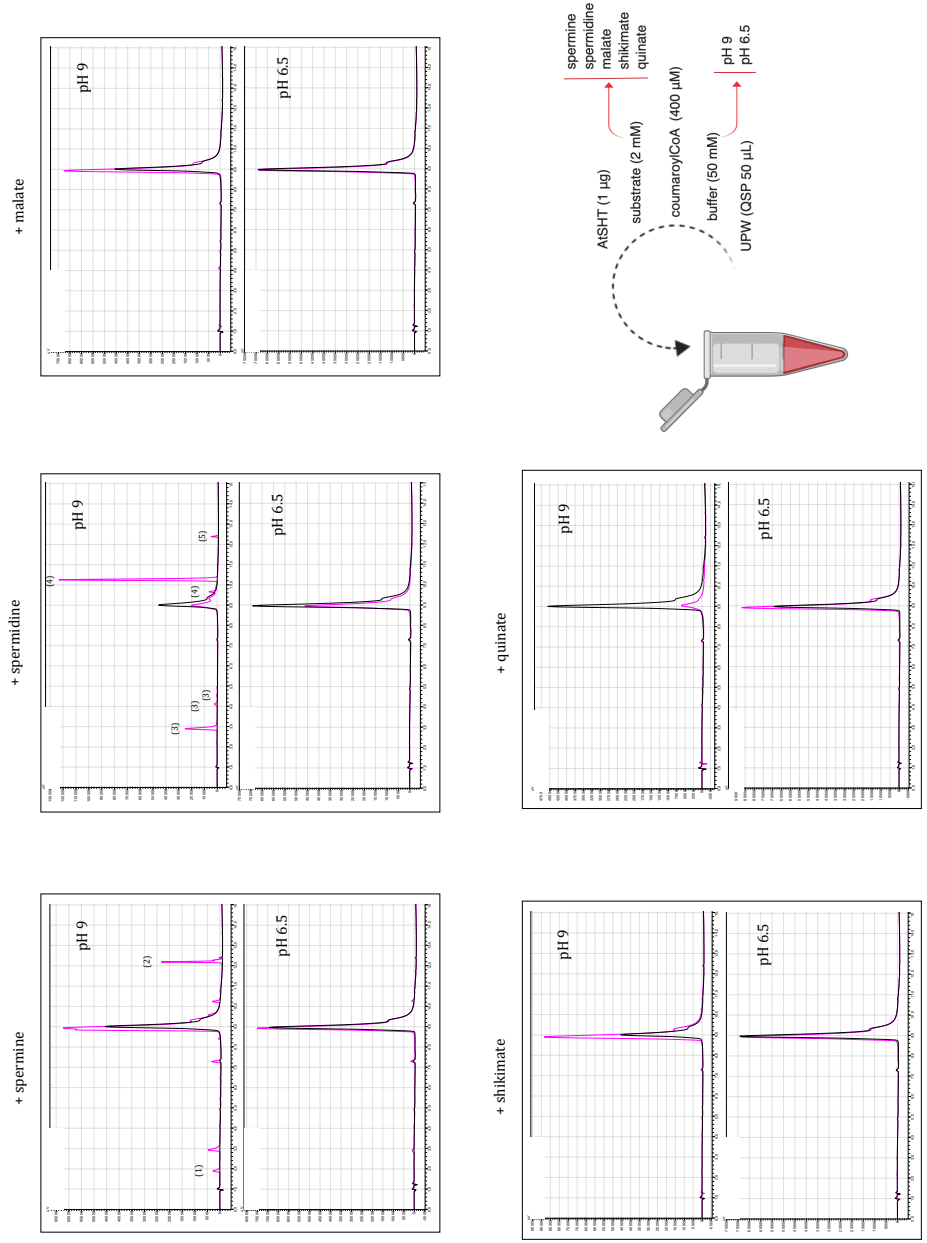


Figure 3.5 – *In vitro* assays of AtSHT activity towards a variety of acyl acceptors at different pH. Assays were incubated 3h at 28°C in obscurity and analyzed in HPLC. Controls performed by omitting the acyl acceptor in the mix are represented in black. (1) monocoumaroyl-spermine (2) tricoumaroyl-spermine (3) monocoumaroyl-spermidine (4) dicoumaroyl-spermidine (5) tricoumaroyl-spermidine

In this *in planta* heterologous system, our proteins of interest metabolic profiles did not yield effective results. So we used a different system, BL21 *E. coli*, to express the mutant TpHCT2*FY and assessed the activity of the purified protein in this model (Figure 3.3). AtSHT*YF was not successfully purified and thus not assayed *in vitro*. TpHCT2*FY surprisingly demonstrated an activity *in vitro*. However this activity was altered compared to the native TpHCT2 (Figure 3.6).

At its optimal pH (6.5), TpHCT2*FY demonstrates an ability to utilize only malate either with coumaroylCoA or caffeoylCoA as acyl donors. At pH 9 a very low activity can be seen when TpHCT2*FY is fed with spermine or spermidine, but this activity seems almost insignificant. It can catalyze the production of derivatives of malate at this pH as well. As a result, the inversion of this consensus motif in TpHCT2 appears to have impaired the versatility and promiscuity properties of the enzyme towards possible substrates and limited them to its preferred *in vivo* acyl acceptor malate.

These directed mutagenesis suggest that the consensus motif FYGN is required in SHTs catalytic abilities, but they were insufficient to determine its full significance in substrate affinities of the proteins. These effects could be attributed to substrates being unable to bind to the modified sites or to an inability to catalyze the acyltransfer on the acyl acceptor with a modified site.

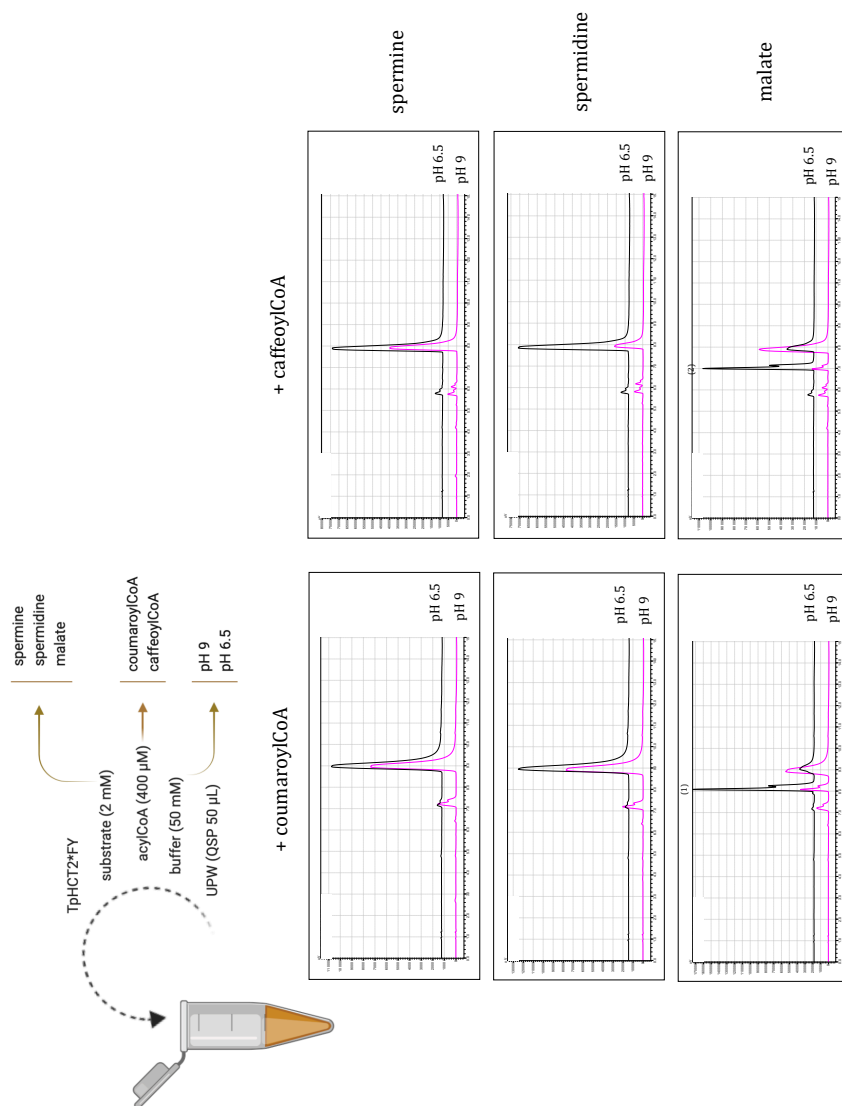


Figure 3.6 – *In vitro* assays of TpHCT2*FY activity towards a variety of acyl acceptors and acyl donors at different pH. Assays were incubated 3h at 28°C in obscurity and analyzed in HPLC. Controls performed by omitting the acyl acceptor (not shown here)

(1) coumaroylmalate (2) caffeoylmalate

3.2.2 *In silico* prediction of protein structure

While sharing 67.8 % of sequence identity, CiSHT1 and CiSHT2 exhibit different catalytic activities. As previously demonstrated, they acylate different substrate. *In silico* tools were used to unravel putative leads as to why they have different substrate affinities. Prediction tools indicate that they do not possess any signal peptides nor putative post-transductional modifications that could alter their conformations and ligand binding pockets.

Suzuki and colleagues (Suzuki, Nakayama, et al. 2004; Suzuki, Nishino, et al. 2007) characterized two MAT¹ proteins from *Dendranthema×morifolium* that possess different functional activities and work consecutively, similarly to CiSHT1 and CiSHT2. They describe the putative acylCoA liaison sites of the DmMAT. MAT enzymes are well represented BAHD enzymes (subclade Ia, Kruse et al. 2020) and have been studied over the last two decades. They are better annotated and characterized. Taking advantage of the homology between BAHDs protein sequences, alignment of a DmMAT with our proteins of interest provides insights regarding putative binding or catalytic sites. Only one DmMAT was used in the alignment for better clarity. The alignment in Figure 3.7 shows the conserved motifs of SHTs, BAHD as well as putative acyl-CoA liaison sites.

A total of seven residues can be identified as potential targets in our search for SHT catalytic sites including conserved sites in BAHD and putative ligand binding sites.

The next objective was to determine what distinguishes SHT1s from SHT2s. As demonstrated in the previous chapter, despite their high sequence identity homology, CiSHT1 and CiSHT2 have very different activities and substrate affinities. Novel sites of interest can be identified by identifying the common characteristics shared by SHT1s or SHT2s of *Asteraceae*. The relationships between SHTs of *Asteraceae* were first assessed using a phylogenetic analysis. The alignment of all SHTs of *Asteraceae* and AtSHT was then generated. In this study, AtSHT was used as a standard for other Eudicotyledons SHT to see if SHT1s

¹ MAT: anthocyanin malonyltransferase



Figure 3.7 – Alignment of CiSHTs with a characterized BAHD to identify putative acyl-CoA liaison sites
 BAHD consensus motifs are framed in orange, SHT consensus motifs are framed in pink and putative acyl-CoA liaison sites are framed in purple.
 Sequences used : Dm3MAT (BAF50706.1), CiSHT1 (AXY93625.1), CiSHT2 (AXY93626.1).

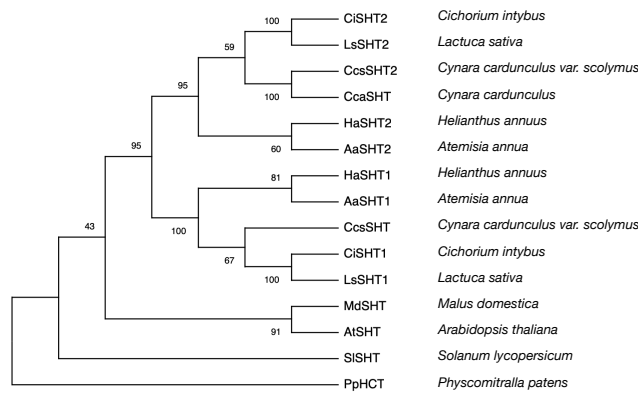
or, more likely, SHT2s shared conserved AA residues with it, which could be involved in their catalytic activity and substrate preferences (Figure 3.8 B.).

The phylogenetic analysis of SHT1 and SHT2 of *Asteraceae* was realized with sequences belonging to *Lactuca sativa*, *Helianthus annuus*, *Artemisia annua*, *Cynara cardunculus var. scolymus*. It was constituted by using the BLAST tool with CiSHT1 and CiSHT2 protein sequences and restricting the search to *Asteraceae* species. It shows a clear dichotomy between the two types of SHT: one subclade is composed of SHT2s like and the second of SHT1s like (Figure 3.8 A.). It appears that both SHT subclades exhibit identical distribution with unchanged evolutive relationships between species, suggesting that all *Asteraceae* presented here have similar evolution stories in the emergence of the duplicated *SHT* gene.

This is reflected also in the Figure 3.8 B. The first observation is that in some positions, there is a dichotomy between conserved AAs in SHT1s and SHT2s. These AA have different physicochemical properties (hydrophobe v/s hydrophile for instance), which could influence the conformation of the proteins. Their conservation among all SHT1s or SHT2s indicates their potential importance in the activities of each protein group. The second observation is that for the most part, these AA are common between SHT2 proteins and AtSHT except for S49 and D450 that are common with SHT1. SHT2s and AtSHT display similar activities as mentioned earlier. Their conservation in SHT2s could implicate their involvement in the catalytic activity of this protein group. Modification between SHT1s and SHT2s of AA close to conserved BAHD sites can be given particular attention since they have a higher probability of weighting on the catalytic activity of the proteins. Other notable differences are a gap in SHT1 compared to SHT2 in position 200-203 and an in/del² mutation in position 413. The analysis of Figure 3.7 and Figure 3.8 also reveals that two of the putative acyl-CoA liaison sites are common to residues of interest highlighted by the study of the dissimilarities between SHT1s and SHT2s in position 45 of CiSHT1 and CiSHT2 and positions 402 of CiSHT1, 404 of CiSHT2 (marked by orange triangle in Figure 3.8).

² in/del: insertion/deletion

A.



B.

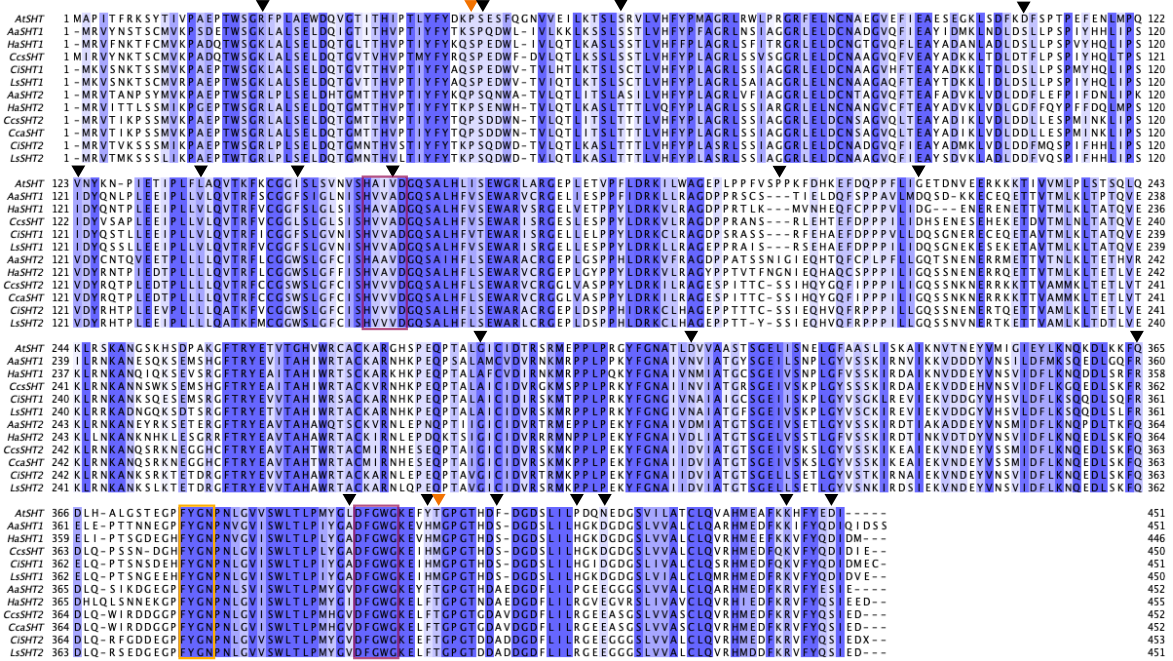


Figure 3.8 – Analysis of the relationship between SHT1s and SHT2s of Asteraceae

A. Phylogenetic tree of SHT1s and SHT2s anchored with a SHT sequence of *P. patens*.

B. Alignment of the protein sequences of SHT1s and SHT2s of Asteraceae to identify conserved sites in each type of proteins. They are marked with triangles. Orange triangle mark putative acyl-CoA liaison sites. BAHD conserved sites are framed in purple. FYGN conserved motif is framed in yellow.

Sequences used : PpHCT (PNR60563.1), SISHT (XP_004243059.1), MdsHT (NP_001306184.1), AtSHT (NP_179497.1), CiSHT1 (AXY93625.1), CiSHT2 (AXY93626.1), LsSHT1 (XP_023737074.1), LsSHT2 (XP_023771219.1), CcsSHT1 (XP_024965677.1), CcsSHT2 (XP_024963804.1), CcaSHT (XP_024965678.1), HaSHT1 (XP_022040251.1), HaSHT2 (XP_021995292.1), AaSHT1 (PWA91131.1), AaSHT2 (PWA65760.1)

3.2.3 Creation of chimeric proteins of CiSHT1 and CiSHT2

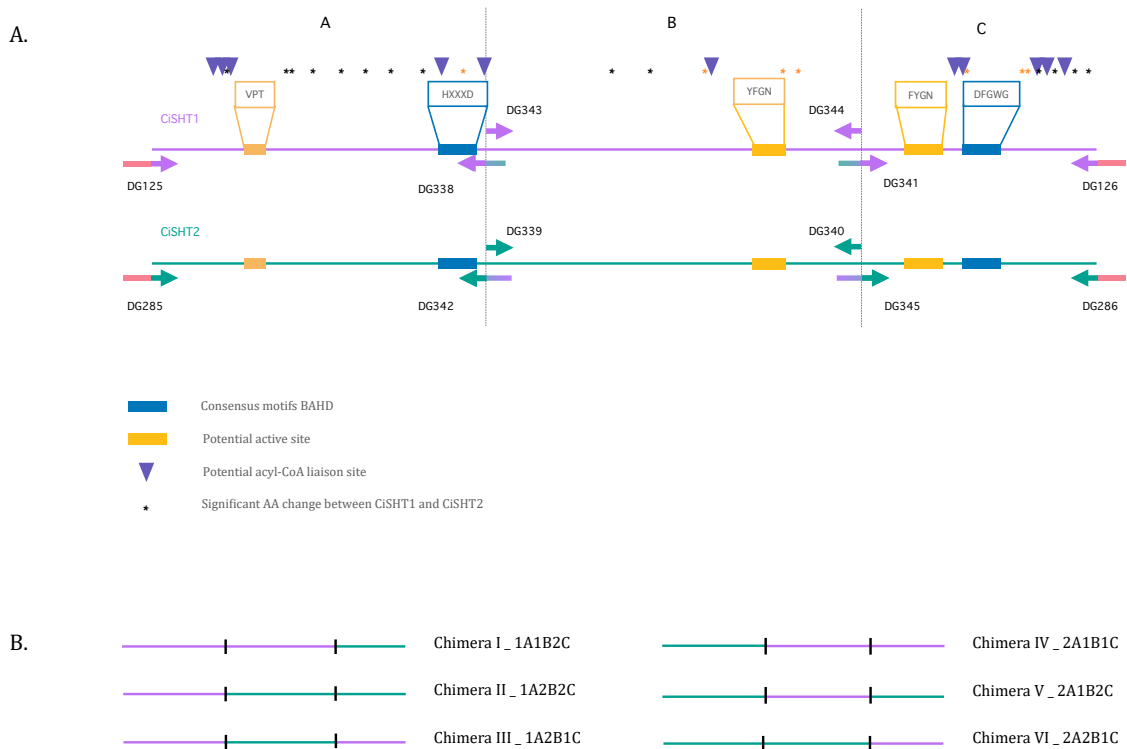


Figure 3.9 – Molecular engineering of chimeric proteins composed of CiSHT1 and CiSHT2

A. Native proteins CiSHT1 (violet) and CiSHT2 (green), their conserved motifs and sites of interest are schematized. In blue, conserved motifs in BAHDs. In yellow, potential active sites. Pointed by a purple arrow, potential acylCoA liaison sites. Marked by a black or orange asterix, significant AA change between CiSHT1 and CiSHT2. The designed primers follow the color code of the proteins.

B. Composition of the six generated CiSHT chimeras

In silico analysis are beneficial but do not provide enough insight to identify which one(s) of the AAs that differ between CiSHT1 and CiSHT2 and play a role in their respective substrate affinities. The strategy used to narrow down the list of putative essential AAs was to create chimeras of CiSHT1 and 2. Proteins were divided into three sections. Each section contained AA of interest determined in the previous section, as well as conserved motifs of BAHD enzyme superfamily. Six chimeric proteins were reconstituted and their activity analyzed using *N. benthamiana* as heterologous system (Figure 3.9). Overlapping primers were

used to generate the proteins. Primers were designed to hybridize at position 174 of CiSHT1 and CiSHT2, and 336 of CiSHT1 or 338 of CiSHT2. Each section was named A, B or C preceded by the number corresponding to the native protein they originate from (*i.e.* 1 or 2).

All these constructions as well as empty plasmid and a plasmid containing the WT sequences were transiently expressed in tobacco leaves. 4 days after agroinfiltration in *N. benthamiana*, transformed leaves were collected, fixed in liquid nitrogen, lyophilized and the phenolamides extracted before HPLC analysis. The metabolic profiles can be found in Figure 3.10.

Different activity behaviors were seen. They were termed as follows. «CiSHT1 activity» shows no accumulation of the phenolamides of interest and is called activity type A. «CiSHT2 activity» with accumulation of the intermediate phenolamides of interest (no fully substituted phenolamides) is called activity type B. «CiSHT1+CiSHT2 activity» with accumulation of all the phenolamides of interest but with low quantities of the intermediate products and a clear shift towards the most substituted products is called activity type C. Thus, we can categorize the activities of the native proteins into these three types and classify them according to the amount and types of substituted phenolamides produced: A>B>C. Those activities are summarized in table 3.2.

Chimeras were overexpressed in *N. benthamiana* leaves alongside with the native proteins and the empty plasmid. One could expect the chimeric proteins to display one of the three activities displayed by the native proteins, alone or combined (A, B or C). Indeed, some chimeras, namely chimera I (1A1B2C), IV (2A1B1C) and V (2A1B2C) exhibited a type A activity similar to CiSHT1 *i.e.* no phenolamide production. Chimera III (1A2B1C) exhibited a type C activity similar to the combined activities of CiSHT1 and CiSHT2. However an unexpected profile was also observed. Chimeras II (1A2B2C) and VI (2A2B1C) catalyzed the production of all the phenolamides of interest with a profile similar to a combination of CiSHT2 alone and of CiSHT1 and 2 together. This novel activity was defined as a BC type. One can also note that no chimeras exhibited

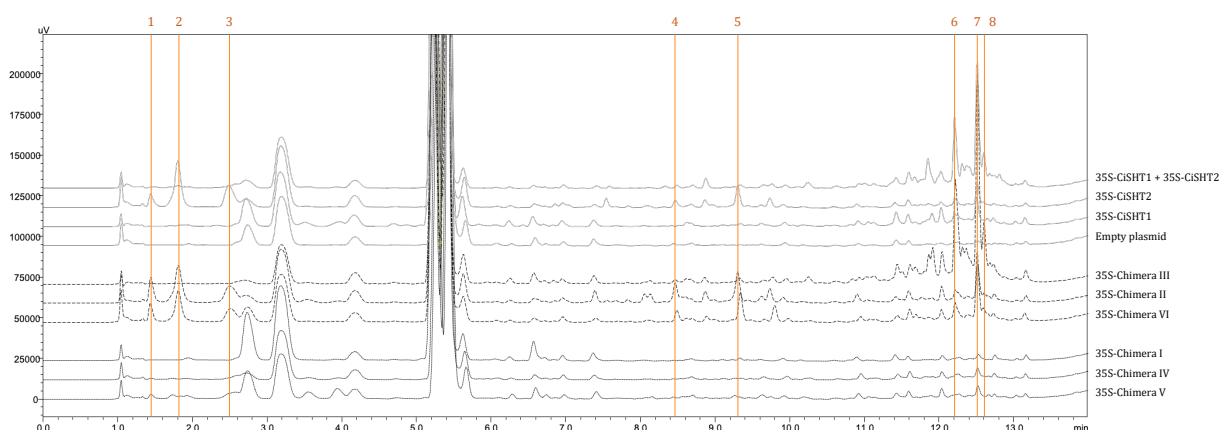


Figure 3.10 – Metabolic profiles of *N. benthamiana* leaves agroinfiltrated with native CiSHT or their Chimeras

(1) monocoumaroyl-spermine ; (2) monocoumaroyl-spermidine ; (3) monocoumaroyl-spermidine ; (4) dicoumaroyl-spermine ; (5) dicoumaroyl-spermidine ; (6) tricoumaroyl-spermine ; (7) tricoumaroyl-spermidine ; (8) tetracoumaroyl-spermine .

a type B activity.

Table 3.2 – Activity types of native CiSHT or CiSHT Chimeras *in vivo* on spermine substrate

Enzyme(s)	monoSpm	diSpm	triSpm	tetraSpm	Activity type	Activity type abbreviation
CiSHT1	×	×	×	×	CiSHT1	A
CiSHT2	✓	✓	✓	×	CiSHT2	B
CiSHT1 + CiSHT2	✓	✓	✓	✓	CiSHT1+CiSHT2	C
Chim I (1A1B2C)	×	×	×	×	CiSHT1	A
Chim II (1A2B2C)	✓	✓	✓	✓	CiSHT2 + (CiSHT1+CiSHT2)	BC
Chim III (1A2B1C)	✓	✓	✓	✓	CiSHT1+CiSHT2	C
Chim IV (2A1B1C)	×	×	×	×	CiSHT1	A
Chim V (2A1B2C)	×	×	×	×	CiSHT1	A
Chim VI (2A2B1C)	✓	✓	✓	✓	CiSHT2 + (CiSHT1+CiSHT2)	BC

Considering those results, an updated classification of the activity types was suggested: A>B>BC>C with BC constituting an original intermediary step towards TetraSpm production.

The three chimeras with type A activity are chimeras I (1A1B2C), IV (2A1B1C) and V (2A1B2C). From those we can conclude that the 2A or 2C sections alone or combined are not sufficient for the protein to either bind to its substrate or

Table 3.3 – Activity types of Chimeras coinfiltrated with native CiSHT proteins *in vivo*

Enzyme(s)	Activity type	Activity type abbreviation
Type A chimeras		
Chim I (1A1B2C)	CiSHT1	A
Chim I + CiSHT2	CiSHT2 + (CiSHT1+CiSHT2)	BC
Chim IV (2A1B1C)	CiSHT1	A
Chim IV + CiSHT2	CiSHT2 + (CiSHT1+CiSHT2)	BC
Chim V (2A1B2C)	CiSHT1	A
Chim V + CiSHT2	CiSHT2 + (CiSHT1+CiSHT2)	BC
Type BC/C chimeras		
Chim II (1A2B2C)	CiSHT2 + (CiSHT1+CiSHT2)	BC
Chim II + CiSHT1	CiSHT1+CiSHT2	C
Chim III (1A2B1C)	CiSHT1+CiSHT2	C
Chim III + CiSHT1	CiSHT1+CiSHT2	C
Chim VI (2A2B1C)	CiSHT2 + (CiSHT1+CiSHT2)	BC
Chim VI + CiSHT1	CiSHT1+CiSHT2	C

perform the acyltransfer on the non-conjugated polyamines or low-substituted phenolamides. Chimera V also proves that the 1B section can not substitute the 2B section in this substrate affinity.

The two chimeras with BC activity, chimeras II (1A2B2C) and VI (2A2B1C) reveal that the presence of respectively 2A2B sections and 2B2C sections allow the protein to use the available substrates. Moreover the presence of respectively 1A or 1C sections provide the catalytic ability to further transform part of the intermediate products into fully substituted phenolamides. However not all of the intermediate products are used as substrates in this reaction, leading to this hybrid BC activity type.

Chimera III (1A2B1C) is the only construction with C activity, encompassing the combined activity of CiSHT1 and CiSHT2 into a unique protein. It shows that only the 2B section is required to have a substrate affinity for non-conjugated polyamines and low-substituted phenolamides and moreover, that when both 1A and 1C sections are combined they can utilize and fully acylate all intermediate products.

From the chimeras I, IV and V we can conclude that with the 2A and 2C sections alone, no type B («CiSHT2») activity can be obtained whereas it is present in chimeras II, III and VI which demonstrates the essential character of the section 2B. Chimeras II and VI present this intermediate BC activity while chimera III has a type C activity. This shows that for a BC activity, section 2B has to be associated with either section 1A and/or section 1C. We can hypothesize that section 2B allows for the intermediate conjugates production and sections 1A/1C bring the necessary catalytic sites to catalyze the production of the fully substituted conjugates. This could be linked to the presence of highly conserved BAHD sites in sections A and C.

We hypothesized that if the inability to acylate the available substrates by type A chimeras is linked to the absence of the essential amino acids in the 1B section, when provided with the proper substituted phenolamides as substrates they could catalyze them into fully substituted products. To test this hypothesis, co-infiltration of type A chimeras with *CiSHT2* were realized (Figure 3.11). A summary of the results can be found in table 3.3 on the previous page.

The introduction of CiSHT2 is responsible for the presence in the metabolic profile of intermediate products (Figure 3.11). For chimeras I (1A1B2C) and V (2A1B2C), only a very low type BC activity can be seen. Sections 1A and 1B do not seem sufficient to grant the complete acyltransferase activity. Another explanation is that the high flexibility of specialized enzymes is responsible for this activity.

Only one combination showed a clear production of fully substituted phenolamides : chimera IV (2A1B1C). The successive action of CiSHT2 and chimera

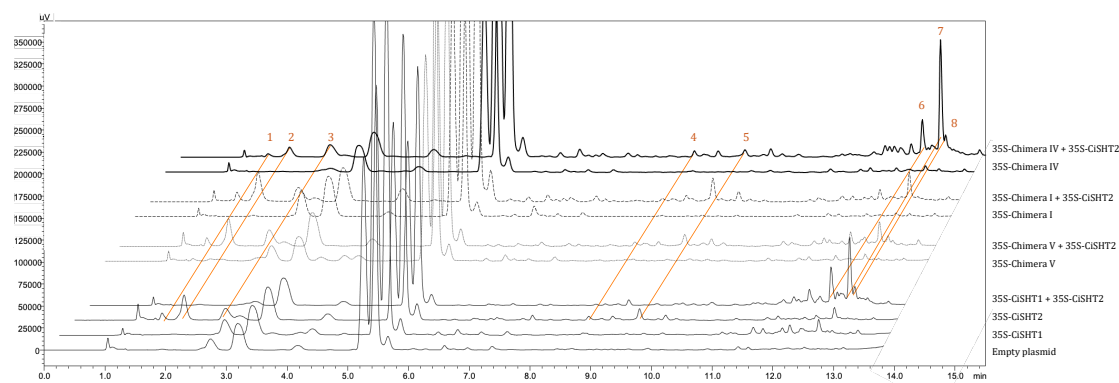


Figure 3.11 – Metabolic profiles of *N. benthamiana* leaves agroinfiltrated with Chimeras of type A activity and the native CiSHT2
 (1) monocoumaroyl-spermine ; (2) monocoumaroyl-spermidine ; (3) monocoumaroyl-spermidine ; (4) dicoumaroyl-spermine ; (5) dicoumaroyl-spermidine ; (6) tricoumaroyl-spermine ; (7) tricoumaroyl-spermidine ; (8) tetracoumaroyl-spermine .

IV had a BC activity profile. The most important AA involved in CiSHT1 acyl-transferase activity seem to be localized in the C section.

3.2.4 Amino acids of interest

Chimeric protein analysis was revealed to be a good tool to detect the regions where important amino acid residues locate. The sequences of CiSHT1, CiSHT2 and AtSHT were aligned to allow identification of putative AA of interest following three criteria : (1) substitution in AA conserved in all *Asteraceae* SHT1s or SHT2s (2) conservation of this AA in SHT2s and in AtSHT considering they share the most similar substrates affinities towards non-conjugated polyamines or low-substituted phenolamides and (3) whether the AA are located near putative catalytic sites or conserved BAHD motifs.

They are listed in table 3.4. The AA of higher interest are marked in orange.

Experimental results suggest that substrate affinity is determined by the presence of AA localized in the B section. Five sequence changes are listed as putative sites involved in substrate binding. Two of them, at positions 288 and 312, were selected because they are conserved in SHT2s and also in AtSHT. The last AA of interest is only present in two of the SHT1s, CiSHT1 and LsSHT1, and in

Table 3.4 – Amino acids of interest and their positions per section in CiSHT1 and CiSHT2

Section	CiSHT1	CiSHT2	AtSHT
A	K21	T21	R22
	SP 45-46	PS 45-46	PS 46-47
	S61	T61	S63
	S107	D107	D109
	I121	V121	V123
	V135	L135	L136
	F146	W146	I147
	A157	V157	V158
B	gap	SS 200-201	SPP 201-203
	D214	G216	G218
	A286	G288	G290
	G307	A309	A311
	N310	D312	D314
C	R361	Q363	Q365
	A392	V394	L396
	H401	F403	Y405
	M402	T404	T406
	S409	A411	F413
	H417	R420	P421
	D420	E423	N424
	K441	R444	K445
D446	S449	D450	

MtSHT. It constitutes a mutation in the conserved motif YFGNA. In those three proteins the Ala is substituted by a Gly. It was demonstrated in a recent paper on BAHD substrate flexibility that this particular AA was essential in the type of substrate(s) that could be accepted by the enzyme (Kruse et al. 2020). Up to now, LsSHT1 and MtSHT remain functionally uncharacterized. Our results also suggest that the acyltransfer activity of CiSHTs is held in sections A and C. Section C seems to have a predominant activity that can be enhanced by the combination with the A section from CiSHT1. They are complementary to unveil a complete acyltransferase activity on all types of substrates, from non-conjugated polyamines to substituted phenolamides.

In section C, 9 AA could be of interest. However, one can note that three of them are localized close to the conserved BAHD motif DFGWG, in positions 394, 403 and 404 (of CiSHT2). Only T404 is also conserved in AtSHT. This AA variation between SHT1s and SHT2s at positions 394 and 403 could play a role in the divergent acyltransferase activity presented by the two enzymes.

In section A, 9 AA of interest can also be found. However, one of them deserve a greater attention. In the BAHD conserved motif HXXXD, a clear discrepancy is present between SHT1s and SHT2s. All SHT1s feature a HXXAD motif whereas all SHT2s and AtSHT have a HXXVD motif. This substitution inside this highly conserved motif could be essential in the difference of activity between CiSHT1 and CiSHT2. The existence of the same motif in AtSHT as in SHT2s constitutes another argument in favor of this hypothesis as they possess similar catalytic activities.

An unexpected results of this structural analysis is the molecular engineering of a unique protein that combines the substrate affinities of CiSHT2 and the acyltransferase abilities of CiSHT1 to produce fully substituted phenolamides in only one step. One could resume the formula for TetraSpm production to section 2B + sections 1A and/or 1C. This Chimera III could be used in other systems such as yeasts to produce TetraSpm more efficiently and in higher quantities for further analysis of the biological properties of this metabolite.

3.2.5 Modelling of CiSHTs: native and chimeric proteins

In CiSHT1 and CiSHT2 three dimensional conformations, the AAs listed in orange Table 3.5 B. are all close to the binding pocket and to the catalytic site. The site in position 157 for both proteins is of particular interest because it belongs to the conserved BAHD site HXXXD and thus is close form the His residue belonging to the catalytic triad His-Ser-Gly of BAHDs. The Ala residue in CiSHT1 is replaced by a Val residue in CiSHT2 (Figure 3.12) and Table 3.5).

In Chimera III (1A2B1C) we can observe that the only sites of interest coming from CiSHT1 is an Ala residue in position 394 flanked to the conserved DFGWG

motif and sites H403 and M402 placed close to the binding pocket (Figure 3.12). The residues G288, A309 and D312 from CiSHT2 are conserved and placed close to the pocket as well (Figure 3.12 and Table 3.5). The Val residue inside the HXXXD conserved motif is also conserved. This is a unique conformation in the chimeric proteins (see Appendix B., figures B.1 and B.2) and justify its particular catalytic activity.

Table 3.5 – Amino Acid of interest in CiSHT1, CiSHT2 and the chimeric protein III (1A2B1C).

Conserved BAHD sites in blue, amino acids of interest in orange.

Section	CiSHT1	CiSHT2	CHIM_III
A	H153	H153	H153
	V154	V154	V154
	V155	V155	V155
	A157	V157	V157
	D158	D158	D158
B	A286	G288	G288
	G307	A309	A309
	N310	D312	D312
C	A392	V394	A394
	D393	D395	D395
	F394	F396	F396
	G395	G397	G397
	W396	W398	W398
	G397	G399	G399
	H401	F403	H403
	M402	T404	M404

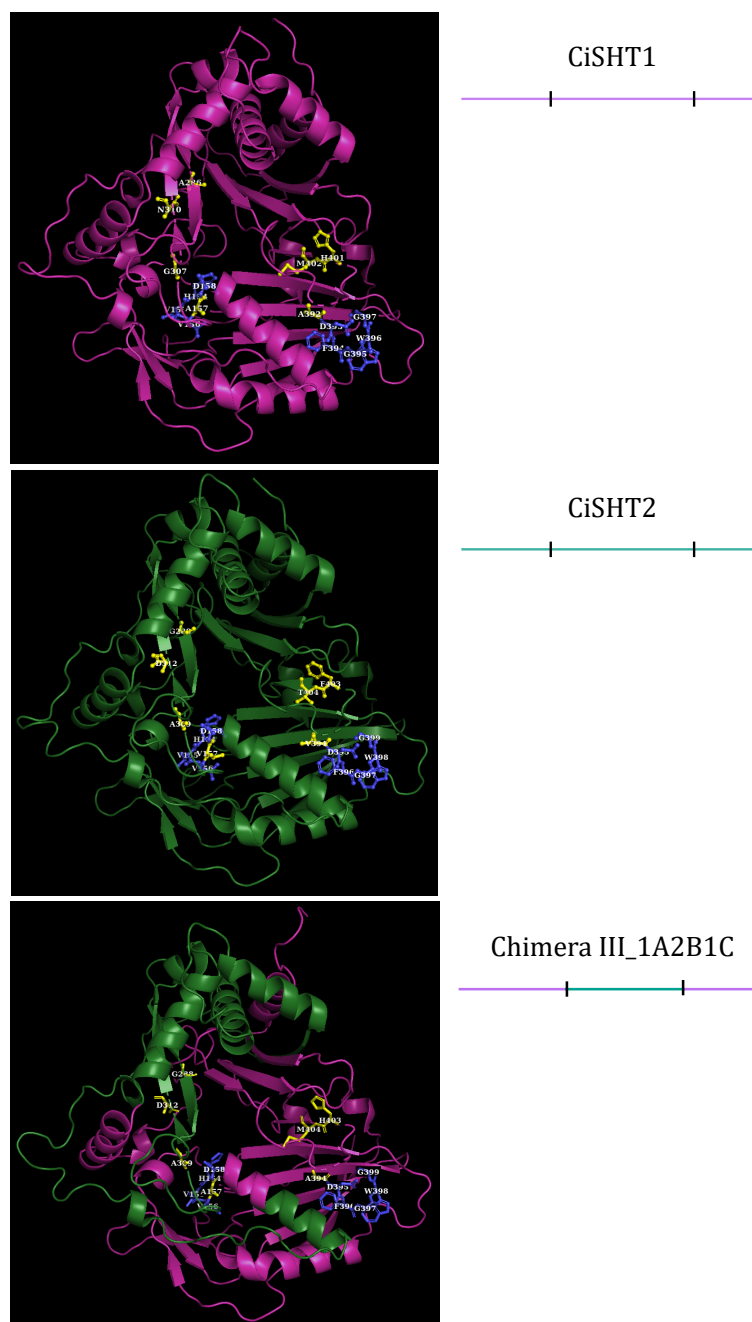


Figure 3.12 – Three dimensional modelling of CiSHT1, CiSHT2 and CHIM_III highlighting the sites of interest. Figure realized using the PyMOL 2.x software (Schrödinger 2021).

Sequences coming from CiSHT1 and CiSHT2 are represented in cartoon form respectively in magenta and green, respectively. Conserved BAHD sites are represented in stick form in blue. Amino acids of interest are represented in stick form in yellow. They are annotated with the letter corresponding to the AA and its position.

3.3 Discussion

In this Chapter, the identification of the essential sites to CiSHT1 and CiSHT2 distinct catalytic activities was undergone. The catalytic sites of SHTs are not well understood, neither is their evolutive history. In the previous chapter, the sequential action of CiSHT2 and CiSHT1 was demonstrated. It was evidenced that this peculiar mechanism is the result of a shift in the ratio of free polyamines in *Asteraceae* floral buds with respect to other Eudicotyledons, as well as a duplication and neofunctionalization of the *SHT* gene in this family (Delporte, Bernard, et al. 2018). Nonetheless, these data did not explain the emergence of such activities and substrates preferences in CiSHTs. conserved motifs of SHT and amino acid residues of interest were studied in this Chapter.

A consensus motif had already been identified by Marianne Delporte (2017) and was our first target. To identify its properties as putative catalytic site, two characterized BAHD, AtSHT and TpHCT2, were chosen for site directed mutagenesis due their activity types and sequence particularities at this precise site (3.1).

The substrate affinities of both AtSHT and TpHCT2 were analyzed in controlled conditions at two pHs (optimal pH of TpHCT2 and AtSHT, respectively) to examine the effects of this experimental set up on this property. *In vitro*, it was demonstrated that TpHCT2 has a wider range of possible acyl acceptors than AtSHT but that the mutation of the conserved YFGN site into FYGN disrupted greatly its catalytic abilities. TpHCT2*FY can not use the free polyamines (spermine and spermidine) as substrates unlike the native protein, only its preferential substrate malate.

In vivo, no activity from the mutated proteins was observed. In *N. benthamiana* fresh leaves, spermine and spermidine are less available than malate (Ali et al. 2020). One can suggest that the conformation of the protein *in vivo* do not allow for protein binding, explaining the absence of metabolite production from the TpHCT2*FY mutant protein.

This work demonstrated the essential nature of this site, but it also demonstrated

that its activity alone is not responsible for substrate binding and catalysis of the acyltransfer, as only a minimal activity was detected in the TpHCT2*FY mutant. Moreover, this is consistent with the work of Peng and colleagues which demonstrated that the FYGN motif is not essential to use spermine as a preferential substrate by spmHT (H. Peng, Meyer, et al. 2019).

Through alignments with characterized BAHD protein sequences, sites of interest in SHTs, specifically SHT1s and SHT2s of *Asteraceae*, were identified *in silico*. To determine the number and degree of potential importance of each identified sites is a difficult task. Thus, it is rather complicated to precisely select amino acid residue(s) for further experiments.

The construction of chimeric proteins of CiSHT1 and CiSHT2 primary goal was to narrow down the list of putative amino acid residues of interest. However, our findings exceeded our expectations. The main hypothesis was that chimeric proteins containing the section conferring substrate affinities to SHTs would present a A or B type activities, regardless of the other sections with which they would be associated. The results showed that no B activity was displayed by the chimeric proteins. Moreover, a new type of activity was exhibited (BC) and one of the chimera had a C activity. This opened new research perspectives. Sections holding complementary aspects of the catalytic activity of SHTs were defined (sections A and C) as well as the section most associated with substrate affinity (section B).

These results show that the consensus motif YFGNX and amino acid residues of interest in the B section of SHTs, as well as the FYGNX motif held in the C section, may be complementary in aspects of substrate binding. This is particularly relevant since the amino acid flanking the FYGN site differs between CiSHT1, CiSHT2 and AtSHT. Moreover, it was demonstrated that the amino acid residue downstream of the YFGN motif had a crucial importance in the types of substrates being able to be utilized by BAHD enzymes (Kruse et al. 2020). The mutation they introduced at that specific site changes the substrate preference of the BAHD enzyme from aromatic compounds to aliphatic com-

pounds. Moreover, they identified a conserved amino acid residue in the A section of their protein of interest that is also essential for the catalytic activity in combination with the aforementioned site, similarly to our conclusions.

From the structural analysis of SHTs, three main perspectives can be suggested. Many targets were identified in this work (see Table 3.4 on page 172). One of the perspective of this work that was already put in motion is to cut into three parts again the B section of Chimeras III (C type activity) and IV (A type activity) to further narrow down our list of targets by analyzing the metabolic profiles of those novel chimeras. Another perspective is to proceed to site-directed mutagenesis on all the targets of high interest (in orange in Table 3.4 on page 172) in chimeras of the three activity types A, BC and C. Most specifically in chimera III (C type activity) since a downregulation of the production of our metabolites of interest or the apparition of more intermediate products would be more easily detected than modifications in the metabolic profiles of the other types of chimeras. One of the most interesting amino acid residues to mutate is the fourth amino acid in the HXXXD conserved BAHD motif, which differs between SHT1s and SHT2s.

One of the potential outcomes of this work is to divide the B section of Chimeras III (C type activity) and IV (A type activity) into three parts again in order to narrow our list of targets even further by analyzing the metabolic profiles of those novel chimeras. The design for this work is already prepared.

A third perspective of this work is to collaborate with teams that specialize in substrate autodocking to perform a *in silico* analysis of the properties of our modelled proteins prior to undertaking wet lab work. This prospect is even more compelling now that the code for AlphaFold2 ([Senior et al. 2020](#)) is freely available on open source platforms like GitHub.

From a metabolic engineering point of view, Chimera III is of the outmost interest. This one-step synthesis of the fully substituted phenolamide of interest can be further characterized in terms of enzymatic properties and activity optimization. Chimera III production in unicellular organisms in bioreactors could

allow for the production of a sufficient amount of TetraSpm to characterize its potential activities, similar to what is done in companies like Cellengo and their branch PAT (Plantes A Traire) on 3,5-diCQA (Miguel et al. 2020).

It would also be interesting to work upstream of the pathway and perform over-expression or silencing of the spermine and spermidine synthases (*SPDS* and *SPMS*) in *C. intybus* to observe *in vivo* the effect of a change in ratio of free polyamines on CiSHTs activity. This work could be complementary to the introduction of *CiSHTs* genes into the *Atsht* KO line of *A. thaliana* and *vice versa*, initiated in the lab and presented in the complementary elements of the previous Chapter.

Computational and physiological characterization of GDSL-lipases like with putative acyltransferase properties in *C. intybus*

4.1 Introduction

Caffeoylquinic acids (CQAs) are the second type of HCA conjugates accumulating in chicory that this work focuses on, particularly 3,5-diCQA. CQAs present great therapeutic properties as exposed in the literature review (Chapter 2). The biosynthesis of CQAs and the species known to produce diCQAs are detailed in this introduction.

4.1.1 Occurrence of diCQA isomers in plants and bioaccumulating species

Our capacity to provide a quality bibliographic overview of diCQA isomer occurrences in plant species is hampered by difficulties with identification and

isomerisation during extraction. Metabolic databases (KNAPSAcK ¹ or Phenol Explorer ², for example) can assist in establishing a groundwork for identifying species accumulating compounds of interest observed in metabolic content research. They do not, however, provide a comprehensive list of all land plants that accumulate diCQA.

An attempt to review diCQA accumulating land plants is provided here and illustrated in a table available in Appendix C (Table C.1). Although it covered 70 species in which diCQA isomers were identified and sometimes quantified by HPLC MS-MS, this review is not exhaustive. It details the organs from which dicaffeoylquinic acids were isolated, as well as the isomers types and contents, if the information is available.

It highlights the need to standardize the nomenclature and extraction procedures when it comes to CQAs because: **(1)** this list may be skewed by the prevalence of Asteraceae in this study and the literature in general; **(2)** the lack of homogeneity in the extraction procedures and of quantification induces a bias in the analysis and identification of diCQA isomers; **(3)** the annotation and thus identification of diCQA isomers is not uniform, some extraction techniques may result in isomerisation which creates confusion in diCQA identification.

The followings are some general observations from this analysis. Among all the plant families present in this study, *Asteraceae* species are the most represented (61% of the species identified) followed by *Apiaceae* (5%), *Caprifoliaceae*, *Solanaceae* and *Rosaceae* (3% each). A total of thirteen plant families were came upon (Table C.1 in Appendix C).

The *Asteraceae* family includes many medicinal plant species that are prone to accumulating active phenolics like diCQAs. A few instances can be found in this review including, but not limited to : *Aster tataricus* L., traditional medicinal plant in Korea, Japan and China utilized for the treatment of urologic symptoms and exhibiting anti-inflammatory properties in benign prostatic hyperplasia (Rho et al. 2020); *Gynura bicolor*, popular in Taiwan and Japan, whose bioactive

¹ http://www.knapsackfamily.com/KNAPSAcK_Family/ ² <http://phenol-explorer.eu>

compounds induce apoptotic and necrotic cell death in human colon carcinoma cells (Teoh et al. 2016); *Achyrocline satureioides* (Lam) DC., traditional medicinal plant of South America with anti inflammatory, sedative and anti atherosclerotic properties and neuroprotective effects (Martínez-Busi et al. 2019); *Achillea millefolium* L. used in traditional medicine of several cultures, with reported antimicrobial, antiphlogistic, hepatoprotective, antispasmodic and calcium antagonist activities (Vitalini et al. 2011); and finally the *Artemisia* genus, among which *A. iwayomogi*, a traditional medicinal plant in Korea used as preventive or therapeutic agent in the treatment of diabetic complications (Y. K. Lee et al. 2017).

Species that stood out for diCQA accumulation are : *Artemisia iwayomogi* (*Asteraceae*) with 68.87 ± 0.19 mg/g dw (dry weight) of 3,5-diCQA extracted from the aerial parts (Y. K. Lee et al. 2017); *Lonicera macranthoides* (*Caprifoliaceae*) with 26.19 ± 1.82 mg/g dw of 3,5-diCQA in flowers and 16.25 ± 0.58 mg/g dw in leaves (M. Yu et al. 2019). Other genera seem to accumulate high amounts of diCQA such as *Ilex* (*Aquifoliaceae*), *Helianthus*, *Cynara*, *Chrysanthemum*, *Rhaponticum*, *Stevia* (*Asteraceae*), *Cuscuta* (*Convolvulaceae*), *Coffea* (*Rubiaceae*) (Peres et al. 2013; B. Peng et al. 2013; Cheevarungnapakul et al. 2019; F. Chen et al. 2014; Azzini et al. 2007; Pandino et al. 2015; Moglia, Acquadro, et al. 2016; Gong et al. 2019; Skala et al. 2015; Karakoöse 2015; Du et al. 2018; Asamenew et al. 2019). Overall, the main isoform of diCQA accumulated is the 3,5-diCQA at 43% of diCQA occurrences, followed by 4,5-diCQA (21%), given that bias may have been induced by the limitations listed above (Table C.1 in Appendix C).

4.1.2 Caffeoylquinic acids biosynthetic pathways and characterized enzymes

The biosynthetic pathway of CQA is well understood. However, the production of di-, tri- or tetra-CQAs is not, and seems to differ according to the species they accumulate in. The more substituted forms of CQAs possess greater activity potential, thus the deciphering of their biosynthetic pathway in accumulating species is essential.

The biosynthesis of CQAs has three main steps that take place in three different compartments of the cell: (1) the main building blocks of our molecules of interest, quinic acid, but also tyrosine and phenylalanine (precursors of *p*-coumaric acid), are synthesized in the plastids; (2) CGA ³, is synthesized in the cytosol via four possible pathways; (3) diCQA isomers are synthesized in the vacuole either from CGA or through acyl migration, as will be discussed in the next section.

4.1.2.a Synthesis of quinic acid, shikimic acid, tyrosine and phenylalanine in the plastid

The first part of the synthesis of CQAs involves the shikimate pathway (Figure 4.1). Quinic acid is synthesized early in the pathway through the reversible reduction of dehydroquinic acid by QDH ⁴. It is then transported in the cytosol through an undetermined mechanism. SDH ⁵, which catalyzes the primary route of the shikimic acid pathway, has a similar structure to QDH. SDH was reported to be universally conserved, but QDH was only identified in seed plants (Carrington et al. 2018). This suggests that QDH originated from SDH's ancestral enzyme based on the phylogenetic study performed by Carrington and colleagues (2018).

Tyrosine as well as phenylalanine are derivatives of arogonate and are synthesized respectively by the catalysis of ADH ⁶ and ADT ⁷, respectively. After being

³ CGA: chlorogenic acid ⁴ QDH: quinic acid dehydrogenase ⁵ SDH: Shikimic acid dehydrogenase ⁶ ADH: alcohol dehydrogenase ⁷ ADT: arogonic acid dehydratase

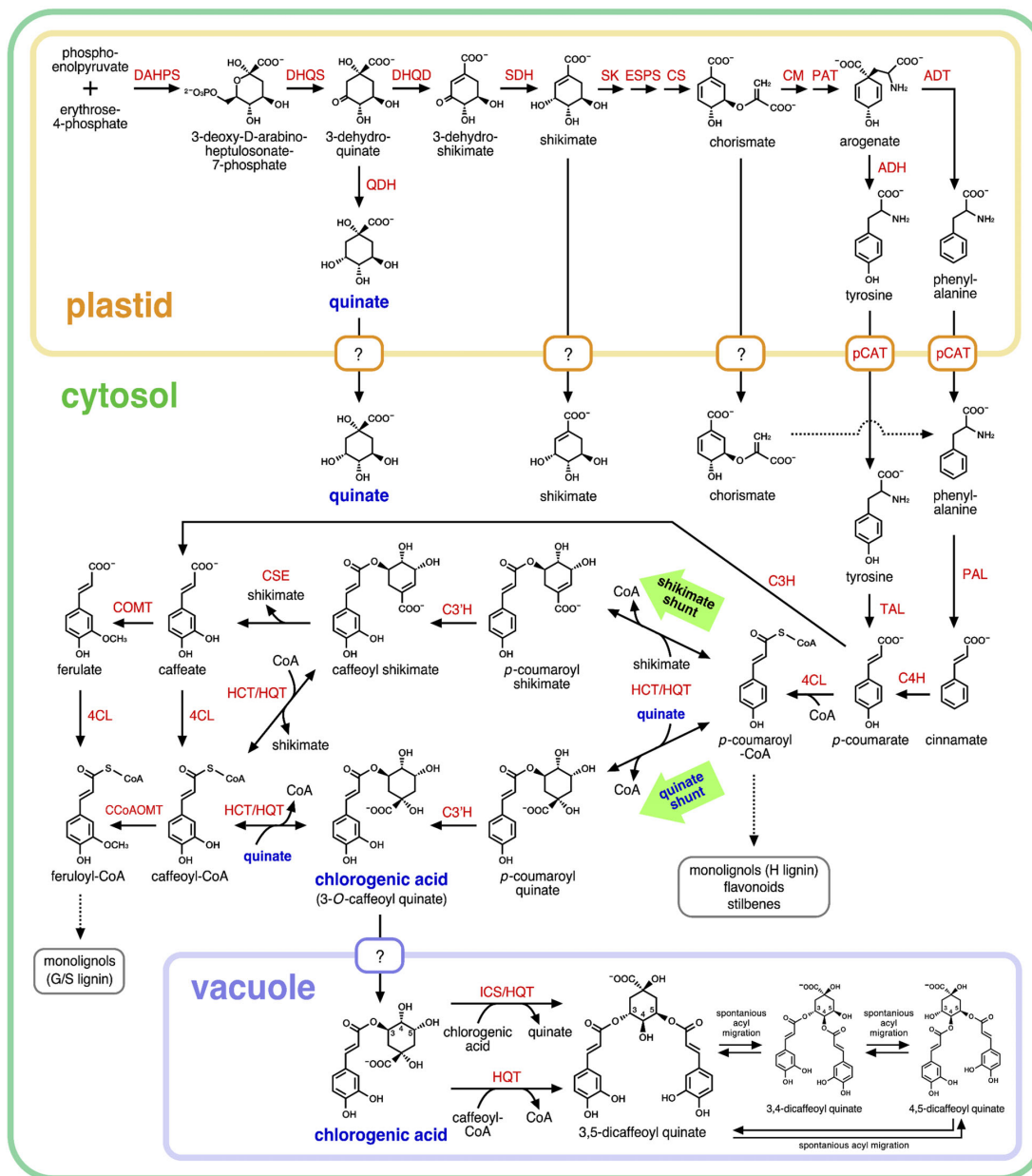


Figure 4.1 – Plant CQA biosynthetic pathway. Figure from Alcázar Magaña et al. 2021.

The shikimic acid pathway, the phenylpropanoid pathway, and dicaffeoyl quinic acid synthesis in plastid, cytosol, and vacuole are shown. Abbreviations: 4CL, 4-coumaric acid:CoA ligase; ADH, alcohol dehydrogenases; ADT, arogenic acid dehydratase; C3H, *p*-coumaric acid 3-hydroxylase; C3'H, *p*-coumaroyl-shikimic acid/quinic acid 3-hydroxylase; C4H, cinnamic acid 4-hydroxylase; CCoAOMT, caffeoyl-CoA O-methyltransferase; COMT, caffeic acid O-methyltransferase; CS, chorismic acid synthase; CSE, caffeoyl shikimic acid esterase; DAHPS, 3-deoxy-7-phosphoheptulonate synthase; DHQD, 3-dehydroquinic acid dehydratase; DHQS, 3-dehydroquinic acid synthase; ESPS, 3-enolpyruvylshikimic acid 5-phosphate synthase; HCT, hydroxycinnamoyl-CoA:shikimic acid/quinic acid hydroxycinnamoyltransferase; HQT, hydroxycinnamoyl-CoA:quinic acid hydroxycinnamoyl-transferase; ICS, isochlorogenic acid synthase; PAL, phenylalanine ammonia-lyase; PAT, prephenic acid aminotransferase; pCAT, plastidial cationic amino acid transporter; QDH, quinic acid dehydrogenase; SDH, shikimic acid 5-dehydrogenase; SK, shikimic acid kinase; TAL, tyrosine ammonia-lyase.

transported in the cytosol by pCAT⁸ they are used as substrates for *p*-coumarate synthesis (Widhalm et al. 2015). Quinic acid, shikimic acid, tyrosine, and phenylalanine as well as chorismic acid are among the compounds synthesized in the plastidial shikimic acid pathway that are transported to the cytosol (Maeda et al. 2012).

4.1.2.b Synthesis of hydroxycinnamoylCoA and chlorogenic acid in the cytosol

In the cytosol, the phenylpropanoid pathway produces a variety of chemicals, leading to the production of CQAs, lignin monomers, flavonoids, and stilbenes (Vogt 2010). Here we focus on two main events of CQAs biosynthesis holding place in the cytosol which are the production of hydroxycinnamoylCoA and of CGA.

p-coumaric acid is generated from either tyrosine through the action of TAL⁹ or from phenylalanine through the consecutive actions of PAL¹⁰ and C4H¹¹. Tyrosine deamination by TAL is an alternate pathway to produce *p*-coumaric acid identified in grasses (Barros, Serrani-Yarce, et al. 2016). *p*-coumaroylCoA is then generated through the ligation of coenzyme A on the resulting *p*-coumaric acid by 4CL¹².

In addition to lignin monomers and CQAs, *p*-coumaroylCoA is at the origin of a significant branch for the production of different phenolic compounds, such as flavonoids and stilbenes. HCT¹³ or HQT¹⁴ use *p*-coumaroyl-CoA to acylate shikimic or quinic acids to produce corresponding esters (Hoffmann et al. 2003). These reactions are reversible, meaning the corresponding CoA ester from the hydroxycinnamoyl esters of shikimic acid and quinic acid can be produced. HCT and HQT catalyze the same reaction, but they have different substrate affinities. HCT has better affinity for shikimate while HQT uses quinate as a preferred substrate (Hoffmann et al. 2003; Niggeweg et al. 2004).

⁸ pCAT: plastidial cationic amino acid transporter ⁹ TAL: tyrosine ammonia-lyase ¹⁰ PAL: phenylalanine ammonia-lyase ¹¹ C4H: cinnamic acid 4-hydroxylase ¹² 4CL: 4-coumaric acid:CoA ligase ¹³ HCT: hydroxycinnamoyl-CoA shikimate hydroxycinnamoyl transferases ¹⁴ HQT: hydroxycinnamoyl-CoA quinate hydroxycinnamoyl transferases

In the CGA synthesis pathway, *p*-coumaroylCoA can be converted into *p*-coumaroyl shikimic acid or *p*-coumaroyl quinic acid, each route being referred as the shikimate or quinate shunts respectively (Ha et al. 2016). Another route produces directly caffeoylCoA after the action of C3H¹⁵ and 4CL, bypassing the shikimate shunt. A focus on the different CGA biosynthetic routes is presented in figure 4.2. All start from *p*-coumaric acid. Route 1, 2 and 4 start with the action of 4CL on *p*-coumaric acid to produce the *p*-coumaroylCoA precursor. Route 1 and 2 share the same start through the shikimate shunt but diverge after the generation of caffeoyl shikimic acid. Route 3 starts with the action of C3H to obtain caffeic acid then acylated by C4L producing a caffeoylCoA precursor.

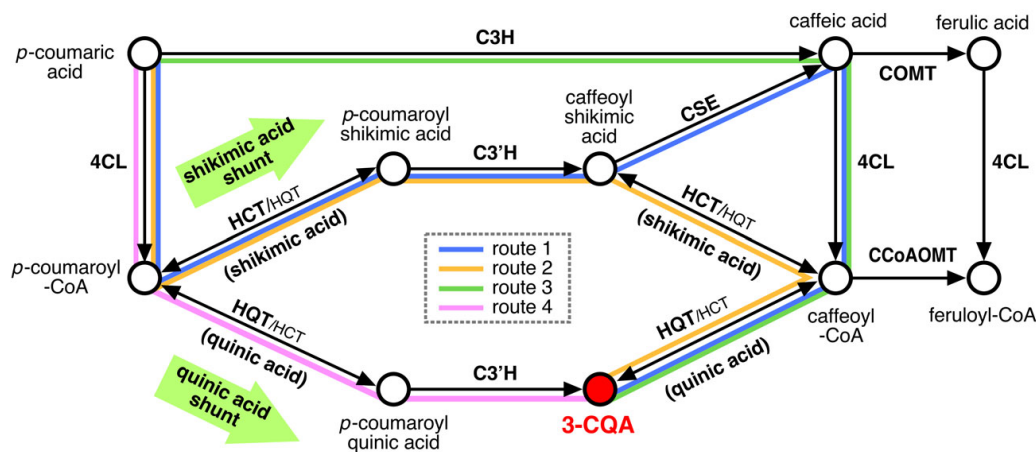


Figure 4.2 – CGA possible biosynthetic routes. Figure from Alcázar Magaña et al. 2021. Four routes are indicated by lines with blue, yellow, green, and pink. Abbreviations: 4CL, 4-coumaric acid:CoA ligase; C3H, *p*-coumaric acid 3-hydroxylase; C3'H, *p*-coumaroyl-shikimic acid/quinic acid 3-hydroxylase; CCoAOMT, caffeoyl-CoA O-methyltransferase; COMT, caffeic acid O-methyltransferase; CSE, caffeoyl shikimic acid esterase; HCT, hydroxycinnamoyl-CoA:shikimic acid/quinic acid hydroxycinnamoyltransferase; HQT, hydroxycinnamoyl-CoA:quinic acid hydroxycinnamoyltransferase; ICS, isochlorogenic acid synthase

The *p*-coumaroylCoA precursor enters either the shikimate (route 1 and 2) or the quinate shunt (route 4). The shikimate shunt consists of the action of a HCT reversibly producing *p*-coumaroyl shikimic acid and then of C3'H¹⁶ oxidizing

¹⁵ C3H: *p*-coumaroyl-3-hydroxylase ¹⁶ C3'H: cytochrome P450- type *p*-coumaric acid ester-specific 3'-hydrolase

the 3' position of *p*-coumaric acid aromatic ring to synthesize caffeoyl shikimic acid .

Routes 1 and 2 both merge with route 3 in the production of caffeoylCoA but through different processes. Route 1 includes the action of CSE ¹⁷ and joins route 3 at the caffeic acid step while route 2 features a HCT and joins route 3 at the caffeoylCoA step.

Most plants are still considered to use route 1 for CGA production. Plants lacking the CSE gene can generate CGA via routes 2 and 3. CSE hydrolyses caffeoyl shikimic acid to produce caffeic acid and shikimic acid . The CSE gene is a well conserved genes, particularly in angiosperms such as *Arabidopsis*, *Medicago*, and *Populus* and gymnosperms such as *Larix kaempferi* (Ha et al. 2016; Saleme et al. 2017; Vanholme, Cesarino, et al. 2013; Xuechun Wang et al. 2019). However, some species do not have the CSE gene such as *Zea mays* or *B. distachyon*. Thus, these species cannot use route 1 for the production of caffeoylCoA and go through other routes for CGA production (Ha et al. 2016). Route 2 generates caffeoylCoA directly from caffeoyl shikimic acid via the action of HCT/HQT. Route 3 was discovered in *B. distachyon* in which a novel C3H was identified that converts *p*-coumaric acid in caffeic acid (Barros, Escamilla-Trevino, et al. 2019). The last step for routes 1, 2 and 3 is the transesterification with quinic acid by a HQT to produce CGA.

The quinate shunt (route 4) is more direct and entails only two steps. First, a HQT transfers a coumaroyl moiety on the quinic acid to produce coumaroylquinic acid that is then oxidized by C3'H to give CGA. Because the shikimic acid shunt is used in the essential lignin biosynthesis pathway, this would constitute a by-pass branch.

A parallel route can be considered in *I. batatas* through cinnamoyl glucose and subsequently caffeoyl-glucose after hydroxylation at positions 3 and 4 of the aromatic ring. A HQT transfers the caffeoyl moiety of caffeoyl glucose on quinic acid to produce CGA (Villegas et al. 1987).

¹⁷ CSE: caffeoyl shikimic acid esterase

The mechanism of transport of CGA into the vacuole is an ongoing research topic. For instance in *C. canephora*, CGA appears to play a role in caffeine storage in the vacuole (Nic-Can et al. 2015). In *L. japonica*, its transport to the vacuole for long-term storage from its place of synthesis is thought to be dependent of the place of synthesis of CGA, *i. e.* via vesicles from the cytoplasm or by direct membrane fusion between the tonoplast and the chloroplast membranes (Yanqun Li et al. 2019).

4.1.2.c Synthesis of diCQAs in the vacuole

There were numerous blind spots in the biosynthetic routes of CQAs, particularly in the synthesis of multisubstituted CQAs. The importance of acyltransferases in multiple phases of the pathway explains the attention given to BAHD enzymes. However, as aforementioned, BAHDs are a rapidly growing specialized metabolism enzymes family. HCT/HQT are thus not always the best candidates in diCQA synthesis pathway.

No enzyme for diCQA synthesis had been found or characterized until the early 2010s. It was theorized that BAHD acyltransferases like HCT/HQT could be involved in the production of diCQA precursors. The hydroxycinnamoyl-CoA shikimate/quinic acid hydroxycinnamoyl transferases are enzymes that reversibly shuttle hydroxycinnamoyl units between their CoA- and shikimate/quinic acid-esterified forms. Both enzymes share a high percentage of sequence identity (over 60%) and can accept a variety of acyl donors, such as *p*-coumaroyl-CoA, caffeoyl-CoA, and feruloyl-CoA, albeit they have different acyl substrate preferences.

Because the mechanisms of diCQA synthesis appear to be analogous to those of its precursor, BAHD acyltransferases were the first candidates to be investigated. However, due to their quickly evolving features and the diversity of their substrates and activities acquired through convergent evolution, we are unable to predict the roles of BAHD enzymes solely based on computational investigations. (Luo, Nishiyama, et al. 2007).

The first enzyme identified as being responsible for the formation of diCQA was, in fact, a HCT of *Coffea canephora* (Lallemand et al. 2012). It was also the first BAHD to have its structure determined by crystallography. This work showed that the capacity of HCT to produce diCQA may be dependent on the position of a specific histidine residue, His-154, as well as the two amino acids following it. Indeed, the activity of this enzyme towards diCQA synthesis increased up to 4-fold when these amino acid residues were directly modified.

In *Solanum lycopersicum*, Moglia and colleagues (2014) identified the second BAHD implicated in the diCQA biosynthetic last step, a HQT. The catalytic activity of this enzyme is dependent on the pH of the subcellular compartment in which it is located. It has a typical HQT activity in the cytoplasm (pH 6–7). It converts to a CCT¹⁸ with irreversible activity in the vacuole (pH 4), resulting in the synthesis of diCQAs in tomato fruits (Figure 4.3). This peculiar activity could be due to the presence of a catalytic histidine, His-276, which allows for CCT action. CCT activity of HCT/HQT would be absent or severely hampered if this residue is not present. For instance, *in vitro* assays with a HQT from globe artichoke yielded unsatisfactory results (Moglia, Lanteri, et al. 2014). This could be related to the presence of a Tyr residue in the location corresponding to His-276 of SlHQT. They speculated that organisms lacking this His residue in HCT/HQT have to rely on alternative biosynthesis pathways.

¹⁸ CCT: chlorogenate:chlorogenate transferase

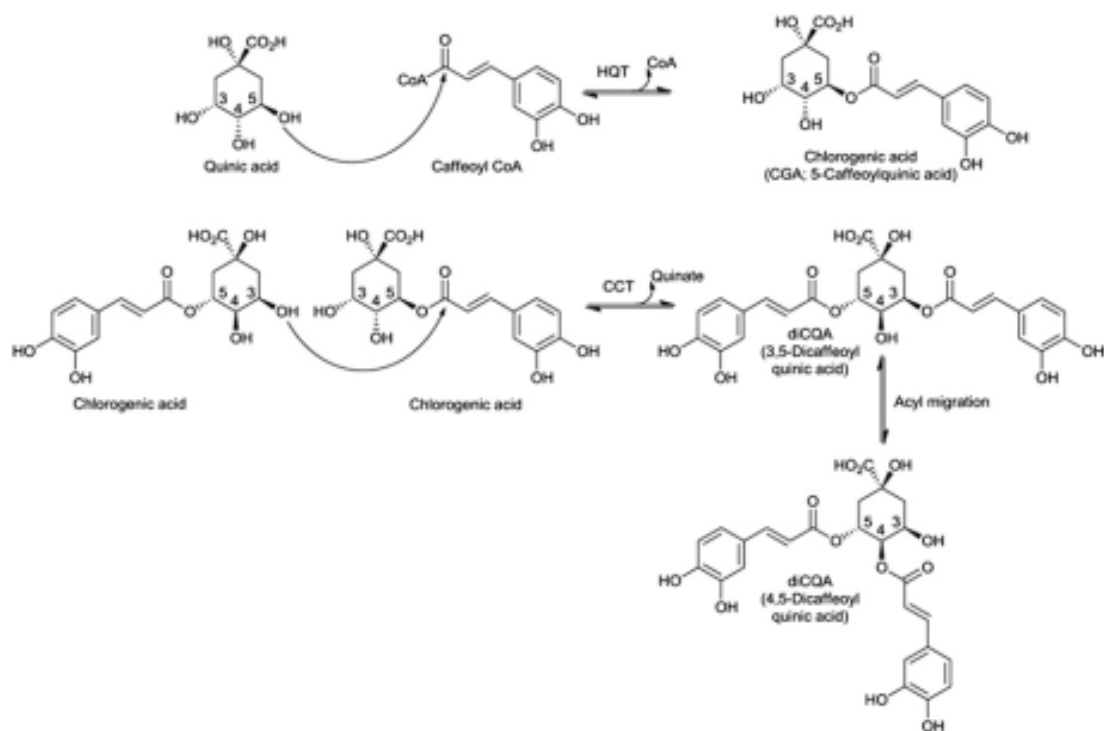


Figure 4.3 – Dual activity of *S. Lycopersicum* HQT. Figure from [Moglia, Lanteri, et al. 2014](#).

Proposed reaction scheme for diCQA synthesis by CCT using CGA as the acyl donor as well as the acyl acceptor compared with the BAHD activity of HQT and HCT using caffeoyl-CoA as the acyl donor and quinate as the acyl acceptor, respectively.

4.1.3 diCQA biosynthetic pathways and characterized enzymes

Up to this point, while considering candidate acyltransferases, the BAHD or SCPL superfamilies were virtually exclusively considered. For instance, recently, a SCPL enzyme was shown to be involved in diCQA synthesis in *Echinacea purpurea* (L.) Moench (*Asteraceae*) (Figure 4.4; [Fu et al. 2021](#)).

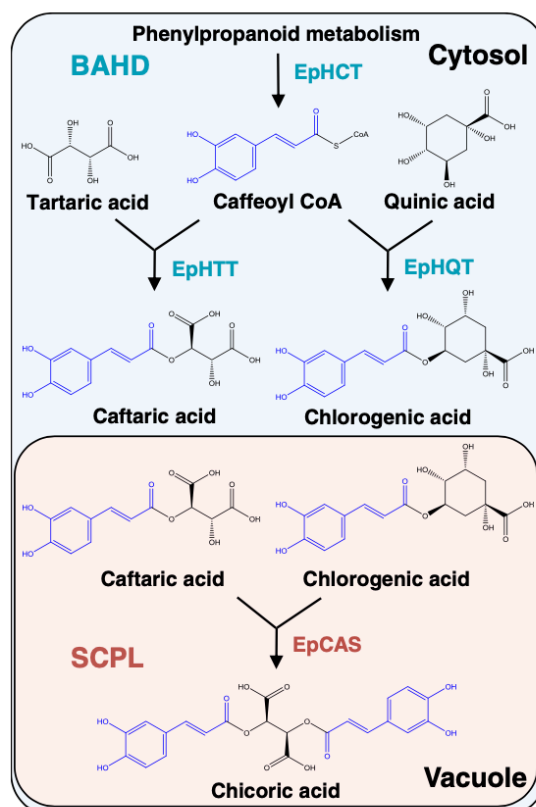


Figure 4.4 – Proposed pathway for chicoric acid in purple coneflower. Figure and legend from [Fu et al. 2021](#).

Two types of acyltransferases distributed in distinct subcellular compartments are involved in the biosynthesis of chicoric acid. In the cytosol, the BAHDs including EpHTT and EpHQT use caffeoyl CoA from phenylpropanoid metabolism as an acyl donor to synthesize caftaric acid and chlorogenic acid, respectively. Both products are then transported into the vacuole. EpCAS, a specialized SCPL acyltransferase, uses CGA as its acyl donor and transfers the caffeoyl group to caftaric acid to generate chicoric acid.

Chicoric acid is naturally accumulated in this species' aerial parts and, to a lesser extent, in its roots. Fu and colleagues goal was to elucidate chicoric acid biosyn-

thetic pathways in purple coneflower. They expected a BAHD to be involved in the last step of this synthesis. However, they discovered that chicoric acid biosynthesis is a three step pathways involving two cytosolic BAHDs, EpHQT and EpHTT, and a vacuolar SCPL, EpCAS (Figure 4.4). SCPL usually prefer 1-O- β -D-glucose esters as acyl donors, whereas EpCAS catalyzes the production of chicoric acid from a molecule of caftaric acid and chlorogenic acid in this case. This pathway mechanisms demonstrates that CGA is a more common acyl donor than previously thought, emphasizing the versatility of acyltransferases.

Indeed, acyltransferase function has been reported in superfamilies even when this is not one of the fundamental features (see section 1.5.5 of the literature review), such as the SGNH¹⁹ superfamily. The enzymes belonging to the SGNH protein family have a catalytic triad of Ser-Asp-His, with serine as the nucleophile of the GDSL motif.

GDSL-lipases are mostly known to be involved in primary metabolism, notably lipid metabolism. However, a few instances of GDSL-lipase like involved in specialized metabolism opened a new research area. One of the first is an esterase involved in the final steps of the ajmaline pathway, a natural molecule found in the roots of *Rauvolfia serpentina* and broadly employed in traditional Indian medicine (Ruppert, Woll, et al. 2005). The acetyl group added previously in the pathway is split off by RsAAE²⁰ either at the antepenultimate or final step of this monoterpenoid indole alkaloid synthesis pathway. Another notable GDSL-lipase enzyme is a sinapine esterase from *Brassica napus* BnSCE3²¹/BnLIP2²² (Clauß, Baumert, et al. 2008) involved in the hydrolysis of sinapine during the early stages of seed germination.

So far we have looked at two cases where the hydrolytic function of GDSL-lipases has been preserved. Several GDSL-lipase-like enzymes lost their original lipase function in favor of a transferase activity. The first characterized

¹⁹ SGNH: named after conserved sites in the superfamily. block I – serine, block II – glycine, block III – asparagine, block V – histidine ²⁰ AAE: acetylajmalan acylesterase ²¹ SCE3: sinapine esterase 3 ²² LIP2: GDSL-lipase like 2

GDSSL-lipase like enzyme identified with no lipase activity was discovered by Teutschbein and colleagues (2010). *S. lycopersicum* has a lipase-like protein that may utilise CGA as an acyl donor and catalyse the transfer of the caffeoyl moiety of CGA to the acceptor molecule glucarate. It was discovered to be a chlorogenate:glucarate caffeoyl-transferase with a high affinity for particular CGA stereoisomers (Teutschbein et al. 2010). While not involved in the last step of the diCQA biosynthesis, the chemical mechanism is very similar. The aptitude of SLCGT²³ to act as an acyltransferase in the formation of hydroxycinnamate esters employs amino acid residues other than those found in the lipase catalytic triad.

These findings renewed the interest for a study conducted by Villegas and colleagues (1987), which identified an enzyme also catalyzing the acyltransfer of a chlorogenate as an acyl donor in *I. batatas*. The product of this reaction is 3,5-diCQA. This enzyme was subsequently named Isochlorogenate Synthase (ICS) but was never functionally characterized (Villegas et al. 1987).

After hitting dead ends with the HCT/HQT candidates studied in chicory for diCQA production, Guillaume Legrand (2016) shifted to this model and conducted a traditional biochemistry approach to identify the gene involved in this pathway. In collaboration with the LAE lab (University of Nancy) and PAT company, IbICS was identified at the molecular level Miguel et al. 2020. They were able to metabolically engineer the biosynthetic pathway of 3,5-diCQA in yeast lines of *Pichia pastoris* using the cloned and characterized *IbICS* and yielded great results: from 2.6 g · L⁻¹ of CGA, 1.2 g · L⁻¹ of 3,5-diCQA were obtained (61.3% mol efficiency). Miguel and colleagues (2020) also identified a HCT capable of producing 3,5-diCQA, but it was significantly less efficient than the *IbICS*. *IbICS* produces 3,5-diCQA through a single acyltransferase reaction involving two CGA molecules (Figure 4.5). Despite the similar substrate affinities and activities, the authors estimated that the *IbICS* they discovered is a different isochlorogenate synthase than the one identified by Villegas and colleagues (1987).

²³ CGT: chlorogenate:glucarate caffeoyl-transferase

Overall, only four enzymes have been identified and characterized for 3,5-diCQA production: two HCTs, one HQT and one GDSL-lipase like. A distinct family or clade is involved in each model: HCT in *Coffea spp.* and *I. batatas*, HQT in *S. lycopersicum* and a GDSL-lipase like in *I. batatas* (Lallemand et al. 2012; Moglia, Lanteri, et al. 2014; Miguel et al. 2020). Two main pathways can be described: **A.** acyltransferase reaction with CGA and caffeoyl-CoA as an acyl donor or **B.** acyltransferase reaction with two CGAs molecules as both the acyl donor and acyl acceptor (Figure 4.5). The two HCTs have a type A activity whereas the GDSL-lipase like IbICS and SIHQT have a B type activity. The production of other isomers of diCQA such as 4,5-diCQA and 3,4-diCQA are more likely the results of acyl migration in the vacuole as a consequence of pH conditions (Moglia, Lanteri, et al. 2014; Alcázar Magaña et al. 2021).

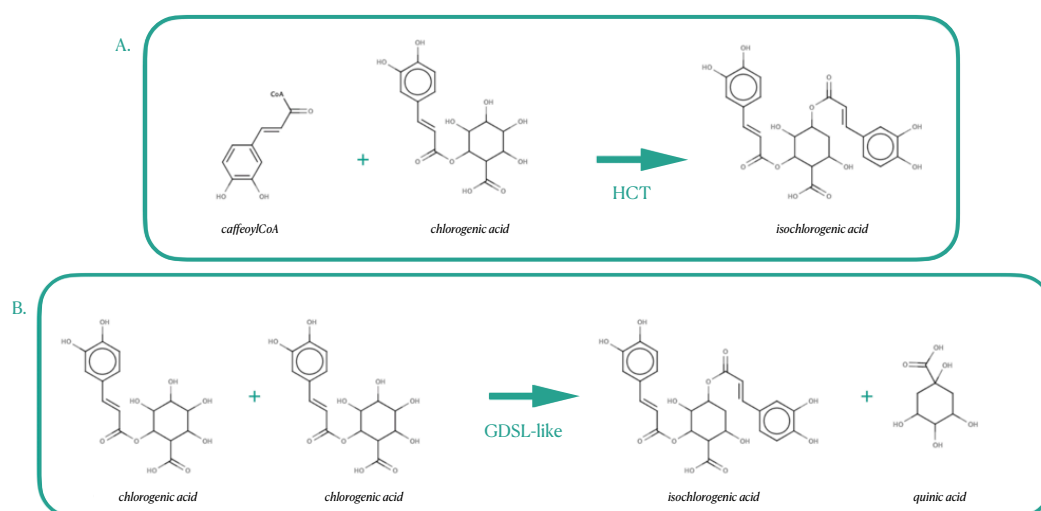


Figure 4.5 – Characterized biosynthetic routes of isochlorogenic acid.

Two types of biosynthetic routes for diCQA have been characterized.

Type **A** : acyltransferase reaction with CGA and caffeoyl-CoA as an acyl donor.

Type **B** : acyltransferase reaction with two CGAs molecules as both the acyl donor and acyl acceptor

The recent research on *IbICS* in *I. batatas* (Miguel et al. 2020) led to a novel hypothesis: GDSL-lipase like might be involved in diCQA production in *C. intybus* as well. Indeed, a BLAST of our EST databases with *IbICS* unveiled the existence of numerous GDSL-lipase like copies in the *C. intybus* genome.

Thus, in this Chapter, two questions were to answer. What is the evolutive history of the identified *CiGDSLs*: are they homologues to the *IbICS* gene following a parallel evolution? Are they correlated to diCQA production in chicory and thus are they good target genes for further characterization?

To answer these questions, *in silico* analysis were performed as well as a preliminary work on the evolutive history of the identified *CiGDSLs* using the current data on characterized GDSL-lipase like (see Table C.2 in Appendix C.). Moreover, their functional characterization to investigate their putative involvement in specialized metabolites production and particularly diCQA production was initiated.

4.2 Material and Methods

4.2.1 Plant Material

Chicory Hairy Root generation and growing conditions

In vitro plants of *C. intybus* L. clone 17, kindly provided by Florimond Desprez SA (Capelle-en-Pévèle, France), were used for this study. They were maintained on H10 solid medium (macro and micro-elements from Heller, Fe-Edta, $10 \text{ g} \cdot \text{L}^{-1}$ sucrose, $6 \text{ g} \cdot \text{L}^{-1}$ agar, pH 5.5) on a 16/8-h light/dark cycle with a temperature of $25 \pm 2^\circ\text{C}$ in the light phase ($37.6 \pm 10.1 \mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$) and at $20^\circ\text{C} \pm 2^\circ\text{C}$ in the dark phase. Clone 17 *in vitro* plants were subcultured every 10 days to 8 weeks in H10 solid medium according to the method described in [Bernard, Alves Dos Santos, et al. 2020](#). Hairy roots were obtained using wild-type *A. rhizogenes* strain 15834 (kindly provided by Marc Buée, INRA, Nancy, France). Independent lines were isolated. Hairy roots were maintained in liquid half strength MS ([Murashige et al. 1962](#)) media added with sucrose ($30 \text{ g} \cdot \text{L}^{-1}$) in flasks on orbital shaker at an agitation speed of 110 rpm with a light source providing about $5 \text{ W} \cdot \text{m}^{-2}$ on a 16/8h light/dark cycle with a temperature of 25°C in the light phase and 20°C in the dark phase.

N. benthamiana for transient expression of our candidate genes

N. benthamiana seeds were sown on soil and cultivated a greenhouse under a 16 h/8 h day/night photoperiod with artificial light ($70 \mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$) at 26°C . Five to six-week-old plants were used after 3–4 weeks for agroinfiltration experiments, as described in [Waadt et al. 2008](#).

4.2.2 Methods

Identification, cloning and sequence analysis of candidate genes for diCQA production in *C. intybus*

To identify sequences of putative homologs of IbICS ([Miguel et al. 2020](#)), its amino acid sequence was used as the query in a tBLASTn search of the in house

EST sequences of *C. intybus*. The three best hits were selected and named *GDSL1*, *GDSL2* and *GDSL3* according to their E value score in the BLAST search.

Full-length cDNAs of the three genes were amplified using a proofreading polymerase (PrimeSTAR HS DNA polymerase, Takara) from a cDNA library generated from mRNA extracted from young leaves, old leaves and roots. The primers used are listed in Table 4.1.

The entry clones (pDONR207_GDSL1, 2 or 3) were obtained through BP recombination of the PCR products with pDONR207 (Invitrogen). For further expression analysis, the open reading frames were introduced respectively into pEAQ-HT_DEST1 expression vector (Invitrogen) by LR recombination to produce pEAQ-HT_DEST1_GDSL1, 2 or 3. pDONR207-W/O-STOP_GDSL1, 2 or 3 were amplified from the aforementioned entry clones with specific primers (Table 4.1) and introduced in the pEAQ-HT_DEST3 expression vector by LR recombination to produce pEAQ-HT_DEST3_GDSL1, 2 or 3 encoding for recombinant proteins with a C-terminal 6xHis-tag.

Computational analysis Conserved motif determination was conducted with the MEME suite (Bailey et al. 2009). The prediction of putative N-terminal signal peptide (SP) was performed using TargetP 2.0 (<http://www.cbs.dtu.dk/services/TargetP/>). To predict putative amino acid sites for post-translational modifications such as glycosylation, we used the ModPred tool (<http://montana.informatics.indiana.edu/ModPred/>). The prediction of the putative subcellular localization of the proteins was done using ChloroP 1.1 (<http://www.cbs.dtu.dk/services/ChloroP/>) and mGOASVM-Loc Plant, V2 (<http://bioinfo.eie.polyu.edu.hk/mGoaSvmServer2>). The alignments and phylogenetic analyses were performed using the MEGA X software (<https://megasoftware.net>; S. Kumar et al. 2018). Alignments were done using the MUSCLE program. To determine the parameters of the phylogenetic studies, a model was generated and gave the following parameters: Maximum Likelihood Tree with WAG model +F +G (F stands for Frequency and G for Gamma Distributed). The chosen heuristic method was Nearest-Neighbor-Interchange (NNI). Phylogenetic trees were reconstructed using this algorithm with 1 000 replications of bootstrap.

Transient expression of CiGDLSL proteins in *N. benthamiana*

Gateway binary vectors were used for transient expression assays (Sainsbury et al. 2009). Vectors pEAQ-HT-DEST1_GDLSL1, 2 or 3 and pEAQ-HT-DEST3_GDLSL1, 2 or 3 as well as the empty vector pEAQ-HT were introduced by electroporation in *Agrobacterium tumefaciens* strain GV2260 selected on Yeast Extract Beef (YEB) medium (10 g·L⁻¹ beef extract, 5 g·L⁻¹ yeast extract, 10 g·L⁻¹ peptone, 15 g·L⁻¹ sucrose, 0.5 g·L⁻¹, MgSO₄, 20 g·L⁻¹ at pH 7.2) supplemented with 100 µg·mL⁻¹ kanamycin. The recombinant strains were grown overnight in YEB medium supplemented with 100 µg·mL⁻¹ kanamycin at 28 °C with shaking at 200 rpm until OD₆₀₀=0.6. The cultures were then pelleted by centrifugation (10 min, 3 000 g, 20°C) and resuspended in infiltration buffer (10 mM MgCl₂, 10 mM MES, pH 5.7, 100 µM acetosyringone) to OD₆₀₀=0.6. Each construct was used to infiltrate the abaxial air space of the two youngest leaves of 6-8 weeks old *N. benthamiana* plants via a needleless syringe (Waadt et al. 2008). After four days, the infiltrated leaves were collected and immediately frozen in liquid nitrogen. The leaves destined to metabolic extraction were freeze-dried before extraction.

Protein extraction from *N. benthamiana* leaves

Total protein extract was obtained by grinding the leaves of three different individuals in presence of extraction buffer (20 mM sodium phosphate, 0.5 M NaCl, pH 7.4) in a 1:2 ratio (g of fresh matter:extraction buffer). The crude plant extract was clarified by centrifugation at 14 000 g for 10 min at 4 °C. The supernatant was further filtered through a Sephadex G25 columns (Sigma Aldrich). Then, 3.5 mL of crude extract were collected and concentrated using centrifugal 10 kDa molecular mass cutoff filters (Amicon® Ultra 0.5 mL Centrifugal Filters -PMNL 10kDa, MILLIPORE). Total protein extract was used immediately for the enzymatic assays (Figure 4.6).

***In vitro* assays of recombinant proteins and analysis of enzymatic properties**

For standard assay of CiGDLSL activity, enzyme solutions were incubated at 28 °C for 30 min to 24h in 35 mM acetate buffer, pH 5.0, containing 1 mM CQA and

10 mM MgCl₂ in a total volume of 100 μ L. Reactions were stopped by adding 300 μ L absolute ethanol (Figure 4.6). The solution was mixed and centrifuged at 14 000g for 10 min at 4°C. After centrifugation, the supernatant was filtered (0.45 μ m filter), and analyzed by HPLC-UV. Reactions were run in triplicates.

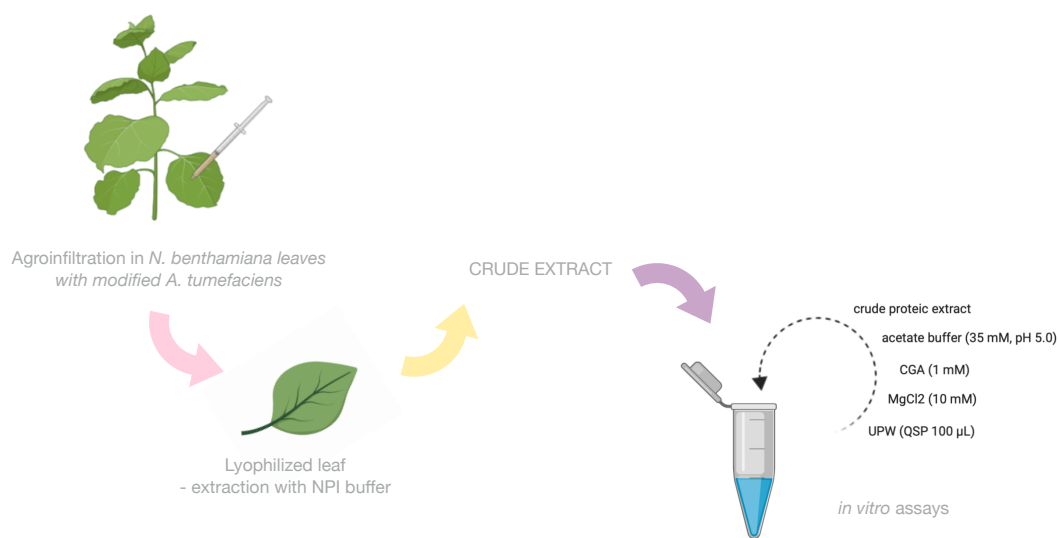


Figure 4.6 – Extraction and activity measurement of the recombinant proteins..

Phenolic compounds extraction and HPLC analysis

The 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). After a 1 h incubation in the dark under agitation at 4 °C, the homogenates were clarified by centrifugation (14 000 g, 4 °C, 10 min) and a second extraction with 1 mL of solvents was done. The pooled supernatants were passed through a 0.45 μ m filter. Metabolite profiles were analyzed by HPLC/DAD as described in section 3.1.2.

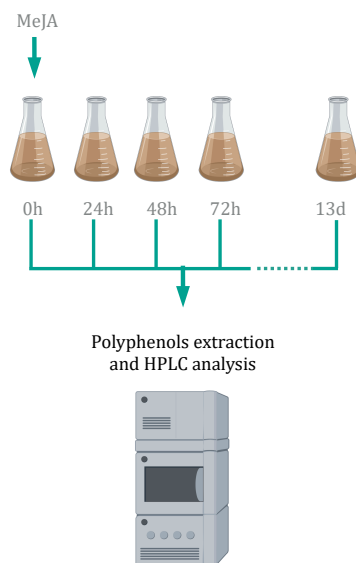


Figure 4.7 – Study of the elicitation of diCQA contents in hairy roots of *Cichorium intybus*.

Elicitation of hairy roots with MeJA

C. Intybus hairy root lines were elicited with MeJA²⁴. Triplicates of subcultures seeded with about 300 mg of root fragments in 20 mL of MS medium were grown for thirteen days. MeJA diluted in 96° ethanol was added in the medium to reach 0.15 mM. As controls, subcultures were either added with the equivalent volume of 96° ethanol or nothing. Roots were sampled and immersed directly in liquid nitrogen 0 h, 24 h, 48 h and 72 h after induction and stored at -80°C until metabolic extraction (Figure 4.7).

²⁴ Methyljasmonate

Table 4.1 – Primers used in GDSLs cloning

Code	Sequence	Target cDNA	Usage	Fwd or Rev
DG462	888gACAAAGTTTGTACAAAAAAGCAGGGCTccaccATGGCGATTTCACCAACTTTTCT	CiGDSL1	Plant overexpression	Fwd
DG463	888gACCACTTTGTACAAGAAAGCTGGGTcCTAGAGGCTTACAAAAGGCTTTGA	CiGDSL1	Plant overexpression	Rev
DG464	888gACAAAGTTTGTACAATAAAGCAGGGCTccaccATGGCGTTTCTTCCAGTTCT	CiGDSL2	Plant overexpression	Fwd
DG465	888gACCACTTTGTACAAGAAAGCTGGGTcTTAGATAGAACTCGATAACCCCTACTCG	CiGDSL2	Plant overexpression	Rev
DG466	888gACAAAGTTTGTACAATAAAGCAGGGCTccaccATGGCGAATTCATCAAACGTTG	CiGDSL3	Plant overexpression	Fwd
DG467	888gACCACTTTGTACAAGAAAGCTGGGTcTTAGATAGAACTTGAGAATCCTCCT	CiGDSL3	Plant overexpression	Rev
DG501	888gACCACTTTGTACAAGAAAGCTGGGTcGAGGCTTACAAAAGGCTTTGA	CiGDSL1 W/O STOP	Protein production	Rev
DG502	888gACCACTTTGTACAAGAAAGCTGGGTcGATAGAACTCGATAACCCCTACTCG	CiGDSL2 W/O STOP	Protein production	Rev
DG503	888gACCACTTTGTACAAGAAAGCTGGGTcGATAGAACTTGAGAATCCTCCT	CiGDSL3 W/O STOP	Protein production	Rev

4.3 Results

4.3.1 *In silico* sequence analysis

Initially diCQA was hypothesized to be synthesized by BAHD enzymes in *C. intybus*, more specifically HCT/HQT as in other models (*i.e.* *S. lycopersicum*; Moglia, Lanteri, et al. 2014). However, a recent study in *I. batatas* highlighted the possibility of a different pathway which we investigated (Miguel et al. 2020). A GDSL-like enzyme which had lost its lipase activities had been identified. We used its protein sequence to perform a BLAST onto the genome of chicory. We focused on the three hits with the highest E values. After identifying our potential genes, annotation of their sequences was realized.

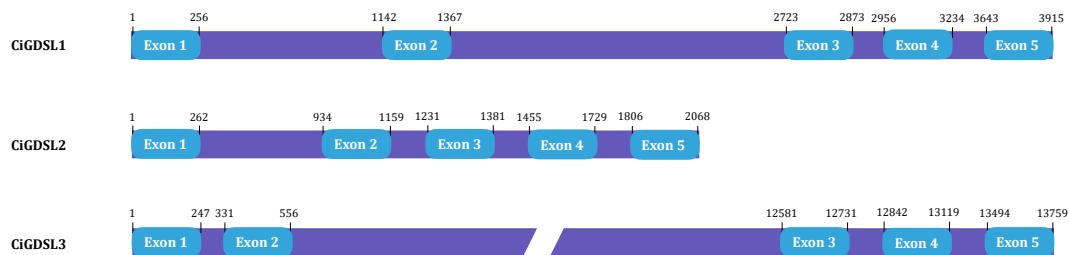


Figure 4.8 – Structure of CiGDSL genes. Introns are represented in purple and exons in blue.

CiGDSL1-3 coding sequences are 1181 pb, 1173 pb and 1164 pb long, respectively. They are all composed of 5 exons and 4 introns (Figure 4.8). *CiGDSL1* is found in linkage group 4 of *C. intybus* L. var. *sativum* genome whereas *CiGDSL2* and *CiGDSL3* are both found in linkage group 6, in a cluster of loci annotated with «SGNH hydrolase-type esterase domain». GDSL lipase are known for being large families with a clustering of the copies, so this result was not surprising. *CiGDSL3* gDNA shows a very peculiar structure. It is present on over 14 kb in the genome because of the 12 kb intron present between exons 2 and 3 (Figure 4.8). This colossal intron presents gaps of great size which were not sequenced and are represented with the letter N standing for undetermined nucleotides. The presence of this unusual intron remains unexplained.

CiGDSL1-3 have deduced protein sequences of 394, 391 and 388 AA with calculated molecular masses of 42.9, 41.8 and 41,7 kDa respectively . Pairwise protein sequence identity percentages are 68 % between *CiGDSL1* and 2, 72,2 % between *CiGDSL1* and 3 and 64,2 % between *CiGDSL2* and 3.

We compared the sequences of our three target genes to known and characterised GDSL-like genes and performed several prediction analysis. Several conserved properties were observed, including conserved motifs, the existence of a signal peptide, and the predicted subcellular localization.

Prediction tools determined that they all possess a N-terminal signal peptide and potential PTM ²⁵. The presence of a signal peptide is coherent with the properties of characterized GDSL-lipase like which are commonly excreted (see Table C.2 in Appendix C). All three *CiGDSLs* are also predicted to be extracellular or secreted proteins (mGOASVM-Loc), which is a common characteristic of GDSL-lipase like (Ding et al. 2019). Among 42 characterized GDSL proteins in the literature, 21 were predicted or demonstrated to use secretory pathways (extracellular or vacuole stored proteins; Table C.2).

CiGDSL1 has a predicted peptide signal of 26 AA depending on the prediction model, generating a 368 AA mature protein. PTM with a high confidence index unraveled by ModPred are amidation and a N-linked glycosylation at position N25 of the uncleaved protein. *CiGDSL2* has a predicted peptide signal of 27 AA, generating a 364 AA mature protein. PTM with a high confidence index are amidation and the SUMOylation at position K306 of the uncleaved protein. *CiGDSL3* has a predicted peptide signal of 21 AA depending on the prediction model, generating a 367 AA mature protein. PTM with a high confidence index are amidation and O-linked glycosylation of positions S329 of the uncleaved protein.

Alignment with characterized GDSL shows the presence of the conserved motifs of the SGNH superfamily in our candidate genes (Figure 4.9).

The alignment is composed of GDSL characterized for using caffeoyl conjugates

²⁵ post-translational modification

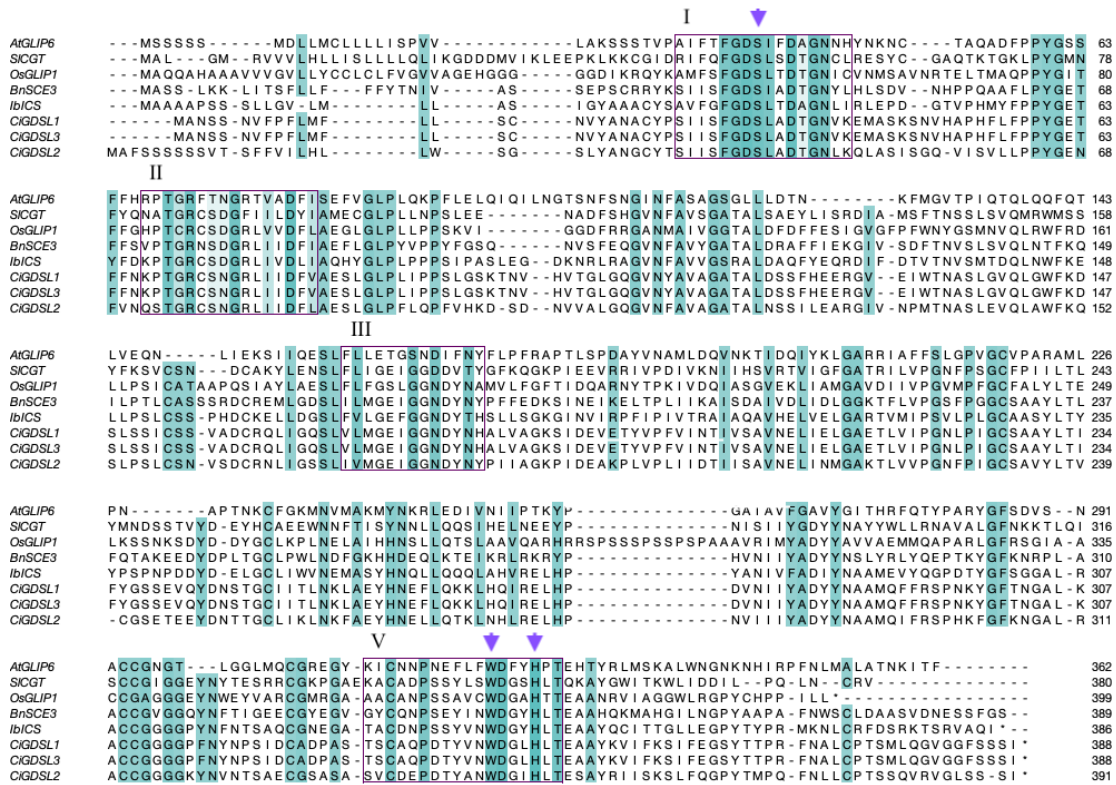


Figure 4.9 – Alignment of our candidate genes with characterized GDSLs. Residues colored in blue are the most conserved. SGNH conserved motifs are framed in black. The residues of the catalytic triad are marked by a purple triangle.

as substrates, while the others are GDSL from model species characterized to have a lipase activity and be involved in lipid catabolism (AtGLIP6) in the modulation of plant immunity through lipid homeostasis (OsGLIP1) with predicted extracellular localization, or to have an esterase activity and be involved in seed development (BnSCE3) with a vacuolar localization (Theologis et al. 2000; M. Gao et al. 2017; Clauß, Baumert, et al. 2008 Clauß, von Roepenack-Lahaye, et al. 2011). These proteins are qualified as GELP²⁶, hydrolyases with broad substrate specificity such as thioesters, aryl esters, amino acids and phospholipids (Akoh et al. 2004). They are characterized by a conserved GDSL motif in the N-terminal part of the protein which distinguishes them from classic lipolytic enzymes which harbor a GxSxG conserved motif (Upton et al. 1995). They are

²⁶ GELP: GDSL-type esterase/lipase protein esterase EC 3.1.1, lipase, EC 3.1.1 ; Chepyshko et al. 2012

also named SGNH hydrolases after the four conserved catalytic residues Ser, Gly, Asn and His present in conserved blocks (I, II, III and V) respectively (Figure 4.9). The amino acids of the catalytic triad of GDSLs can be found in block I (Serine) and block V (Aspartate and Histidine) mark by a triangle in Figure 4.9. The conservation of these motifs in *C. intybus* GDSLs confirms that they belong to the SGNH enzyme family, more specifically to the GELP family.

4.3.2 Elicitation of phenolics production in Chicory Hairy-Roots

To investigate the role of *CiGDSL* genes in diCQA biosynthesis, contrasted diCQA accumulation conditions were searched. Thereafter, the levels of transcripts from the three *CiGDSL* genes and the contents of diCQA may be investigated to potentially be correlated. To achieve these conditions, it was necessary to establish a model in which diCQA accumulation could be easily quantified and elicited.

Chicory hairy roots are the model most suitable to find these conditions. After elicitation with MeJA, contrasted accumulation of specialized metabolites can usually be observed (Bernard, Alves Dos Santos, et al. 2020). Thus, a kinetic study of the accumulation of CQA and diCQA over a period of 13 days after elicitation with MeJA was carried out (Figure 4.10). Elicitation was performed using MeJA with a final concentration of 0.15 mM and controls consisted of either no alteration of the culture conditions (control) or with the addition of 96 % EtOH (solvent of the MeJA solution used in the study). This kinetic allowed to pinpoint which time point would be more suitable for our study.

The control and EtOH conditions are similar for both molecules as they show no significant increase in the contents of CQA or diCQA in the chicory hairy roots (Figure 4.10). When elicited with 0.15 mM of MeJA, CQA (diCQA putative precursor) accumulates in the hairy roots only after 2 days and reaches its maximum concentration after 3 days. After that time period, CQA concentrations drop at 6 days and 9 days, before increasing again after 13 days of incubation (Figure 4.10 A.). For diCQA, a different accumulation profile is observed. After 24 h, an increase of its contents is already detectable. diCQA content reached its

maximum after 72 h and then remains quite stable (Figure 4.10 B.).

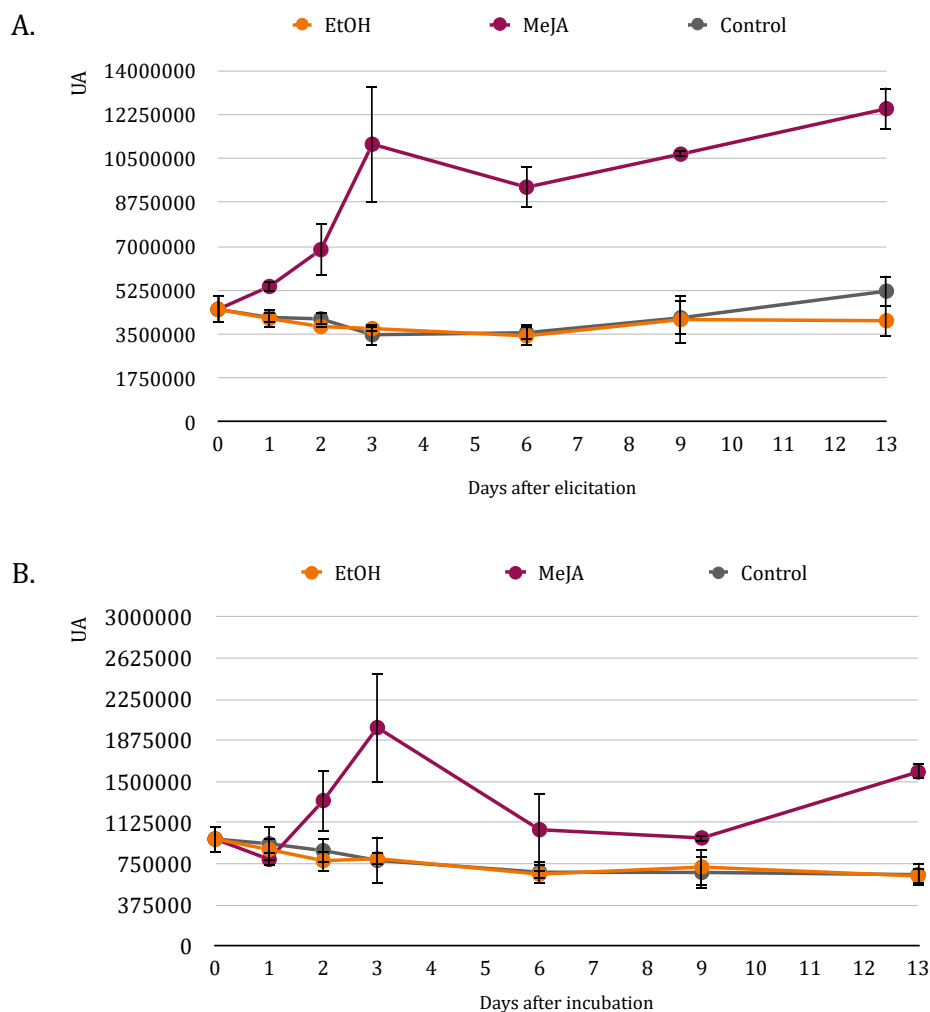


Figure 4.10 – Kinetic study of CQA (A.) and diCQA (B.) accumulation in chicory Hairy Roots after elicitation with MeJA

Elicitation was performed by adding MeJA at a final concentration of 0.15 mM (MeJA in the figure). As controls, the experiments were performed without any addition (Control) or with the addition of 96° EtOH (EtOH), the solvent of the MeJA solution.

It is thus likely that the expression of the genes involved in diCQA production will be overexpressed after 48 hours of incubation following MeJA elicitation. The perspectives of this research are to perform RT-qPCR on the GDSL genes after 48 hours to see if they are overexpressed and thus have more evidence of

their involvement in diCQA production.

4.3.3 Transient expression in *N. benthamiana* leaves and protein purification

The potential activity of CiGDSL recombinant proteins towards CGA was investigated. The initial step was to see if CiGDSLs could use the available substrates and make CGA derivatives when overexpressed *in planta*. We then assayed their activity after extraction *in vitro*.

The purpose of this study was to explore if our candidate proteins could catalyze the synthesis of diCQA in an *in vivo* system with the available non- or low-substituted CQAs. Because it has been shown that unicellular models are not always efficient when it comes to GDSL-lipase like expression, we performed transient expression assays in tobacco rather than BL21 *E. coli* or *S. cerevisiae*. Indeed, neither SLCGT (*S. lycopersicum*) nor IbICS (*I. batatas*), two GDSL-lipase-like enzymes that use chlorogenate as a substrate, were successfully produced in these systems, possibly due to a lack of post-transductional modifications in these unicellular systems or poor protein solubilization, as the authors suggest (Teutschbein et al. 2010; Miguel et al. 2020).

To that extent we cloned the *CiGDSLs* genes in pEAQ-HT-DEST1 vectors with no tags to avoid potential interference with active sites of the proteins. We then used the properties of *A. tumefaciens* to insert our genes into the leaf plant cells genome. After a 4 days incubation period, transformed leaves were sampled, lyophilized and the phenolics extracted. The extracts were analyzed by HPLC (Figure 4.11). The empty plasmid profile shows the naturally occurring CQAs in *N. benthamiana*. We are in consequence searching for a shift in the metabolic profile rather than new peaks. When *CiGDSLs* are overexpressed, we see an increase in the area of several peaks with spectra corresponding to CQA molecules.

Overexpression of *CiGDSL1* induces few metabolic changes but slight increases of peaks annotated as diCQA derivatives (annotated (2) at retention

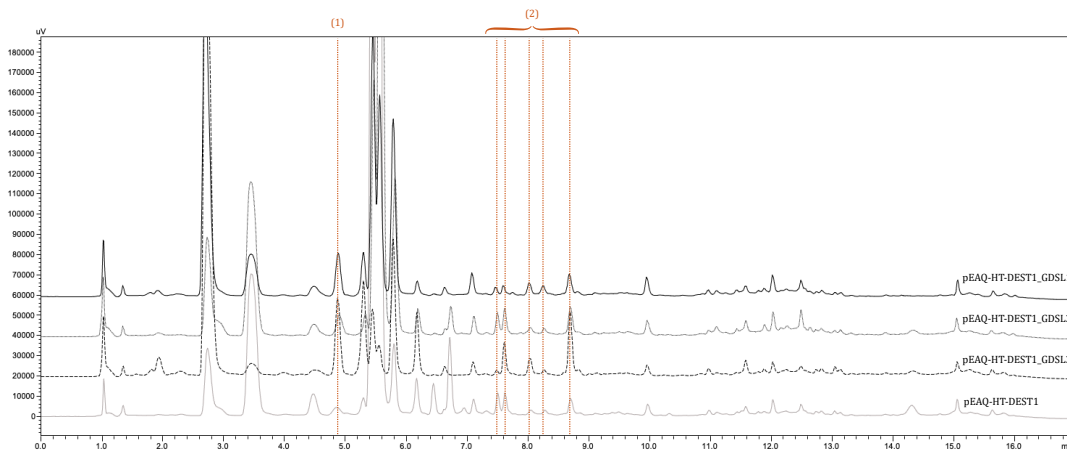


Figure 4.11 – Metabolic profiles of *N. benthamiana* leaves agroinfiltrated with pEAQ-HT-DEST1_CiGDSLs

Peak (1) was identified as a CQA derivative and peaks annotated (2) as diCQA derivatives according to their retention times.

times 8.05 and 8.30 min, Figure 4.11). A similar influence can be observed by *CiGDSL2*, with a slight increase of a different diCQA derivatives (peak annotated (2) at retention time 7.65 min; Figure 4.11). Overexpression of *CiGDSL3* induces the most visible and numerous changes in the metabolic profile compared to the empty plasmid control. We can observe a decrease of the peak at retention time 7.50 min and increases of three diCQA derivatives (peaks annotated (2) at retention times 7.65, 8.05 as well as 8.75 min) with the last peak showing the most substantial change (Figure 4.11). Overall, all CiGDSLs induced diCQA derivatives production in this model but it seems of different isomeric nature according to each protein. When *CiGDSLs* were overexpressed in *N. benthamiana* tissues, we presume distinct isomers of diCQA or CQA derivatives were generated. Because of the natural presence of CQA derivatives in tobacco leaves, the action of CiGDSLs is difficult to distinguish in this model.

The main conclusions of this work are that CiGDSLs seem able to utilize CGA as a substrate, however the exact identity of their product remains undetermined and need to be identified by MS/MS. This preliminary work is promising in the characterization of these three candidate genes in the production of diCQA. However, these encouraging results *in vivo* led to further studying of those

recombinant protein properties in a more controlled environment.

4.3.4 *In vitro* assays of enzymatic activities from crude or purified extracts

Crude protein extracts of transformed *N. benthamiana* leaves were used in enzymatic assays. The figure 4.12 illustrates the HPLC analysis of *in vitro* assays in which crude extracts of leaves transformed either with *pEAQ-HT-DEST1-CiGDSLs* or the empty plasmid were incubated with CQA at 28°C in the dark for 5h.

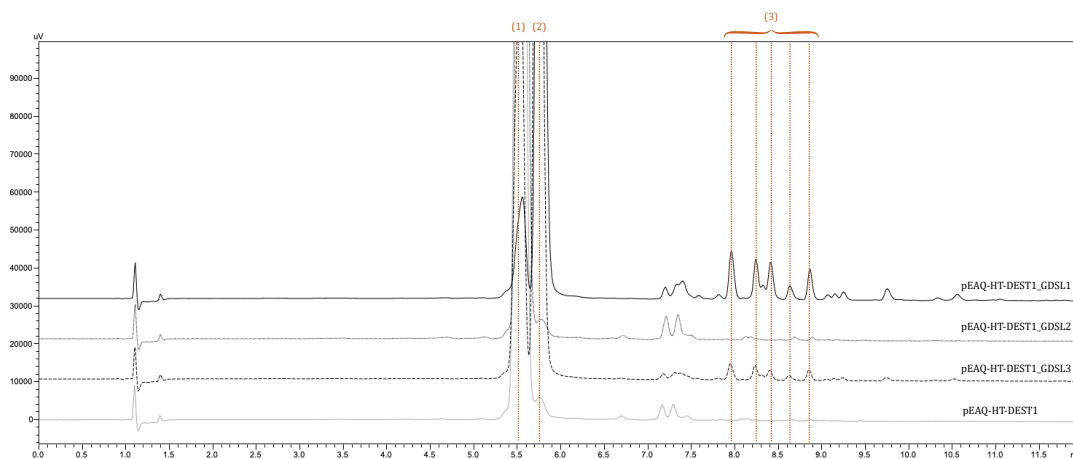


Figure 4.12 – Enzymatic assays with crude proteic extracts of *N. benthamiana* leaves agroinfiltrated with *pEAQ-HT-DEST1_CiGDSLs*

Peak (1) is the CGA added as a substrate in all assays. Peak (2) was identified by MS/MS as caffeic acid. Peaks (3) were identified as CQA derivatives according to their retention times and the nature of the substrate.

In the tested conditions *in vitro* CiGDSL2 shows poor activity whereas CiGDSL1 and CiGDSL3 are both active.

One can note the expected presence of CGA at retention time 5.50 min, the used substrate in all metabolic profiles (peak annotated (1); Figure 4.12). The production of caffeic acid (peak annotated (2) and identified by MS/MS, data not shown) is clearly observed in presence of the crude protein extracts of CiGDSL1 and CiGDSL3 (Figure 4.12). Both proteins exhibit the same metabolic profiles

and appear to catalyze the production of CQA derivatives (peaks annotated **(3)**; Figure 4.12). In both assays, diCQA isomers were identified by MS/MS analysis but their exact nature remains unclear (data not shown). This activity is reminiscent of the demonstrated catalytic ability of EpCAS, a SCPL enzyme, to also use chlorogenic acid as substrate in dicaffeoyltartric acid production (Fu *et al.* 2021). Moreover, this pathway also uses two HCA as substrates to form a di-substituted cinnamic acid.

CiGDSLs were also cloned in pEAQ-HT-DEST3 vectors, conferring the recombinant proteins a HIS-tag in C-terminal position for future purification. This would be the next logical step along further identification MS/MS of the characterization of *CiGDSLs* activities towards CQAs.

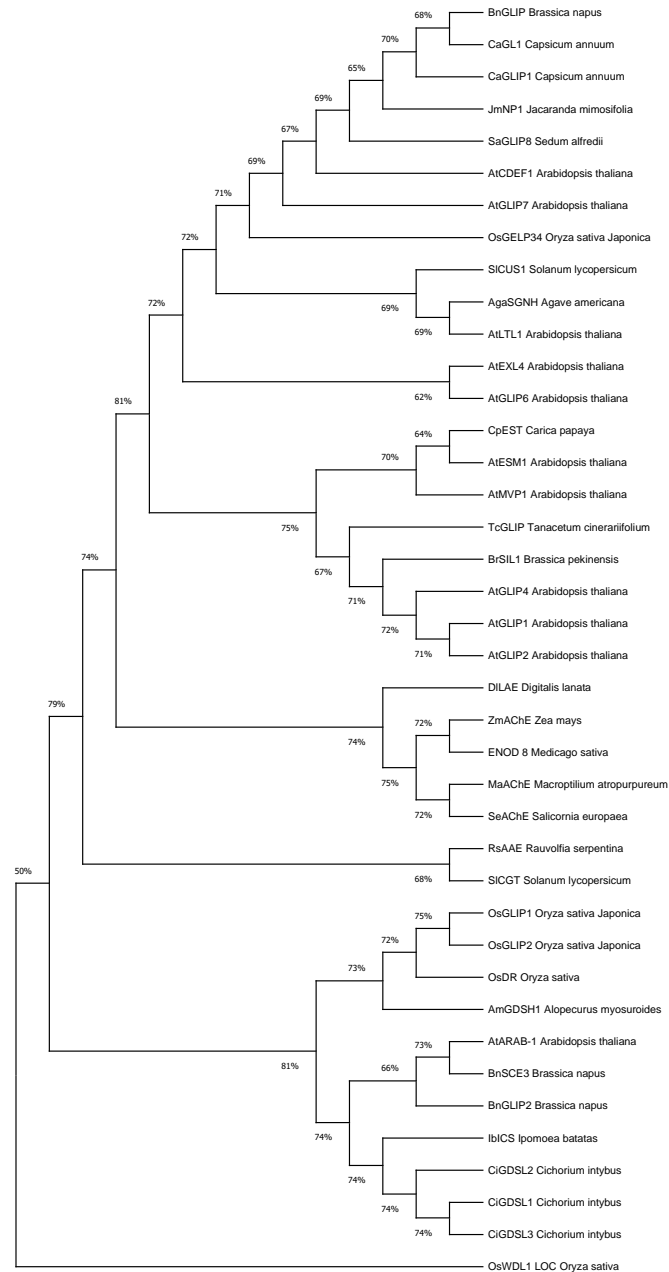


Figure 4.13 – Phylogenetic tree of characterized GDSL-like lipase/esterase and transferase protein sequences listed in table C.2

The tree was constructed with the Mega X software using the Maximum likelihood method with depth of analysis a of 1000 bootstrap.

4.3.5 Evolution history of CiGDSLs and diCQA production

As previously mentioned, the pathways of diCQA production can differ from one species to the next. The evolutive history of the enzymes involved in its production can unveil the putative type of biosynthesis present in the species under study. We were particularly interested in the relationship between the GDSL-like of *C. intybus* and *I. batatas*, which seem to be both involved in the synthesis of diCQA of B type (see section 4.5). It appears that this novel activity might have been acquired independently. Thus, we presume that they could be homologous genes. However, the lack of data on characterized GDSL-lipase like compels us to treat the following work with caution regarding its conclusions.

To infer this phylogenetic relationship, we assembled a tree of all characterized GDSL-lipase like we were aware of (Figure 4.13). Their catalytic activity, localisation and function are summarized in Table C.2. We can observe that *CiGDSLs* cluster closely with *IbICS*. The rest of their clade is composed of *AtARAB-1*, *BnSCE3* and *BnGLIP2* which all have an esterase activity. The next closest *GDSLs* are lipases *OsGLIP1* and *2*, and *AmGDSH-1* a hydrolase and esterase. Other transferases or acyltransferases can be found in this tree: *SICGT* of course but also *OsGELP34*, *SICUS1*, *AgaSGNH*, *TcGLIP* for instance. GDSL-lipase like don't seem to cluster only according to their function, highlighting their great adaptive flexibility in terms of function and substrate preferences. One limitation of this tree is that it is composed of paralogs and not only homologs of GDSL-lipase like. As aforementioned, GDSL-lipase like constitute clusters of great number of copies which have or can not be all characterized. For these reasons, predicting GDSL-lipase like family evolution history is not yet possible. However, the present work allows us to gain insight in diCQA production pathways evolutive history by identifying species in which GDSL-lipase like are involved in its metabolism.

Figure 4.14 represents species in which GDSL-lipase like with a transferase activity was identified. When we look at the five GDSL-lipase like able to use chlorogonate as a substrate namely *CiGDSLs*, *IbICS* and *SICGT*, we can see that

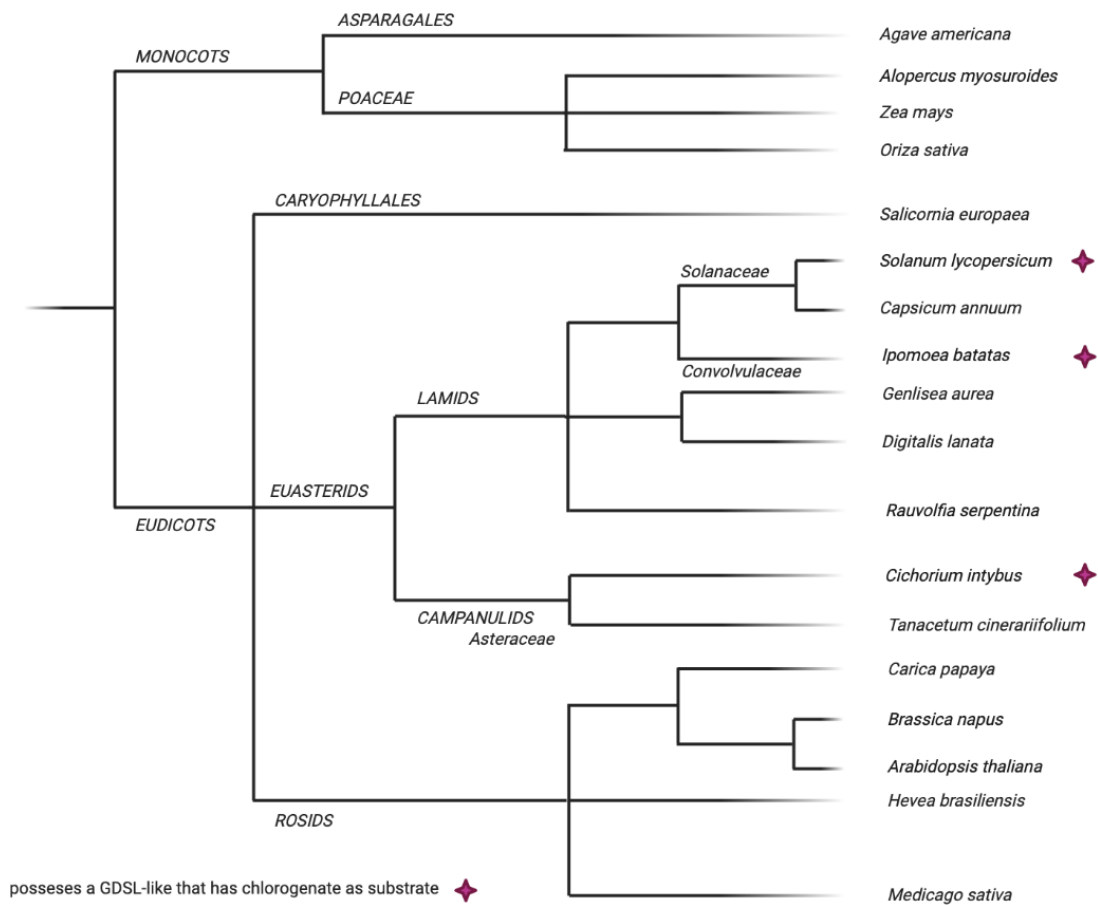


Figure 4.14 – Occurrence of GDSL-lipase like proteins with a transferase activity in land plant species

Species that possess a GDSL-like lipase using chlorogenate as a substrate are marked with a purple star.

the species in which they are found (marked by a star symbol) are not closely related.

This trait occurrence would then classify as an homoplasy meaning that it was not inherited from a common ancestor. They are three forms of homoplasy: convergence; parallelism and reversion. They are represented in Figure 4.15. The term parallel evolution refers to when ancestral descendants with different biochemical activities but a common structural lineage evolve to synthesize the same metabolite at the same time (Figure 4.15 A). The term convergent evolution is used when different protein structures with no structural resemblance catalyze

the synthesis of the same metabolite (Figure 4.15 A). Reversion concerns traits going back to their ancestral state after a mutation, recovering their primary function (Figure 4.15 B). The mechanisms involved can range from mutations leading to similar protein sequences to a horizontal transfer of genes, or the presence of a similar catalytic triad.

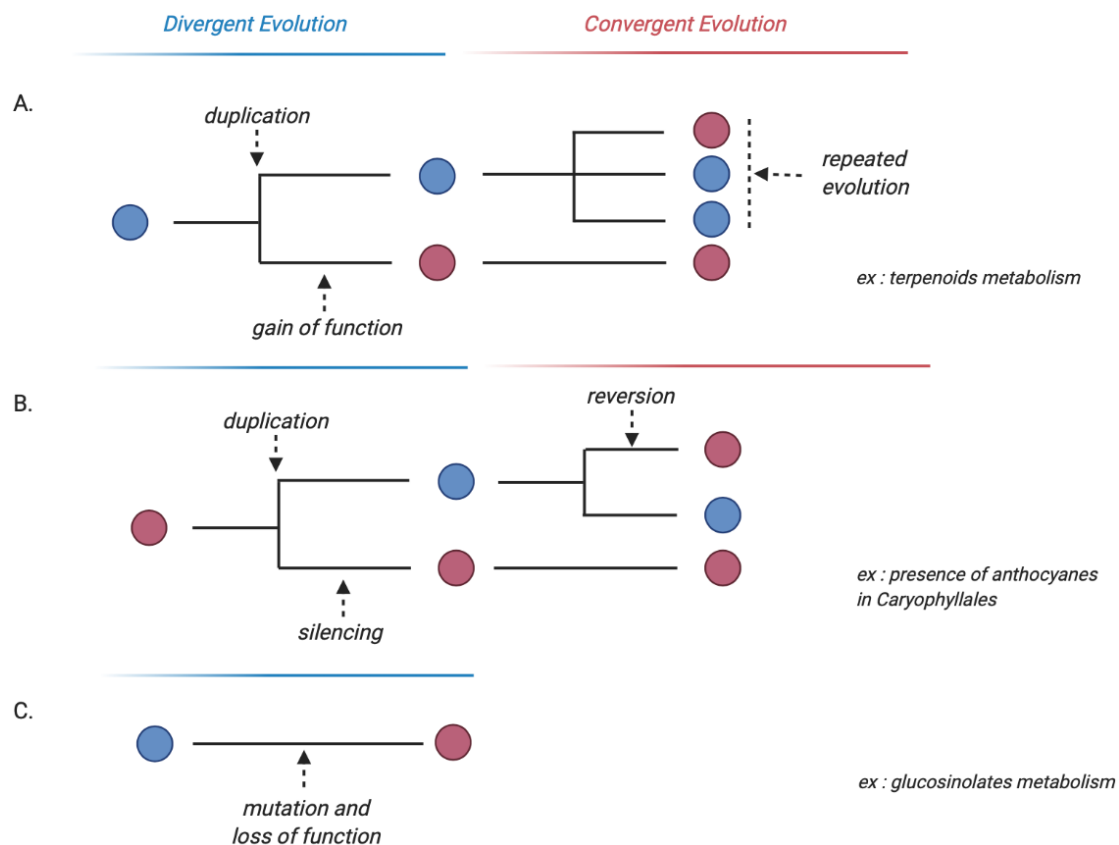


Figure 4.15 – Existing evolution mechanisms that can lead to homoplasy or homology. Examples are respectively from [Matsuba et al. 2013](#) and [Brockington et al. 2015](#)

The parsimony principle states that the evolution route with the fewest evolution events is the most plausible, so we can consider that the use of chlorogenate by GDSL-like with transferase activity is a gain of function in these three species and that their closest common ancestor did not have this ability. Hence the definition of parallel evolution homoplasy would most fit the current data. Moreover, the high protein sequence similarity between IbICS and CiGDSLs suggests that a homology relationship is plausible. Hence, to this day a clear conclusion on the

evolutionary link between *IbICS* and *CiGDSLs* is not achievable.

As described in section 4.5, other synthetic routes of diCQA are described involving BAHD acyltransferases, HCT/HQT. We can make the assumption that both routes might exist in parallel in most diCQA accumulating species and in some both are active (*e.g.* type A and B routes in *I. batatas*), whereas some favor one route over the other one which might be silenced (route A - HCT/HQT in *Coffea spp.* and *S. lycopersicum* or possibly route B - GDSL-like in *C. intybus*).

Further work is needed, particularly using available genomes and proteomes to identify other candidates for diCQA production in both routes and gain hindsight in the evolution history of diCQA synthesis. Moreover, identification by MS as well as protein purification work are essential to confirm the role of the identified *CiGDSLs* in the synthesis of diCQA in *C. intybus*.

4.4 Discussion

Few biosynthetic pathways leading to the production of CQAs and in our case diCQA have been successfully characterized to date. In past studies (Legrand 2015) BAHD enzymes were thought to be the main candidates for this synthesis (Introduction of Chapter 4.1 on page 183). However, GELP became new candidate genes with the work of Teutschbein and colleagues (2010). Moreover, Miguel and colleagues demonstrated the role of a GDSL-lipase like in diCQA production in their model, *I. batatas* (Miguel et al. 2020). A bibliographic overview of characterized members of this newly studied superfamily was carried out and demonstrates the wide array of activities and functions of GDSL-lipases like or GELP (Table C.2 in Appendix C.). Several copies of GDSL-like lipase were determined as good candidates in the chicory genome. However, numerous loci were annotated as GDSL-lipases like. This is common to this enzyme family as demonstrated in other models such as soybean and grape berry (Su et al. 2020; Ni et al. 2020). Because it is impossible to characterize all copies, only the three best matches were chosen for this study.

After identification of candidate genes in the chicory genome, a computational analysis of their sequences was conducted and a functional study using a heterologous expression method was initiated. The *in silico* analysis of those sequences confirmed that they had the characteristics of GDSL with esterase activity. The phylogenetic analysis showed that the production of diCQA by GELP was shared in several species that are not closely related: *S. Lycopersicum*, *I. batatas* and *C. intybus*. Parsimony suggests that parallel evolution led to these events and that *IbICS* and *CiGDSLs* are homologous genes if they indeed encode a similar activity. Until further data is available, the hypothesis of a reversion can not be examined.

The diversity in the physiological processes and in the organ localization of the GDSL family member is reflected in the various ways they can be elicited. Some may be induced by phytohormones, as in our case, while others may be induced by other stimuli, such as biotic or abiotic stresses (Table C.2 in Appendix C.)

(Ding et al. 2019). This work demonstrated that chicory hairy roots accumulate high levels of diCQA and that elicitation with MeJA in this model resulted in contrasted accumulation of this molecule of interest, which is ideal for further characterization studies.

cDNA *CiGDSL* sequences were amplified from chicory cDNA banks and cloned into expression vectors. It has been demonstrated that protein expression in prokaryotic systems can lead to improperly conformed proteins or proteins lacking the necessary post translational modifications. Yet, the predictions studies for CiGDSLs show that such PTM are conceivable. Hence, the most appropriate models for their heterologous expression are *N. benthamiana* and *P. pastoris* (Ling 2008; X. Li et al. 2013). This justifies the use of tobacco leaves as our heterologous system in this work as well as explains possible differences in activities *in vivo* or *in vitro*. The HPLC metabolic profiles obtained after extracting the infiltrated leaves were conclusive in demonstrating that CQAs were generated when our candidate genes were overexpressed. Enzymatic assays performed using crude protein extracts from *N. benthamiana* leaves and CQA as a substrate were consistent for two of the candidate genes in the tested conditions.

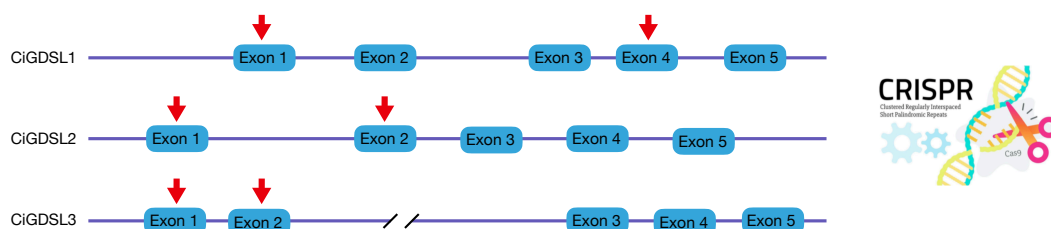


Figure 4.16 – Design for the creation of GDSL KO lines using CRISPR-Cas9 technology.

Many experiments were put in motion to complete this work, especially regarding the functional characterization of the three CiGDSLs. The determination of Chicory Hairy Roots as our model will allow to perform the designed RT-qPCR studies after 48 hours of MeJA elicitation. In the same model, *CiGDSL*-KO lines using CRISPR-Cas9 technology are being selected. Those KO lines will help

determine if *CiGDSLs* are indeed involved in diCQA production and if their actions are redundant or not. This redundancy could be expected due to the importance of this pathway in the plant specialized metabolism. The cloning of the three genes of interest in expression vectors suitable for protein purification and sub-cellular localization was also realized. These experiments constitute the main perspectives of the presented work.

GDSL-like lipases have great value in many industries such as food, cosmetics and medicine for they catalyze natural products of high interest and possess catalytic activities that can be valorized in these fields (Ding et al. 2019). The work presented in this chapter contributed to highlight the presence and importance of GDSL-like lipases in plant molecular processes, as well as gain insights into their evolutionary history and into the biosynthetic pathway of a highly coveted natural product.

General Conclusions and Perspectives

The rising of heat waves and extreme weather events endangers plant survival and food security on a global scale. The FAO ¹ urges the search for sustainable agriculture solutions. Understanding plant molecular responses and physiological acclimations to such constraints is essential to a stable future and contributes to the sustainable development goals of the United Nations.

Natural products like specialized metabolites are one way to enhance plant resilience to these constraints. Specialized metabolites great diversity in structure, roles and functions picked the interest of numerous plant scientists. Originally studied towards medicinal or industrial applications, their interest in plant physiology is now recognized and valorized.

Industrial chicory is emblematic of the North of France. Its durable cultivation is dependent on varieties selection resistant to novel constraints as well as carrying organoleptic qualities in line with the consumers demand. In both these aspects, the deciphering of specialized metabolite biosynthetic pathway is needed. Fortunately, chicory is a compliant species to biotechnological approaches, particularly for the production of hairy roots from which one is able to regenerate plantlets. Moreover, its study for agronomic purposes led to the sequencing of its genome, crucial to molecular cloning and engineering.

¹ FAO: Food and Agriculture Organization of the United Nations

This makes chicory a useful model for the study of the molecular pathways leading to the accumulation of natural products of interest in its tissues. Some of these metabolites, CTAs, CQAs and phenolamides, are produced through acylation reactions. One of the main enzyme superfamily to perform such catalysis in plants are BAHDs. The team identified seven BAHD in chicory belonging to the clade V and characterized five of them during the thesis of Guillaume Legrand and Marianne Delporte (Legrand 2015; Delporte 2017).

Two of the seven identified BAHD remained to be fully characterized, a project that was commonly pursued in Guillaume Bernard's thesis and the present work (Bernard 2018). These BAHD were annotated as SHTs and correlated to the production of an unusual phenolamide found in the pollen coat of chicory, TetraSpm. However, their roles in the biosynthesis of this metabolite were unclear. In collaboration with Guillaume Bernard, the deciphering of the mechanism of its production as well as its validation *in vivo* were performed. The generation of *Cisht* KO mutant lines with the CRISPR-Cas9 technology provided sufficient insight to establish a new hypothetical production pathway. The sequential actions of CiSHT2 and CiSHT1 was demonstrated *in vivo* in two different heterologous systems (Figure 5.1). Moreover, RMN analysis brought light on the catalytic preferences of CiSHT2. Indeed, secondary amine groups are acylated first on the spermine backbone. CiSHT1 acylates primary amine groups at the extremity of the spermine molecule.

The promiscuity of *CiSHTs* (particularly *CiSHT2*) and *AtSHT* in sequence, activity and phylogenetically rises the question of their affinities for different polyamines and the production of different final products *in planta*. Two non exclusive hypothesis are to be considered. The first is an influence of the available free polyamines *in vivo* on the activity of the enzymes. The second is an intrinsic preference for a polyamine over another originating from a difference in their sequences. The ratio of spermine to spermidine were investigated in the floral buds of *C. intybus* and compared to the ration in *A. thaliana* floral buds (Fellenberg, Ziegler, et al. 2012), confirming the correlation between the polyamine most available *in planta* and the main phenolamides accumulated in each model. In addition, *in vitro* assays as well as complementation experiments

of KO mutants seem to lead to the same conclusion: SHTs activities of *A. thaliana* and *C. intybus* are influenced by the nature of the available free polyamines.

The perspectives of this part of the presented work are the following. The analysis by MS of the complemented lines of *atsht* KO *A. thaliana* with the *CiSHTs* will provide further data to confirm or infirm this hypothesis. Moreover, the parallel work in double *Cigdsl* KO lines of *C. intybus* complemented with *AtSHT* has been performed. The analysis of the phenolic composition of the floral buds of these transformed lines will enable to strengthened the conclusion towards the influence of free polyamines on the activity of SHTs. An other *in planta* approach is the creation of mutant lines in which the contents of the free polyamines, here spermine and spermidine, could be modulated or completely altered. This could be achieved by working upstream of the pathway on the polyamines synthases, SPMS and SPDS respectively. The analysis by HPLC MS/MS of the phenolamides accumulated in these mutant lines of either *C. intybus* or *A. thaliana* would facilitate an unequivocal characterization of the polyamine ratio influence on the activity of SHTs.

A *in vitro* characterization with the purified intermediate products of the pathway for further assays of the *CiSHTs* and *AtSHT* activities would allow to assess their substrate affinities towards each of them and precise the mechanisms of fully substituted phenolamide production.

From an evolutive perspective, the screening of the *Asteraceae* species and closely related family for their polyamine contents seems crucial to pinpoint the timing of the event leading to this shift and possibly *SHT* gene duplication within this family.

Nonetheless, the shift in the polyamine ratio in the *Asteraceae* family does not fully explain the differential activities and products of *AtSHT*, *CiSHT2* and *CiSHT1*. In order to investigate the cause of those differences, a structural analysis work was undertaken to identify catalytic sites and motifs essential to substrates affinities. During Marianne Delporte's thesis, a conserved motif in SHTs was identified: FYGN (Delporte 2017). It was the first motif we were interested in mutating to confirm or infirm its involvement in SHT activity. It

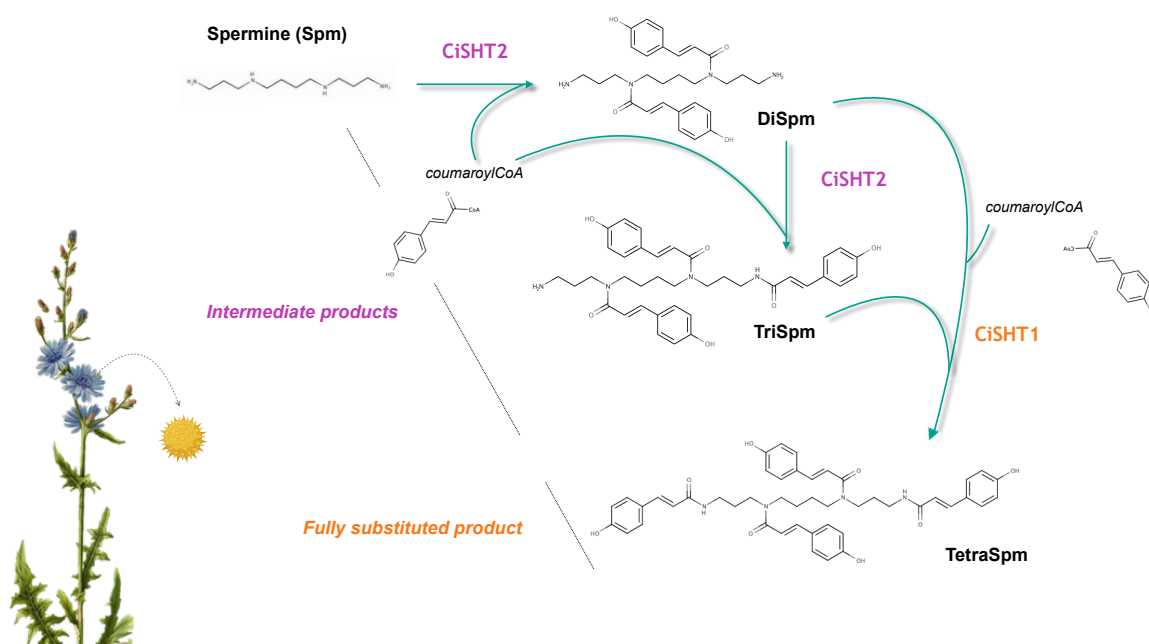


Figure 5.1 – Proposed Tetracoumaroyl-spermine biosynthetic pathway in *Cichorium intybus*

mono-, di- and tri- substituted spermine are designated as intermediate products in this biosynthetic pathway. Spm stands for coumaroyl-spermine here.

so happens that TpHCT2, also a member of BAHD clade V, exhibits similar transferases activities but on different substrates. Moreover, an inversion of the Phe and Tyr amino acid can be observed in this conserved motif. After assaying the *in vitro* activities of AtSHT and TpHCT2 towards an array of acyl acceptors, this motif was mutated in both proteins. The inactivity of the produced mutant showcased the essential character of this consensus motif. However, its presence alone does not seem to be responsible for substrate binding.

In silico analysis and annotation of the protein sequences of CiSHTs highlighted numerous sites of putative interest in their distinct substrate preferences. Blindly performing site-directed mutagenesis on all of these sites being costly and time consuming with no guarantee of results, chimeras proteins of CiSHTs were generated to narrow down our search. This led to a shorter list of sites of interest, of which the positions seem coherent with the predicted conformations produced by homology modelling. In addition to these expected results, an unforeseen

outcome was produced. One of the chimera of CiSHTs, chimera III (1A2B1C), has the catalytic ability to produce TetraSpm in one step instead of the two required *in planta*. This chimera III thus constitutes a remarkable tool in the production of TetraSpm in heterologous systems or in bioreactors, and might alleviate difficulties to produce this natural compound in sufficient quantities for activity assays.

The perspectives of this work include the site-directed mutagenesis of the shorter list of sites of interest. This can be considered for further characterization, potentially with modelling and autodocking analysis to validate their relevance beforehand. However, the collaboration with bioinformatics experts or with the creators of the unprecedented AlphaFold2 tool seems necessary to achieve accurate and precise predictions of 3D protein modelling.

In vivo, the overexpression in chicory hairy roots of both *CiSHTs* was already contemplated for TetraSpm production and purification. The overexpression chimera III can reduce the workload and the potential off target effects of introducing several genes into this model. The expression of native or mutated SHT proteins of chicory in combination with other enzymes to produce novel compounds is a compelling perspective. The work of Perrin and colleagues (2021) demonstrated that, even if not always fruitful, the construction of pathways with enzymes from different species can lead to the production of unique metabolites in *S. cerevisiae* or *P. pastoris*. One could take advantage of the successive actions of the two CiSHTs and attempt to create spermine derivatives with a broader range of acyl donors and thus new to nature compounds.

This thesis also focused on another hydroxycinnamic acid conjugate accumulated in chicory for which the biosynthetic pathway remained unclear despite the work done on the seven identified BAHD. Indeed, HCTs/HQTs of chicory appeared to not be involved in diCQA production in our model (Legrand 2015). The work of Miguel and colleagues (2020) in collaboration with our team led to the characterization of a novel pathway requiring GDSL-lipases like for diCQA production in *I. batatas*. Hence, GDSL-lipases like were identified, cloned and assayed in chicory in order to determine if they play a role in diCQA production

in our model as well. Additionally, their evolutive history was investigated. The current available data on GELP allowed for computational analysis of the targeted CiGDSLs. They exhibit similar characteristics to GDSL-lipases like with esterase and acyltransferase activities. From an evolutive point of view, the production of diCQA through their action would be the result of a convergent evolution between *I. batatas* and *C. intybus*.

This work demonstrated a clear correlation between CiGDSLs overexpression and CQA conjugates production (Figure 5.2). They represent robust target genes in this pathway and will be further characterized within the team. *In vitro* assays with purified enzymatic extracts, subcellular localization as well as generation of KO lines using the CRISPR-Cas9 technology are the next perspectives of this study to confirm the roles of these CiGDSLs in diCQA production and their potential functional redundancy. These experiments constitute some of the perspectives of this chapter, alongside the following experiments.

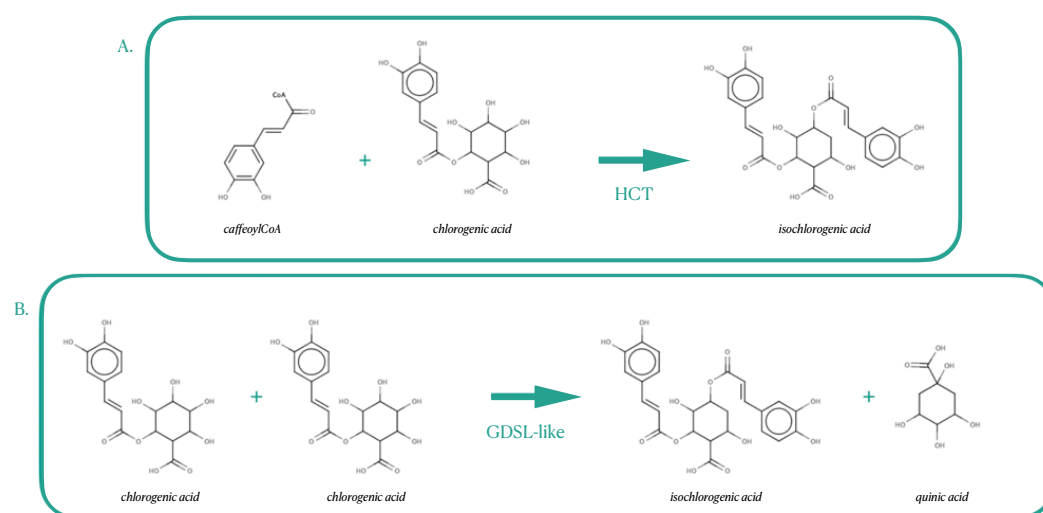


Figure 5.2 – Characterized biosynthetic routes of isochlorogenic acid.

Two types of biosynthetic routes for diCQA have been characterized.

Type **A** : acyltransferase reaction with 3-CQA and caffeoyl-CoA as an acyl donor.

Type **B** : acyltransferase reaction with two 3-CQAs molecules as both the acyl donor and acyl acceptor

The prospect of a functional redundancy is conceivable for a metabolite with such strong activities, as a fail-safe in the event of a loss of one copy or for

concentration modulation purposes. Here, several copies of GDSL-lipases like in chicory have been linked to CQA metabolism. However, this redundancy could hide the impact of a KO mutagenesis of one copy on the overall diCQA production and thus impair the characterization of the role of each copy. A quantitative work could alleviate such limits. Additionally, the analysis of the molecular profiles and phenotypes of these *Cigdsl* KO-lines would benefit the characterization of the physiological role of these natural products in our model. The characterization of these GDSL-lipase like is essential in two aspects. The first is the production of the potent and coveted diCQA for instance in bioreactors if the GDSL-lipase like genes are confirmed to be involved in its biosynthesis. However, potential PTM could cause setbacks in this enterprise. The second is to contribute to definition of the roles and functions of GDSL-lipase like, notably GELP, in plants. This enzymatic family knows an emergent notoriety and the participation of its members in such diverse metabolic pathways supports its putative importance in plant physiology (Table C.2; [Ding et al. 2019](#)).

Overall, this thesis led to the validation of the biosynthetic pathway of TetraSpm, to the further characterization of the enzymes involved in its pathway as well as initiated the characterization of target genes involved in the production of diCQA. The phylogenetic history of both pathways was investigated. Moreover, this work provided new tools for metabolic engineering studies of these pathways. It also contributed to the recognition of acyltransferase families other than BAHD and SCPL in plants.

Most importantly, it led the team a step closer to the physiological characterization of these two specialized metabolites *in planta* which can not be assessed before deciphering their production pathways. Finally, these molecules may be of great interest in human applications or in plant resilience to biotic and/or abiotic stresses, which is highly relevant in the global climate change context.

Appendix **A**

Biotechnological tools for the study of the genes of interest

In order to investigate the roles of our target genes, traditional techniques of functional characterization were used: overexpression *in planta* or in other *in vivo* models or silencing. Both of these methods take advantage of the properties of agrobacteria phytopathogens.

Agrobacterium transformed with modified viral plasmids (Sainsbury et al. 2009) are widely used for transient recombinant protein expression or stable transformation of an individual and thus lineage. *Agrobacterium tumefaciens* was the first agrobacteria to be used for such purposes. This telluric phytopathogen is responsible for the crown-gall disease resulting from the trans-kingdom transfer of a Ti¹ plasmid containing a T-DNA segment integrated into the host cell's genome.

When a mechanical wounding occurs, plants emit phenolic compounds attracting the bacteria at the site of the injury. The Ti plasmid is constituted of a region containing the virulence genes (*vir* genes) and a region containing the T-DNA that will be transmitted as a single strand DNA and integrated into the host cell genome. The plasmid also comprise genes necessary for opines synthesis and phytohormones elicitation (auxins and cytokinins, involved in callogenesis and cell growth and replication, forming the tumors). This allows the bacteria to profit from the host cell molecular machinery for the replication of its T-DNA and results in the production of opines by the host cell, sources of carbon and nitrogen for the bacteria (Figure A.1). The T-DNA extremities are essential for its excision, transfer and integration, allowed also through the action of the VIR proteins. Those two 24 pb segments are called LB (Left Border) and RB (Right

¹ Ti: Tumor inducing

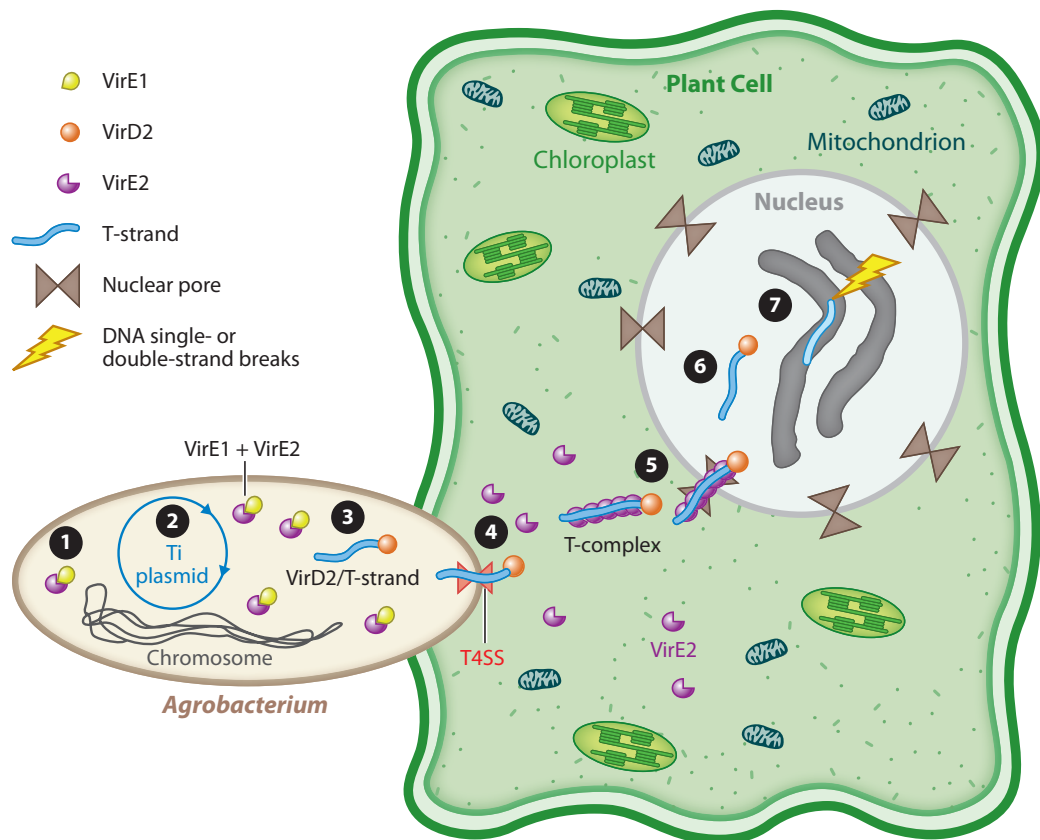


Figure A.1 – *Agrobacterium* T-DNA integration into a plant cell's genome. Figure and legend from Gelvin 2017.

Many components of the model remain speculative (*e.g.*, the presence of T-complexes in the plant and where in the plant cell VirE2 may be removed from T-strands). From left to right, the hypothesized steps include: (1) plant phenolic and sugar molecules are perceived by the VirA-VirG two-component regulatory system, resulting in the induction of *vir* genes and the production of Vir proteins. (2) VirD1 and VirD2 nick the T-DNA region of the Ti plasmid at border repeat sequences. (3) VirD2 covalently attaches to the 5' end of one T-strand, forming a VirD2/T-strand complex. (4) VirD2/T-strands and Vir effector proteins exit the bacterium using a T4SS. Although VirE2 associates with its chaperone VirE1 in the bacterium, only VirE2 is transported to the plant. (5) Within the plant, VirE2 protein may associate with T-strands, and the T-complex traffics through the plant cytoplasm and enters the nucleus through nuclear pores. Not shown are other *Agrobacterium* and plant proteins that likely associate with T-complexes. (6) VirE2 protein dissociates from T-strands. (7) T-strands integrate into plant chromosomes, where the encoded transgenes may be expressed.

Abbreviations: T-complex, VirD2/T-strand/ VirE2 complex; T-DNA, transfer DNA; Ti plasmid, tumor-inducing plasmid; T-strand, a single-strand form of transfer DNA; T4SS, type IV secretion system; *vir*, virulence gene; Vir, Virulence protein.

Border), they are at the origin of molecular cloning techniques implementation in plants such as the Gateway technique (Hartley et al. 2000). The complete molecular system of T-DNA integration and transmission is not completely understood to this day and a lot of questions remains unanswered (Gelvin 2021).

Two methods for molecular cloning into modified Ti-plasmid (non virulent) have been developed: (1) a *cis*- system in which the gene is incorporated by homologous recombination inside the already existing T-DNA, (2) a *trans*- system or binary system in which the gene of interest is cloned in between the LB and RB sequences like in the Gateway method. These properties are used for transient enzyme production when used on somatic cells which has the advantages of being fast, flexible and not influenced by the position in the genome in which the T-DNA is inserted (Verpoorte et al. 2000). However, they can also be used on germinal cells for stable transformation (like the floral dip method; Clough et al. 1998). The main method used in this work is the transient protein expression in *Nicotiana benthamiana* leaves with the needle less syringe agroinfiltration technique. Agroinfiltration is performed on the abaxial side of the leaves on account of the higher number of stomata and the absence of cuticle which would impair the infiltration process. Leaves are slightly wetted a few minutes beforehand to allow the stomata aperture to open and greater yield and facility of infiltration (Figure A.2).

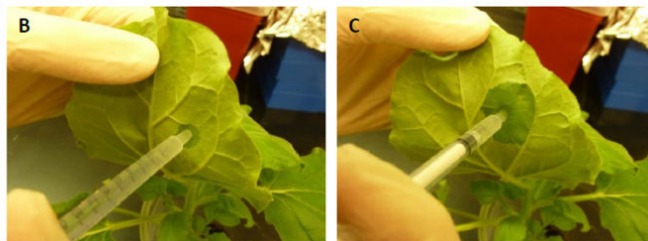


Figure A.2 – Pictures of the needleless syringe agroinfiltration technique. Figure and legend adapted from Q. Chen et al. 2013.

Syringe agroinfiltration of *N. benthamiana* leaves with *Agrobacterium tumefaciens* harboring the gene of interest was resuspended in infiltration buffer and loaded into a syringe without a needle. *Agrobacteria* were injected into the interstitial space of the leaf (B and C)

Agrobacterium rhizogenes, another phytopathogen, through a very similar mechanism of T-DNA transfer provokes the production of opines and induces a peculiar phenotype: the emergence of roots in an anarchical manner. This gave the name to the plasmid inducing this transfer and phenotype Ri for Root inducing (Figure A.3). This phenotype is induced by the proteins encoded by the

rol oncogenes *A*, *B*, *C* and *D*. *rolB* has been particularly investigated because the action of this oncogene alone can induce root formation, however many aspects of its actions and regulation are still under inquiry (Mauro et al. 2021).

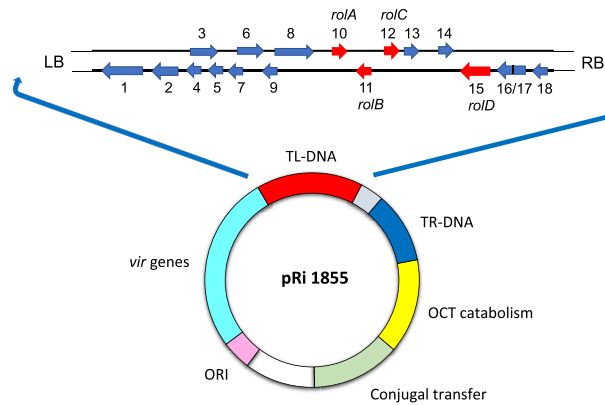


Figure A.3 – Schematic representation of agropine-type Ri plasmid of *A. rhizogenes*. Figure from Mauro et al. 2021.

OCT, octopine; ORI, origin of replication; LB, left border; RB, right border.

This phenotype is of great interest in biotechnical applications. It is the cause of the hairy root syndrome, which allows for the production of mutant lines in the form of fast-growing roots, which have the advantages of being easy to cultivate in vitro and producing a large amount of biomass (Bourgaud et al. 2001; Steingroewer et al. 2012). Moreover, burgeons can develop from hairy root of chicory thanks to the totipotence² of plant cells (Sun, Monneuse, et al. 1991). We can thus regenerate fully grown plants and stable mutant lines with this model.

² totipotence: capacity of plant cells to dedifferentiate from they attributed role and differentiate again in a new cell type and regenerate a complete individual identical to the parent plant.

Appendix **B**

Appendix of Chapter 3 on the next page.

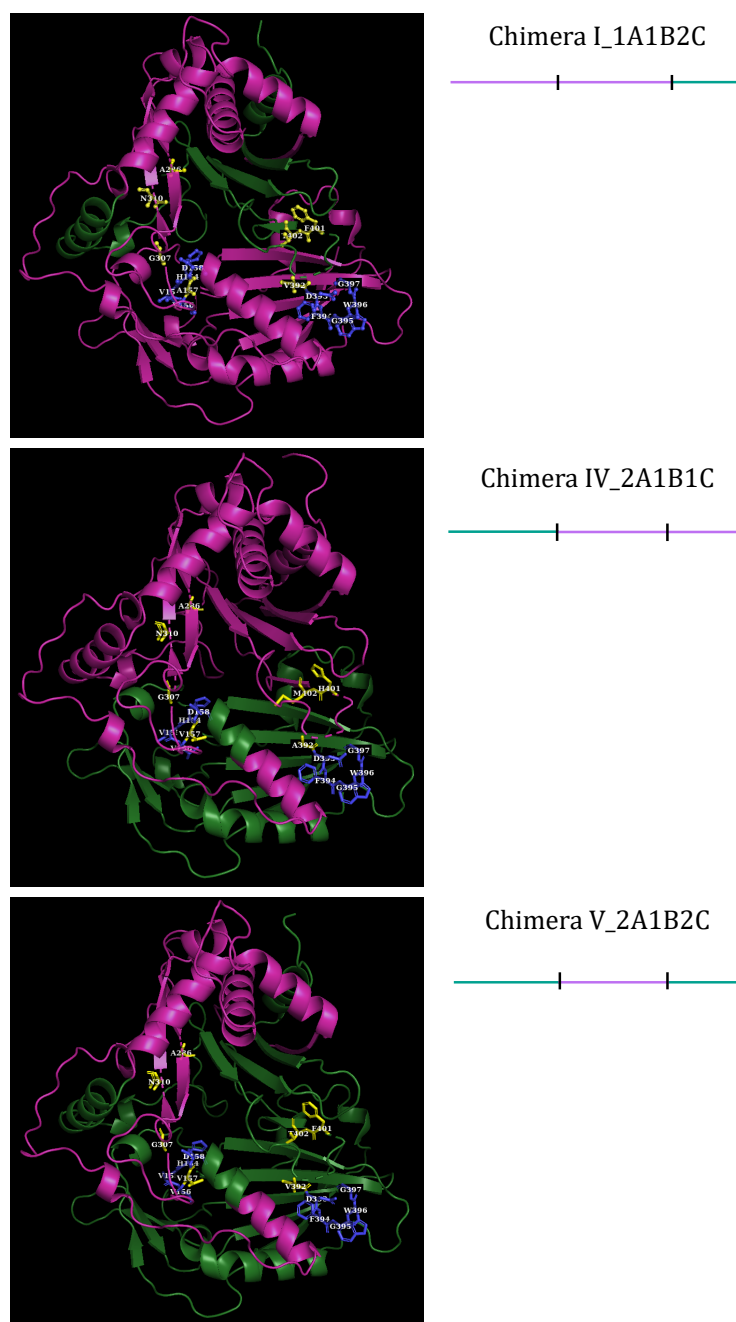


Figure B.1 – Three dimensional models of CiSHT1, CiSHT2 and their chimeras of type A highlighting the sites of interest. Figure realized using the PyMOL 2.x software (Schrödinger 2021)

Sequences coming from CiSHT1 and CiSHT2 are represented in cartoon form respectively in magenta and green. Conserved BAHD sites are represented in stick form in blue. Amino acid of interest are represented in stick form in yellow. Schematization of the sequences of the chimeras with sequences from CiSHT1 represented in violet and sequences from CiSHT2 represented in green are next to each 3D model.

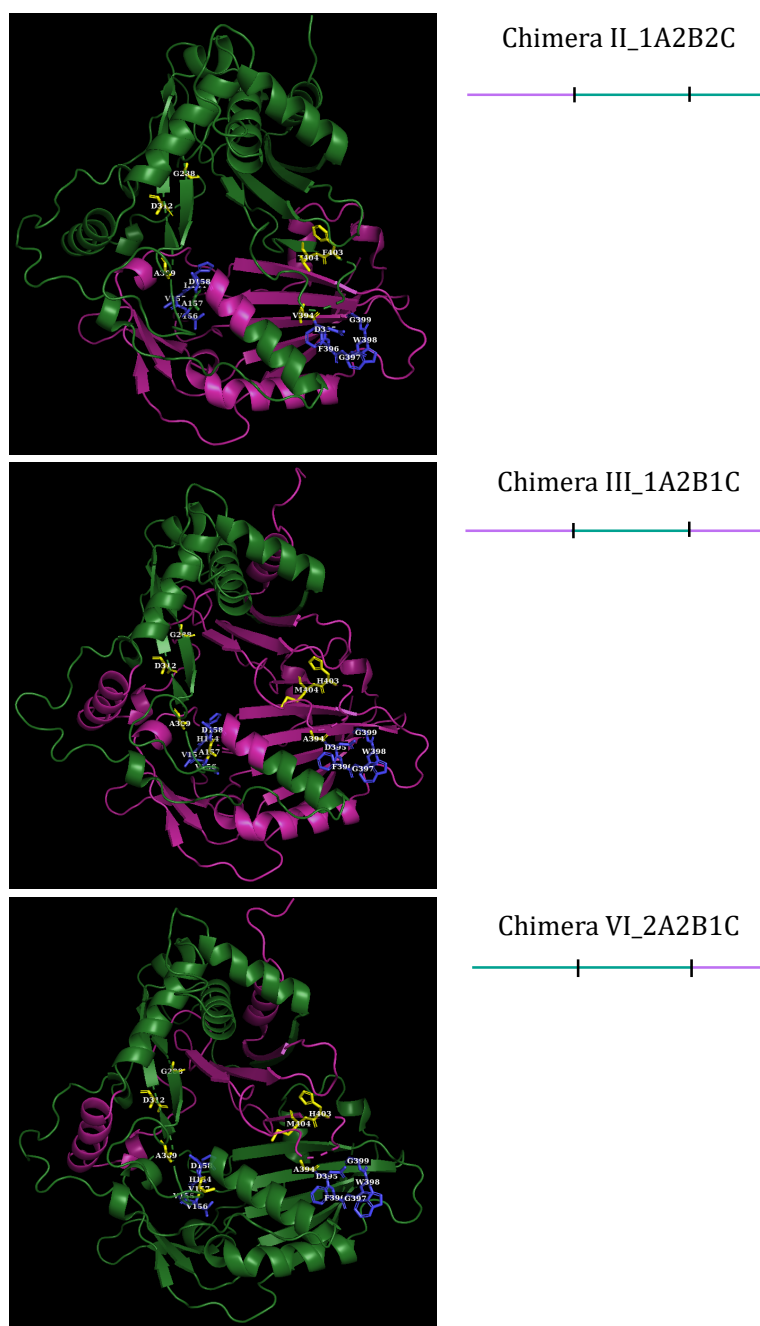


Figure B.2 – Three dimensional models of CiSHT1, CiSHT2 and their chimeras of type BC and C highlighting the sites of interest. Figure realized using the PyMOL 2.x software (Schrödinger 2021)

Sequences coming from CiSHT1 and CiSHT2 are represented in cartoon form respectively in magenta and green. Conserved BAHD sites are represented in stick form in blue. Amino acid of interest are represented in stick form in yellow. Schematization of the sequences of the chimeras with sequences from CiSHT1 represented in violet and sequences from CiSHT2 represented in green are next to each 3D model.

Appendix **C**

Appendix of Chapter 4 on the next page.

Table C.1 – Overview of diCQA accumulating species available in the literature

Family	Species	Extract origin	Identified molecule	Content	Reference	
Apiaceae	<i>Centella asiatica</i>	Water extracts	CQA 1,5-CQA 3,4-dtCQA 3,5-dtCQA	NA	Nora E Gray et al. 2014	
	<i>Centella asiatica</i>	Aerial parts	1,4-dtCQA 3,4-dtCQA 3,5-dtCQA	NA	Long et al. 2012	
	<i>Centella glabrata</i>	Aerial parts	3,4-dtCQA 3,5-dtCQA 4,5-dtCQA	NA	Long et al. 2012	
	<i>Daucus carota</i>	After 12h in cold	CQA 4,5-dtCQA	0,398 mg/g dw 0,0882 mg/g dw 0,086,2 mg/g dw	Becerra-Moreno et al. 2015	
	<i>Foeniculum Vulgare</i> Mill	Aerial parts	1,3-dtCQA 1,4-dtCQA 1,5-dtCQA 3,4-dtCQA 3,5-dtCQA 4,5-dtCQA	NA	Faudale et al. 2008	
	<i>Angelicae Pubescentis Radix</i>	Whole plant	4,5-dtCQA	NA	L. Yang et al. 2020	
	<i>Ilex hainanensis</i> Merr.	Leaves	4,5-dtCQA 3,5-dtCQA 3-CQA	1,71 mg/g 5,51 mg/g 190 µg/mL	B. Peng et al. 2013; Gan et al. 2018	
	<i>Ilex paraguariensis</i>	Water extract	5-CQA 3,5-dtCQA 4,5-dtCQA	210 µg/mL 130 µg/mL 250 µg/mL	Peres et al. 2013; Gan et al. 2018	
	Aquifoliaceae	<i>Helianthus annuus</i>	Sprouts	5-CQA 1,5-CQA dtCQA	7 mg/g dw 14 mg/g dw 3,5 ± 0,4 mg/g	Cheevrungapakul et al. 2019
		<i>Onopordum acanthium</i> L.	Fruits	4,5-dtCQA	NA	Daci et al. 2014
<i>Artemisia capillaris</i>			3-CQA	7,752 ± 2,872 mg/g dw	Tabassum et al. 2016	
<i>Helianthus tuberosus</i> L.		Leaves	3,4-dtCQA 3,5-dtCQA 1,5-dtCQA 4,5-dtCQA	0,618 ± 0,215 mg/g dw 4,900 ± 1,492 mg/g dw 1,733 ± 0,567 mg/g dw 5,633 ± 2,990 mg/g dw	F. Chen et al. 2014; Showkat et al. 2019; Chen et al. 2019	
<i>Cynara scolymus</i> L.		Raw artichoke	CQA dtCQA	2,76 mg/g 3,1 mg/g	Azzini et al. 2007	
<i>Cynara cardunculus</i>		Romanesco C3 - leaves Altisil 41 - leaves	5-CQA 1,5-dtCQA 5-CQA 1,5-dtCQA CQA	0,37 mg/g dw 0,25 mg/g dw 1,02 mg/g dw 0,4 mg/g dw	Pandino et al. 2015	
<i>Cynara cardunculus</i> L. var. <i>scolymus</i>			3,5-dtCQA	NA	Moglia, Acquadro, et al. 2016	
<i>Leptocarpha rivularis</i> DC.		Aerial parts	CQA 3,4-dtCQA 3,5-dtCQA	NA	Jiménez-González et al. 2018	
<i>Vernonia fastigiata</i>			CQA 3,4-dtCQA 3,5-dtCQA 4,5-dtCQA	NA	Masike et al. 2017	
<i>Chrysanthemum morifolium</i>			CQA (maj 5-CQA) 3,4-dtCQA 3,5-dtCQA 4,5-dtCQA	0,6 to 2 mg/g dw 0,2 to 0,7 mg/g dw 5 to 7 mg/g dw 3 to 5 mg/g dw	Gong et al. 2019	
<i>Helichrysum italicum</i> subsp. <i>picardii</i> Itaneres			3,4-dtCQA 3,5-dtCQA	2,03 ± 0,44 mg/g dw 14,48 ± 2,84 mg/g dw	Gonçalves, Moreira, Grosso, et al. 2017	
<i>Erigeron annuus</i>		Leaves	4,5-dtCQA 3,5-dtCQA	8,86 ± 1,60 mg/g dw 0,02 mg/g dw	Jang et al. 2010	

Table C.1 continued from previous page

Family	Species	Extract origin	Identified molecule	Content	Reference
	<i>Gymnaster koratensis</i>	Tea	3,5-dfCOA	NA	Iho et al. 2013
	<i>Coreopsis tinctoria</i>		3,5-dfCOA	NA	Shing-Chung Lam et al. 2016
	<i>Artemisia iwayomagi</i>	Aerial parts	3,4-dfCOA	8.18 ± 0.10 mg/g dw	Y. K. Lee et al. 2017
			3,5-dfCOA	68.87 ± 0.19 mg/g dw	
	<i>Aster glehnii</i>	Aerial parts	3,4-dfCOA	NA	Y.-J. Lee et al. 2017
			3,5-dfCOA	NA	
	<i>Tithonia diversifolia</i>	Aerial parts	4,5-dfCOA	NA	G.-J. Zhao et al. 2012
	<i>Erigeron breviscapus</i>	Whole plant	3,5-dfCOA	0.004 mg/g dw	X. Li et al. 2013
			3,4-dfCOA	NA	
	<i>Artemisia frigida</i>	Tea	3,5-dfCOA	0.82 ± 0.02 mg/100mL	
			4,5-dfCOA	16.35 ± 0.43 mg/100mL	Oleinnikov et al. 2019
			1,3-dfCOA	2.87 ± 0.08 mg/100mL	
			1,5-dfCOA	NA	
			1,3-dfCOA	NA	
			1,5-dfCOA	NA	
	<i>Dittrichia viscosa</i> L.	Leaves	3,4-dfCOA	NA	Rhimi et al. 2019
			3,5-dfCOA	NA	
			4,5-dfCOA	NA	
	<i>Rhaponticum carthamoides</i>	Roots	3,4-dfCOA	0.52 (2.62) mg/g dw	
			3,5-dfCOA	8.47 (0.51) mg/g dw	Skala et al. 2015
			1,5-dfCOA	1.44 (2.75) mg/g dw	
	<i>Carlina acutis</i> L.	Roots	4,5-dfCOA	1.04 (2.05) mg/g dw	Strzemiński et al. 2019
		Leaves	3,5-dfCOA	1.57 ± 0.11 mg/g dw	Bessada et al. 2015
	<i>Achillea millefolium</i> L.		1,3-dfCOA	0.91 ± 0.13 mg/g dw	
			1,4-dfCOA	NA	
			3,4-dfCOA	NA	
			3,5-dfCOA	NA	
	<i>Lactuca sativa</i> L.	Leaves	3,5-dfCOA	NA	X. Yang et al. 2017
			1,3-dfCOA	NA	
	<i>Erigeron multiradiatus</i>	Whole plant	3,4-dfCOA	NA	Zhi-feng Zhang et al. 2016
			3,5-dfCOA	NA	
			4,5-dfCOA	NA	
	<i>Achyrocline satureioides</i> (Lam) DC.	Aerial parts	3,5-dfCOA	NA	Martinez-Busi et al. 2019
			1,5-dfCOA	NA	
	<i>Anthemis tinctoria</i>	Aerial parts	3,4-dfCOA	NA	Orlando et al. 2019
			4,5-dfCOA	NA	
	<i>Aster tataricus</i> L.	Roots and rhizomes	3,5-dfCOA	NA	Rho et al. 2020
			3,4-dfCOA	NA	
	<i>Espeletia barclayana</i>	Leaves	1,5-dfCOA	NA	Padilla-Gonzalez et al. 2017
			3,4-dfCOA	NA	
	<i>Dithaldea nervosa</i>	Roots	1,3-dfCOA	NA	L. Liu et al. 2018
			1,5-dfCOA	NA	
			3,4-dfCOA	NA	
			3,5-dfCOA	NA	
	<i>Xerolekia speciosissima</i> L. Anderb.	Leaves	4,5-dfCOA	1.286 ± 0.342 % dw	Kleczeek et al. 2020
			3,4-dfCOA	0.718 ± 0.242 % dw	

Table C.1 continued from previous page

Family	Species	Extract origin	Identified molecule	Content	Reference
	<i>Helichrysum arenarium</i> (L.) Moench	Flowers	4,5-dtCOA 3,4-dtCOA 3,5-dtCOA 4,5-dtCOA 3,4-dtCOA 3,5-dtCOA 4,5-dtCOA 3,4-dtCOA 3,5-dtCOA 4,5-dtCOA 3,4-dtCOA 3,5-dtCOA 3,5-dtCOA 1,3-dtCOA 1,5-dtCOA 3,4-dtCOA 3,4-dtCOA 3,5-dtCOA 4,5-dtCOA 3,5-dtCOA 4,5-dtCOA 1,5-dtCOA	0.029 ± 0.007 % dw NA NA NA NA NA NA NA NA NA NA NA NA 13.15 ± 2.1 mg/g dw ND	J. Yang et al. 2021 Kongkiatpaiboon et al. 2018 Pantoja Pulido et al. 2017 S. Hong et al. 2015 Danino et al. 2009 J. Chen et al. 2014 Teoh et al. 2016 Karakošec 2015 J. Kim et al. 2020
	<i>Pluchea indica</i>	Leaves	3,4-dtCOA 3,5-dtCOA 4,5-dtCOA 3,4-dtCOA 3,5-dtCOA 4,5-dtCOA	NA NA NA NA NA NA	Simonovska et al. 2003
	<i>Tithonia diversifolia</i>	Leaves	3,4-dtCOA 3,5-dtCOA 4,5-dtCOA	NA NA NA	Tardugno et al. 2018; Miliala et al. 2009
	<i>Ligularia fischeri</i>	Leaves	3,4-dtCOA 3,5-dtCOA 4,5-dtCOA	NA NA NA	Petropoulos et al. 2018
	<i>Inula viscosa</i>	Leaves	3,4-dtCOA 3,5-dtCOA 4,5-dtCOA	NA NA NA	Pasko et al. 2018
	<i>Gynura divaricata</i>	Aerial parts	3,4-dtCOA 3,5-dtCOA 4,5-dtCOA	NA NA NA	Stefani et al. 2019
	<i>Gynura bicolor</i>	Leaves	3,4-dtCOA 3,5-dtCOA 4,5-dtCOA	NA NA NA	Yan et al. 2016
	<i>Stevia rebaudiana</i>	Leaves	3,4-dtCOA 3,5-dtCOA 4,5-dtCOA	7 mg/g dw 2 mg/g dw NA	J. Lee et al. 2019
	<i>Carthamus turkestanicus and lanatus</i>	Flowers	3,4-dtCOA 3,5-dtCOA 4,5-dtCOA	NA NA NA	M. Yu et al. 2019
	<i>Smilanthus sonchifolius</i>	Leaves	3,4-dtCOA 3,5-dtCOA 4,5-dtCOA	NA NA NA	T. Ma et al. 2018
	<i>Cichorium intybus</i> L.	Leaves	3,4-dtCOA 3,5-dtCOA 4,5-dtCOA	NA NA NA	S.-N. Wang et al. 2021
	<i>Cichorium spmosum</i> L.	Leaves	3,4-dtCOA 3,5-dtCOA 4,5-dtCOA	NA NA NA	Cho et al. 2016
Brassicaceae	<i>Brassica oleracea</i> L. var. <i>italica</i>	Sprouts Florets	COA dtCOA CQA dtCOA	0.37 mg/g dw NA NA 0.388 mg/g dw	
Bromeliaceae	<i>Hechtia glomerata</i> Zucc.	Water extract	3,4-dtCOA	2.06% extract	
Caprifoliaceae	<i>Lonicera japonica</i> Thunb.	Leaves	3-COA 3,5-COA 1,5-COA 3,4-dtCOA 3,5-dtCOA 4,5-dtCOA	14 mg/g dw 7 mg/g dw 2 mg/g dw NA NA NA	
	<i>Lonicera japonica</i>	See reference	NA	NA	
	<i>Lonicera macranthoides</i>	See reference	NA	NA	
	<i>Lonicerae japonicae</i>	Floral buds	3,5-dtCOA 4,5-dtCOA 3,5-dtCOA 3,4-dtCOA 4,5-dtCOA	NA NA NA NA NA	
	<i>Valeriana jatamansi</i> Jones	Roots and rhizomes	3,4-dtCOA 3,5-dtCOA	NA NA	
Chenopodiaceae	<i>Salicornia herbacea</i> L.	Aerial parts	3,5-dtCOA	NA	
	<i>Cuscuta chinensis</i> Lam.	Leaves	CQA 3,5-dtCOA 3,4-dtCOA 4,5-dtCOA 3,4-dtCOA 3,5-dtCOA 4,5-dtCOA 3,4-dtCOA 3,5-dtCOA 4,5-dtCOA	12.4 mg/g 4.6 mg/g 0.7 mg/g 1.8 mg/g 11.64 ± 0.45 mg/g of dw 18.69 ± 1.34 mg/g of dw 3.40 ± 0.77 mg/g of dw 0.38 ± 0.05 mg/g dw 0.50 ± 0.02 mg/g dw	Du et al. 2018 C. Zhang et al. 2019 Ghasemzadeh et al. 2016
Convolvulaceae	<i>Ipomoea batatas</i> L.	Leaves	3,5-dtCOA 4,5-dtCOA	NA NA	
	<i>Ipomoea batatas</i> L.	Roots	3,5-dtCOA 4,5-dtCOA	37.25 mg/g dw 0.28 mg/g dw	
Juglandaceae	<i>Juglans regia</i> L.	Leaves	CQA 3,4-dtCOA	NA NA	Luanna Gutierrez-Ottiz et al. 2018

Table C.1 continued from previous page

Family	Species	Extract origin	Identified molecule	Content	Reference
Pandanaaceae	<i>Pandanus tectorius</i>	Fruits	3,5-dfCOA	0,31 mg/g dw	X. Zhang et al. 2013
			4,5-dfCOA	0,25 mg/g dw	
			1,3-dfCOA	NA	
			1,5-dfCOA	NA	
			3,4-dfCOA	NA	
Polygonaceae	<i>Fallopia sachalinensis</i>	Roots	3,5-dfCOA	0,01 mg/g dw	Lachowicz et al. 2019
			3,5-dfCOA	0,01 mg/g dw	
			4,5-dfCOA	0,03 mg/g dw	
			1,5-dfCOA	NA	
			3,5-dfCOA	NA	
Solanaceae	<i>Solanum lycopersicum</i>	Fruits (crude extracts)	3,5-dfCOA	NA, main	Moglia, Lanteri, et al. 2014
			4,5-dfCOA	NA	
			CQA	0,19 mg/g dw	
			1,3-dfCOA	0,04 mg/g dw	
			3,4-dfCOA	0,03 mg/g dw	
	<i>Nicotiana benthamiana</i>	Leaves	3,5-dfCOA	0,03 mg/g dw	Moglia, Acquadro, et al. 2016
			4,5-dfCOA	0,03 mg/g dw	
			3-CQA	0,2 mg/g fw	
			4-CQA	0,6 mg/g fw	
			5-CQA	0,75 mg/g fw	
	<i>Nicotiana benthamiana</i>	Leaves	1,3-dfCOA	0,005 mg/g fw	Cheevrungratpakul et al. 2019
			1,5-dfCOA	0,003 mg/g fw	
			4,5-dfCOA	0,002 mg/g fw	
			3,4-dfCOA	NA	
			3,5-dfCOA	NA	
<i>Nicotiana tabacum</i>	Leaves	3,5-dfCOA	NA	Ncube et al. 2014	
		1,5-dfCOA	NA		
		3,5-dfCOA	NA		
		4,5-dfCOA	NA		
		3,5-dfCOA	NA		
Saxifragaceae	<i>Saxifraga umbellulata</i>	Aerial parts	3,5-dfCOA	NA	A. Yang et al. 2018
Tiliaceae	<i>Corechorus oltorius</i> L.	Leaves	1,5-dfCOA	NA	Wagdy et al. 2019
			3,5-dfCOA	NA	
Rosaceae	<i>Aronia melanocarpa</i>	Young leaves	Undetermined isomer	1,836,1 ± 0,026 mg/g of fw	J. E. Lee et al. 2014
		Matured leaves		0,1284 ± 0,024 mg/g of fw	
		Aged leaves		0,0386 ± 0,001 mg/g of fw	
		Leaves		NA	
Rosaceae	<i>Cydonia oblonga</i> Miller	Aerial parts	3,5-dfCOA	NA	A. P. Oliveira et al. 2007
			3,5-dfCOA	1,10 ± 0,23 mg/g dw	
			CQA	50 mg/g dw	
Rubiaceae	<i>Coffea arabica et robusta</i>	Green beans	dfCOA	10 mg/g dw	Asamenew et al. 2019
			COA	10 mg/g dw	
			dfCOA	0,7 mg/g dw	

Table C.2 – Characterized GDSL available in the literature

Species	Gene Name	Accession ID	Expression characteristics	Localization	Biochemical activities	Biological function	Functional classification	References
<i>Agave americana</i>	AgaSGNH	AA575127.1 / Q517N0	Leaf epidermis	Epidermal cells	Esterase and acyltransferase activities	Plant cutin biosynthesis	Plant Development and Morphogenesis	Reina et al. 2007
<i>Alopecurus myosuroides</i>	AmGDSHI	CAG27610	Seedling leaf	Extracellular space	Hydrolase and esterase activities	Herbicide bioactivation in crops and weeds	Plant Development and Morphogenesis	Cummins et al. 2004
<i>Arabidopsis thaliana</i>	AtFXG1	Q9FXE5.1	Young leaves, apical region of the inflorescence stem	Apoplast	Hydrolase and esterase activities	Hydrolyzes α -1,2-linked fucose	Specialized Metabolism	de la Torre et al. 2002
<i>Arabidopsis thaliana</i>	AtARAB-1	Q38894	Rosette, stem, seed	Extracellular space / secreted	Hydrolase and esterase activities	Lipid catabolism	Specialized Metabolism	Ling 2008
<i>Arabidopsis thaliana</i>	AtESM1	Q9LJG3	Rosette, flower, seed	Extracellular space / secreted	Hydrolase and esterase activities	Represses or inhibits nitriles production from methionine-derived and indol-3-ylmethyl glucosinolates. Favors isothiocyanate production.	Plant Defense	Burrow et al. 2008; Zhiyong Zhang et al. 2006; Mergner et al. 2020
<i>Arabidopsis thaliana</i>	AtMVP1	Q7XA74	Seedling, rosette leaves, roots, inflorescence and imbibed seed, but not in pollen, induced by MeJA and pathogen infection	Vacuole and ER bodies	Hydrolase activity	Organization of the endomembrane system, endoplasmic reticulum morphology and organelle distribution	Plant Defense	Agee et al. 2010
<i>Arabidopsis thaliana</i>	AtGLIP4	Q9LJPI	Cotyledons; flower; rosette; root	NA (extracellular space)	Lipase activity	Lipid catabolism	Plant Defense	Salanoubat et al. 2000; Mergner et al. 2020
<i>Arabidopsis thaliana</i>	AtGLIP1	At5g40990 / Q9ELN0	Seedling, leaf, root, stem and flower	Extracellular or cell wall	Lipase activity	Enhance resistance to pathogenic fungi and bacteria	Plant Defense	Oh et al. 2005; Kwon et al. 2009; Sunghwan Kim et al. 2012; H. J. Kim et al. 2014
<i>Arabidopsis thaliana</i>	AtGLIP2	At1g53940 / Q9SYF0	Seedling, stem and root; Induced by SA, JA and ET	NA (extracellular space)	Lipase activity	Resistance to bacterium <i>Erwinia carotovora</i>	Plant Defense	D. S. Lee et al. 2009
<i>Arabidopsis thaliana</i>	AtEXL4	Q0WUV7	Pollen coat	Extracellular space / secreted	Hydrolase activity	Promoting pollen hydration on the stigma	Plant development and Morphogenesis	Updegraff et al. 2009; Ling 2008
<i>Arabidopsis thaliana</i>	AtGLIP6	Q9C996	Germinating seeds	NA (extracellular space)	Lipase activity	Lipid catabolism	Plant development and Morphogenesis	Theologis et al. 2000; Pignatta et al. 2014
<i>Arabidopsis thaliana</i>	AtGLIP7	Q8LFI9	Flower	NA (extracellular space)	Lipase activity	Lipid catabolism	Plant development and Morphogenesis	Oh et al. 2005; Mergner et al. 2020
<i>Arabidopsis thaliana</i>	AtLTL1	Q9M8Y5	Flowers, reproductive stems and rosette leaves. Roots to a lower extent. Induced by SA.	Extracellular space / secreted	Hydrolase and esterase activities	Salt tolerance	Plant Defense	Naranjo et al. 2006
<i>Arabidopsis thaliana</i>	AtCDF1	Q9SZW7	Flower, root	Extracellular space / secreted	Hydrolase activity	Cutinase	Plant Development and Morphogenesis	Ling 2008; Mergner et al. 2020

Table C.2 continued from previous page

Species	Gene Name	Accession ID	Expression characteristics	Localization	Biochemical activities	Biological function	Functional classification	References
<i>Brassica napus</i>	BnGLIP2	AY870270	Germinating seeds	Vacuoles of germinating seeds	Esterase activity	Rapeseed germination, morphogenesis, flowering	Plant Development and Morphogenesis	Ling et al. 2006
<i>Brassica napus</i>	BnSCE3	AY870270	Developing seeds	Vacuoles of germinating seeds	Esterase activity	Seed development	Plant Development and Morphogenesis	Clauß, Baumert, et al. 2008; Clauß, von Roepenack-Lahaye, et al. 2011
<i>Brassica napus</i>	BnGLIP	AG183937.1 / M9VY51	Seed in development	NA	Lipase activity	NA	Plant Defense	X. Tan et al. 2014; L. Wan et al. 2002
<i>Brassica pekinensis</i>	BnSIL1	Q8L8G1 / AY101366	Leaves and stems, induced by SA	NA (extracellular space)	Lipase activity	SA and pathogen response	Plant Defense	K.-A. Lee et al. 2003
<i>Capsicum annuum</i>	CaGL1	Q0R4F7	Induced by MeJA	Cell wall or extracellular space	Lipase activity	JA signaling pathway or wound response	Plant Defense	K.-J. Kim et al. 2008
<i>Capsicum annuum</i>	CaGLIP1	Q08ET5	Induced by SA, ET, Me-JA, SNP, methyl viologen, salt, dehydration and wounding	Vascular tissues	Hydrolase activity	Disease susceptibility and abiotic stress tolerance	Plant Defense	J.-K. Hong et al. 2008
<i>Carica papaya</i>	CpEST	P86276	Latex	NA (extracellular space)	Hydrolase and esterase activities	Lipolytic activity towards triacylglycerols, vinyl esters and phospholipid.	Specialized Metabolism	Abdelkafi et al. 2009
<i>Cichorium intybus</i>	CiGDSL1	NA	NA	NA	NA	NA	Specialized Metabolism	Kandzia et al. 1997
<i>Cichorium intybus</i>	CiGDSL2	NA	NA	NA	NA	NA	Specialized Metabolism	Arif et al. 2004
<i>Cichorium intybus</i>	CiGDSL3	NA	NA	NA	Hydrolase and esterase activities	cardenolide- transforming enzyme, lanatoside 15'-O-acetyltransferase	Specialized Metabolism	Miguel et al. 2020
<i>Digitalis lanata</i>	DILAE	O82681	Cell wall	NA	NA	NA	Specialized Metabolism	Kram et al. 2008
<i>Hevea brasiliensis</i>	Hev b 13	Q7Y1X1.1	NA	NA	Lipase and esterase activities	Allergenic protein binding to IgE	Plant Defense	K. Yamamoto, Oguri, and Momonoki 2008
<i>Ipomoea batatas</i>	IbICS	QOH99319.1	NA	NA (extracellular space)	Acyltransferase activity	Isochlorogenate biosynthesis	Specialized Metabolism	Pringle et al. 2004
<i>Jacaranda mimosifolia</i>	JmNP1	B0FTZ8	Floral nectar	Extracellular space / secreted	Lipase and esterase activities	Modulate lipid content of flower nectar	Plant Development and Morphogenesis	J.-J. Park et al. 2010
<i>Macropodium atropurpureum</i>	MaAcHe	B0M183	NA	NA (extracellular space)	Hydrolase and esterase activities	Hydrolyse acetylthiocholine and propionylthiocholine	Specialized Metabolism	Huihui Zhang et al. 2020; Matsumoto et al. 2005
<i>Medicago sativa</i>	ENOD8	AAB41547 / L18899	Root nodule	Cell wall	Esterase activity	Nodule formation and nitrogen fixation, active on acetyl and butyryl esters	Plant development and Morphogenesis	Y. Yu et al. 2020
<i>Oryza sativa</i>	OsWDL1 LOC	Q7XXR3	Epidermal cell layers	Endoplasmic reticulum	Transferase and hydrolase activities	Wax and keratin synthesis	Plant Development and Morphogenesis	M. Gao et al. 2017
<i>Oryza sativa</i>	OsGELP34	Os02g0290900	Anthers during pollen exine formation	Endoplasmic reticulum	Acyltransferase activity	Exine formation	Plant Development and Morphogenesis	
<i>Oryza sativa</i>	OsDR	LOC_Os02g15230	NA	NA	NA	Silica deposition in leaves	Plant Development and Morphogenesis	
<i>Oryza sativa</i>	OsGLIP1	KU350624	Leaf and leaf sheath	Endomembranes of ER and of cytoplasmic vesicles (apoplast)	Lipase activity	Modulate plant immunity through lipid homeostasis	Plant Defense	

Table C.2 continued from previous page

Species	Gene Name	Accession ID	Expression characteristics	Localization	Biochemical activities	Biological function	Functional classification	References
<i>Oryza sativa</i>	OsGLIP2	NM_001063391	Elongating internodes	Endomembranes of ER and of cytoplasmic vesicles (apoplast)	Lipase activity	Modulate plant immunity through lipid homeostasis	Plant Defense	M. Gao et al. 2017
<i>Rauvolfia serpentina</i>	RsAAE	Q3MKY2	Leaves	NA	Esterase activity	Ajmaline biosynthesis	Specialized Metabolism	Ruppert, Xueyan, Ma, et al. 2005
<i>Salicornia europaea</i>	SeAChE	C7G362	Cortex of roots and stems	NA (extracellular space)	Hydrolase and esterase activities	Cell to cell transport, acetylcholinesterase	Specialized Metabolism	K. Yamamoto, Oguri, Chiba, et al. 2009
<i>Sedum alfredii</i>	SaGLIP8	MK440746.1 / Sa13F.102	High expression level in all tissues	Extracellular space	NA	Increase Cadmium tolerance and accumulation	Plant Defense	T. Li et al. 2019
<i>Solanum lycopersicum</i>	SlCCT	E7AIM3	Leaves	Apoplast	Hydrolase and transferase activities	Caffeoylglucuronate biosynthesis	Specialized Metabolism	Teuschbein et al. 2010
<i>Solanum lycopersicum</i>	SlCUS1 / SlGDSL1	SGN-U585129	Epidermis of growing fruit	NA (extracellular space)	Hydrolase and acyltransferase activities	Extracellular deposition of the cutin polyester in the tomato fruit cuticle	Plant Development and Morphogenesis	Girard et al. 2012; Yeats et al. 2014
<i>Tanacetum cinerariifolium</i>	TcGLIP	AFI04755.1 / JN418994	Leaves, seedlings, buds and flowers	Extracellular space	Transferase and esterase activities	Herbivore resistance, pyrethrin production	Plant Defense	Kikuta, Ueda, et al. 2012; Kikuta, Yamada, et al. 2013
<i>Zea mays</i>	ZmAChE	O5FC14 / AB093208	Root, seedlings	Cell wall	Hydrolase and esterase activities	Cholinester-hydrolyzing	Specialized Metabolism	Sagane et al. 2005

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Abstract

Metabolism and Roles of Hydroxycinnamic Acids in *Cichorium intybus* L.

Industrial chicory, *Cichorium intybus* L. var. *sativum*, accumulates a wide array of potent specialized metabolites. These metabolites exhibit very diverse structures, activities and functions that are remarkable from a physiological standpoint, but also for their applications such as in human and animal health, agronomy and other diverse industries. This work focused on two specialized metabolites of *C. intybus*: an unusual phenolamide tetracoumaroyl-spermine (TetraSpm) and 3,5-isochlorogenic acid (diCQA). This research contributes to the biochemical and physiological characterization as well as the investigation of the evolutive history of the enzymes involved in the biosynthesis of these two hydroxycinnamic acid conjugates. We used computational analysis and molecular engineering approaches. We demonstrated the sequential action of the two CiSHTs (Spermine Hydroxycinnamoyl Transferases) *in vivo* in the biosynthetic pathway of TetraSpm. We investigated the influence of free polyamine availability and structural features on the catalytic activities of SHT proteins. Furthermore, we engineered a chimeric protein able to catalyze the production of TetraSpm in a single step. The study of diCQA metabolism in *C. intybus* involved the identification and preliminary characterization of target genes belonging to the GDSL-lipase like enzymatic family. Moreover, it emphasized the diversity of plant acyltransferases involved in specialized metabolism. The physiological roles and functions of TetraSpm and diCQA are still to be determined. However, the conservation of TetraSpm among the *Asteraceae* family suggests that it plays a significant role in their evolution, and the therapeutic properties of diCQA are of great interest in human health. Thus, deciphering these biosynthetic pathways is crucial for their reconstitution in more efficient systems *via* molecular engineering and consequently the valorization of these compounds.

Keywords: *Cichorium intybus* L., Specialized metabolism, Molecular engineering, Acyltransferases, Hydroxycinnamic acids, Heterologous expression

Résumé

Métabolisme et Rôles d'Acides Hydroxycinnamiques chez *Cichorium intybus* L.

La chicorée industrielle, *Cichorium intybus* L. var. *sativum*, accumule une grande diversité de métabolites spécialisés. Ces métabolites montrent une grande variété de structures, activités et fonctions qui sont considérables d'un point de vue physiologique, mais aussi pour des applications humaines telle qu'en santé humaine et animale, en agronomie et autres diverses industries. Ce travail se concentre sur deux métabolites spécialisés de *C. intybus*: une phénolamide inhabituelle, la tétracoumaroyl-spermine (TetraSpm), et l'acide isochlorogénique (3,5-diCQA). Il constitue une contribution aux caractérisations biochimique, physiologique ainsi qu'à l'étude de l'histoire évolutive d'enzymes impliquées dans la biosynthèse de deux acides hydroxycinnamiques conjugués. Nous avons utilisé à ces fins des approches bioinformatiques et d'ingénierie moléculaires. Concernant la TetraSpm, nous avons démontré l'action successive des deux CiSHTs (Spermine Hydroxycinnamoyl Transférases) *in vivo* dans sa voie de biosynthèse. Nous avons exploré l'influence du ratio de polyamines libres disponibles et des caractéristiques structurelles des protéines SHT sur leurs activités catalytiques. En outre, nous avons produit une protéine chimérique capable de catalyser la production de TetraSpm en une seule étape. L'étude du métabolisme du diCQA chez *C. intybus* a impliqué l'identification et la caractérisation préliminaire de gènes cibles appartenant à la famille enzymatique des GDSL-lipases like. De plus, cela a mis en évidence la diversité des acyltransférases impliquées dans le métabolisme spécialisé des plantes. Les rôles physiologiques et fonctions de la TetraSpm et du diCQA restent à déterminer. Cependant, la conservation de la TetraSpm au sein de la famille des Astéracées suggère son importance dans leur histoire évolutive, et les propriétés thérapeutiques du diCQA sont d'un grand intérêt en santé humaine. Ainsi, la compréhension de leurs voies de production permettra de leurs reconstitutions dans des systèmes hétérologues plus efficaces grâce à l'ingénierie moléculaire et par conséquent à la valorisation de ces composés.

Mots-clés: *Cichorium intybus* L., Métabolisme spécialisé, Ingénierie moléculaire, Acyltransférases, Acides hydroxycinnamiques, Expression hétérologue