Biosynthesis and function of hydroxycinnamic acid amides in flowers of *Arabidopsis thaliana*

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Science is wonderfully equipped to answer the question "How?"

but it gets terribly confused when you ask the question "Why?"

Erwin Chargaff (1905-2002)

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Summary

Hydroxycinnamic acid amides (HCAAs) are a diverse group of plant secondary metabolites and found in many plant species. Although their distribution in different plant organs was described and their variable chemical structures have been well analyzed, HCAA biosynthesis, its regulation and the physiological role in plants remain largely unknown. The present study focused primarily on the elucidation of a universal HCAA biosynthetic pathway unique to anthers, more specifically in tapetal cells of *Arabidopsis thaliana* and their biological function on the pollen grain surface.

Based on the identification of a final HCAA-decorating methylation step performed by a CCoAOMT-like *O*-methyltransferase, AtTSM1, which is specifically localized in the tapetum of young flower buds, we identified several genes and corresponding enzymes participating to HCAA formation in *A. thaliana* anthers. Among these, AtTSM1 co-expressed genes have been selected and characterized regarding their localization and contribution to HCAA accumulation. Beside AtTSM1 a total of six additional genes encoding HCAA-biosynthetic enzymes have been identified and correlated to altered phenolic profiles of mature pollen grains. These include an early methylation step in the phenylpropanoid pathway, converting caffeoyl-CoA into feruloyl-CoA catalyzed by CCoAOMT1, otherwise associated with lignin monomer formation in vascular tissue. Hydroxycinnamoyl CoA esters (HCAs) are then linked to polyamines (PAs) in particular spermidine by two BAHD-like acyltransferases, SHT (spermidine hydroxycinnamoyl transferase) and SDT (spermidine disinapoyl transferase) in part subsequently modified by a cytochrome P450 (CYP98A8) dependent hydroxylation and by the previously mentioned AtTSM1. SAM-decarboxylase 1 and spermidine synthase 1 appear also to be involved in HCAA-formation in anthers. Their absence in knock out lines leads to reduced HCAA-accumulation. All identified enzymes contribute to a flower bud specific pathway localized in the tapetum of developing anthers, resulting in a diverse profile of HCAAs in the pollen tryphine of *A. thaliana*.

Knock out of both key acyl transferase steps performed by SHT and SDT, linking HCA and PA biosynthesis, does not lead to an increased accumulation of parent compounds, since neither HCA accumulation nor enhanced PA levels are observed in these mutants. Thus a tight regulation of HCAA precursors is required, which apparently does not take place predominantly at the transcription levels.

The biological relevance of pollen HCAAs is still obscure. Comparing *A. thaliana* wild type and HCAA deficient *sht* and *sht sdt* pollen regarding functionality, physiological changes and abiotic/biotic stress response did not show any effect on pollen fertility, reproduction

efficiency, or physiological properties. Temperature- and UV-stress was exposed to pollen grains, but did not provide conclusive evidence for a protective role of these compounds. If there is an incorporation of HCAAs into pollen walls, which might results in a significant contribution to pollen wall stability, is currently investigated. At present, the biological function of HCAAs as key components of the angiosperm pollen surface remains puzzling.

Zusammenfassung

Hydroxyzimtsäureamide (HCAAs) sind universelle pflanzliche Sekundärmetabolite, welche in vielen Pflanzenspezies nachgewiesen wurden. Obwohl sie bereits zahlreich beschrieben wurden und ihre chemische Struktur gut untersucht ist, bleibt die HCAA Biosynthese, deren Regulation und physiologische Funktion in Pflanzen unklar. Ziel dieser Arbeit war es, die HCAA Biosynthese im Tapetum der Antheren von *Arabidopsis thaliana* aufzuklären und deren biologische Funktion auf der Pollenoberfläche zu untersuchen.

Basierend auf der Identifizierung einer enzymatischen HCAA-Methylierung, durchgeführt von einer CCoAOMT-ähnlichen *O*-Methyltransferse (AtTSM1), welche spezifisch im Tapetum der Antheren lokalisiert ist, wurden weitere HCAA synthetisierende Enzyme und deren kodierende Gene identifiziert. AtTSM1 co-exprimierte Gene wurden selektiert und bezüglich ihrer Lokalisation und ihrer Beteiligung an der HCAA Biosynthese analysiert. Insgesamt sechs weitere Enzyme konnten identifiziert werden, deren *knockout* Mutation mit veränderten HCAA-Profilen der Pollen korrelierte und somit Rückschlüsse auf die HCAA Biosynthese ermöglichte. Die Biosynthese von HCAAs beinhaltet einen frühen Methylierungsschritt im Phenylpropanstoffwechsel, welcher Kaffeoyl-CoA in Feruloyl-CoA überführt und durch die CCoAOMT1 katalysiert wird, einem Enzym ebenfalls bekannt aus der Ligninbiosynthese. Gebildete Hydroxyzimtsäure-CoA-Ester (HCAs) werden anschließend mit Polyaminen verknüpft, in diesem Fall Spermidin. Dieser Transfer wird durch zwei BAHD-ähnliche Acyltransferasen SHT (Spermidin Hydroxyzimtsäure Transferase) und SDT (Spermidin Biscoumaroyl Transferase) katalysiert. Resultierende HCAAs werden anschließend teilweise, durch eine Cytochrom P450 (CYP98A8) abhängige Hydroxylierung und durch die bereits erwähnte AtTSM1 modifiziert. SAM-Decarboxylase 1 und Spermidin Synthase 1 sind ebenfalls an der HCAA Biosynthese in Antheren beteiligt. Ihr Fehlen in entsprechenden *knockout* Linien führt zu einer Reduktion der HCAAs. Sämtliche identifizierten Enzyme tragen zu einem blüten-spezifischem Stoffwechselweg bei, welcher im Tapetum der Antheren lokalisiert ist und in einer Vielfalt an HCAAs des Pollenkits resultiert.

Die Verknüpfung von HCA und PA Biosynthese und somit die Bildung von HCAAs wird durch zwei Acyltransferasen, SHT und SDT, im Tapetum von *A. thaliana* katalysiert. Der *knockout*, beider kodierender Gene, führt nicht zu einer Anreicherung der HCAA-Vorläufermoleküle. Weder HCA-Akkumulation, noch erhöhte PA Mengen wurden in diesen Linien beobachtet. Was auf eine strenge Regulation beider Vorstufen schließen lässt, welche jedoch nicht vorrangig auf transkriptioneller Ebene stattfindet.

Die Untersuchung der biologischen Bedeutung dieser pollenspezifischen HCAAs, war ein weiterer Gegenstand der vorliegenden Arbeit. Ein Vergleich von *A. thaliana* Wildtyp und HCAA-defizienter *sht* und *sht sdt* Pollen bezüglich Funktionalität, physiologischer Veränderungen und abiotischer bzw. biotischer Stressantwort zeigte keine Unterschiede auf Pollenfertilität, Fortpflanzungsrate oder adhäsiver Eigenschaften. Behandlung mit Temperatur- und UV-Stress erbrachte keine Hinweise auf mögliche Schutzfunktionen dieser Verbindungen auf der Pollenoberfläche. Ob HCAAs in die Pollenwand integriert werden und somit deren Stabilität beeinflussen, ist Gegenstand laufender Untersuchungen. Somit bleibt gegenwärtig die biologische Funktion der HCAAs als Bestandteil der Pollenoberfläche bedecktsamiger Pflanzen weiterhin rätselhaft.

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List of abbreviations

°C	degree Celsius
ADC	arginine decarboxylase
AtTSM1	*Arabidopsis thaliana* tapetum specific *O*-methyltransferase 1
BAHD	family of acyltransferases named according the first letter of each of the first four characterized enzymes (BEAT, AHCT, HCBT, DAT)
CCoAOMT1	caffeoyl coenzyme A *O*-methyltransferase 1
COMT1	caffeic acid *O*-methyltransferase 1
CYP98A8/9	cytochrome P450 monooxygenases belonging to clade CYP98A
HCA	hydroxycinnamic acid
HCAA	hydroxycinnamic acid amide
HPLC	high-performance liquid chromatography
LC MS/MS	liquid chromatography tandem mass spectrometry
ODC	ornithine decarboxylase
PA	polyamine
SAM	*S*-adenosyl-L-methionine
SAMDC	SAM decarboxylase
SCT	spermidine dicoumaroyl transferase
SDT	spermidine disinapoyl transferase
SHT	spermidine hydroxycinnamoyl transferase
UV	ultraviolet
WT	wild type

1 Introduction

Plant constituents and metabolism have been intensively studied for decades. In addition to essential primary metabolites (carbohydrates, lipids and amino acids), higher plants are also able to synthesize a large number of low molecular weight organic compounds called secondary metabolites. It is presumed that the plant produces these substances in response to biotic and abiotic stimuli and that they have UV protective, attractant (pollinating insects, seed dispersing animals) and defensive (against viruses, bacteria, fungi, competing plants and predators) properties. The three main groups of plant secondary metabolites are: terpenoids, alkaloids and phenolics. Terpenoids, derived from the C5 precursor isopentenyl diphosphate (IPP), form the largest class of natural products and are involved in defense against predators, pathogens and competitors and serve as information messengers (Gershenzon and Dudareva 2007). Alkaloids are derived from amino acids and are involved in plant defense and provide aroma and flavor; many have pharmacological properties (De Luca and Laflamme 2001). Phenolics are derived from the shikimate pathway (Wink 2003). Phenolic compounds like hydroxycinnamic acids (HCAs) can also be linked to primary metabolites like polyamines and are referred to as phenolamides or hydroxycinnamic acid amides (HCAAs). These HCAAs have been described for several decades, and they occur throughout the plant kingdom (Bassard *et al.,* 2010). With a focus on HCAAs, this introduction aims to give an overview about the biosynthesis of the corresponding precursors, particularly polyamines (PAs) and HCAs, and the linkage of both pathways leading to the formation of the resulting HCAAs. Due to their predominant presence in plant reproductive organs, anther and pollen development will be introduced.

1.1 Polyamines: important for plant development

PAs are small organic molecules containing more than one amino group and they are found in a wide range of organisms from bacteria to plants and animals. The tetramine spermine was first discovered in aging human semen by van Leeuwenhoek (1678), while the diamines putrescine and cadaverine were identified in putrefying cadavers more than two hundred years later (Brieger 1885). The chemical composition and structure of spermidine and spermine was resolved in the 1920's by Dundley and coworkers (1926; 1927). The major plant PAs, putrescine, spermidine and spermine (for structures see Figure 1-1), are found in every plant cell in titers ranging from approximately 10 μM up to millimolar concentrations (Fuell *et al.,* 2010). Similar, but less abundant plant PAs include cadaverine, present in several leguminous plants (Smith and Wilshire 1975), and thermospermine, first identified from the thermophilic bacterium *Thermus thermophiles* but also found in higher plants like pea, alfalfa (*Medicago sativa*) and *A. thaliana* (Kakehi *et al.,* 2008; Oshima 1979). The unusual tetraamine canavalmine was identified in the seeds of *Canavalia gladiata* (Fujihara *et al.,* 1982).

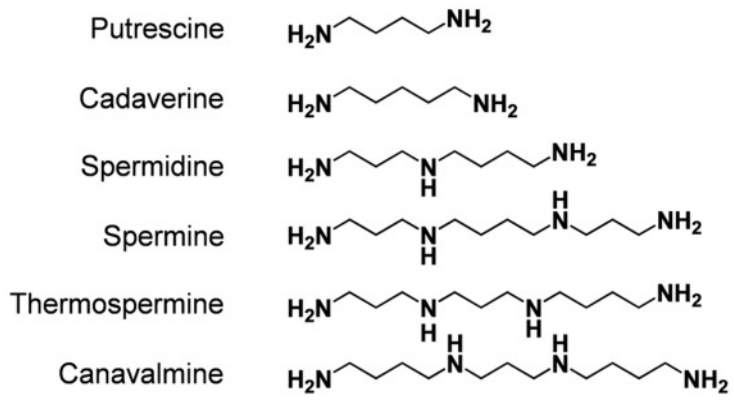

Figure 1-1: Structures of common plant polyamines.

1.1.1 Biosynthesis of polyamines

The biosynthetic pathway for putrescine, spermidine and spemine is well investigated (Bagni and Tassoni 2001; Fuell *et al.*, 2010). This pathway was first studied in prokaryotes, but, in general the same biosynthetic steps have been found in eukaryotes. The simplest PA, putrescine, is derived either directly from ornithine by ornithine decarboxylase (ODC) or from arginine through several steps catalyzed by arginine decarboxylase (ADC), agmatine iminohydrolase (AIH) and *N*-carbamoylputrescine amidohydrolase (CPA) (Figure 1-2). Higher molecular weight polyamines like spermidine and spermine are formed by sequential addition of aminopropyl groups to putrescine and spermidine by the activity of spermidine synthase and spermine synthase. Spermidine is also converted to thermospermine, a structural isomer of spermine, by thermospermine synthase. Both latter reactions require decarboxylated *S*-adenosyl-*L*-methionine (dcSAM) as an aminopropyl donor that is produced by SAM decarboxylase (SAMDC). Interestingly, in addition to its role as a methyl group donor, SAM is also a common precursor for both polyamines and ethylene which is produced via 1-amino-cyclopropane-1-carboxylic acid (ACC). Therefore, SAMDC could regulate both biosynthetic pathways (Evans and Malmberg 1989). The diamine cadaverine is derived from the amino acid lysine by decarboxylation catalyzed by the enzyme lysine decarboxylase (Bunsupa *et al.*, 2012).

Genes encoding some of these PA biosynthetic enzymes have been cloned and characterized in different plant species. In the case of the model organism *A. thaliana*, all genes encoding the above-mentioned enzymatic activities except for ODC have been identified and characterized in detail. *A. thaliana* is the only known plant and one of two eukaryotic organisms that have been demonstrated to lack ODC activity (Hanfrey *et al.*, 2001). This implies that *A. thaliana* is totally dependent on the ADC pathway for putrescine biosynthesis. The *A. thaliana* genome has two genes encoding ADC (ADC1: *At2g16500*, ADC2: *At4g34710*) (Galloway *et al.*, 1998; Watson and Malmberg 1996) and a single gene for AIH (*At5g08170*) and CPA (*At2g27450*) (Illingworth *et al.*, 2003; Janowitz *et al.*, 2003; Piotrowski *et al.*, 2003). There are two

spermidine synthases (SPDS1: *At1g70310* and SPDS2: *At1g23820*), one spermine synthase (SPMS: At5g53120), one thermospermine synthase (ACL5: *At5g19530*) (Hanzawa *et al.*, 2002; Kakehi *et al.*, 2008; Panicot *et al.*, 2002) and at least four SAM decarboxylases (SAMDC1: *At3g02470*, SAMDC2: *At5g15950*, SAMDC3: *At3g25570*, SAMDC4: *At5g18930*) (Franceschetti *et al.*, 2001; Ge *et al.*, 2006).

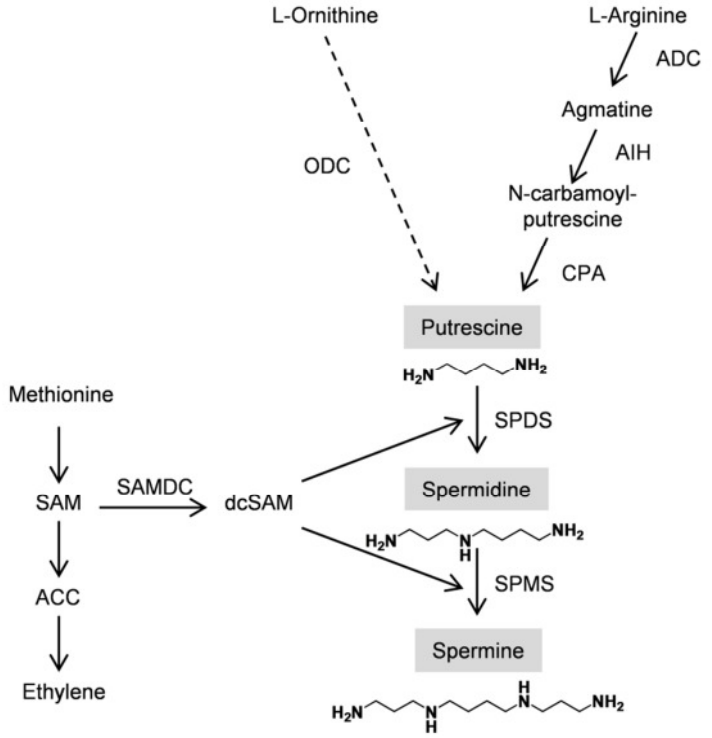

Figure 1-2: Biosynthesis of the most common plant polyamines. ACC: 1-amino-cyclopropane-1-carboxylic-acid, ADC: arginine decarboxylase, AIH: agmatine iminohydrolase, CPA: *N*-carbamoylputrescine amidohydrolase, dcSAM: decarboxylated *S*-adenosyl-L-methionine, ODC: ornithine decarboxylase, SAM: *S*-adenosyl-L-methionine, SAMDC: S-adenosyl-L-methionine decarboxylase, SPDS: spermidine synthase, SPMS: spermine synthase. Dashed arrow indicates a step which does not occur in *A. thaliana*.

In plants, PAs occur not only as free bases but they can be further modified and enter secondary metabolism. For example cadaverine can be converted into quinolizidine alkaloids whereas homospermidine, synthesized by transferring an amino butyl moiety in a NAD^+ dependent reaction to the diamine putrescine, serves as an essential precursor in the synthesis of pyyrolizidine alkaloids (Ober and Kaltenegger 2009). Putrescine also serves as a precursor for the biosynthesis of nicotine, tropane and nortropane alkaloids. The first decisive metabolic step is catalyzed by a putrescine *N*-methyltransferase (PMT). This SAM-dependent methyltransferase evolved from spermidine synthase and, besides sequence identities, shares a similar enzymatic mechanism (Biastoff *et al.*, 2009). Both enzymes accept the same substrate, putrescine and analogous co-substrates, SAM and decarboxylated SAM (dcSAM). Compared to spermidine synthase, where an amino propyl group is transferred from dcSAM to putrescine to form spermidine, PMT catalyzes the methyl transfer from SAM to putrescine to

form *N*-methylputrescine, committing putrescine to alkaloid biosynthesis (Biastoff *et al.,* 2009). Additionally, PAs also occur in a conjugated form, associated with small molecules derived from the shikimic acid and phenylpropanoid pathway, leading to the HCAAs already mentioned above.

1.1.2 Function of plant polyamines

The cationic nature of PAs at physiological pH is one of the main properties believed to mediate their biological activity. They are able to interact with negatively charged macromolecules, such as DNA, RNA, proteins (Bachrach 2005), membrane phospholipids and proteins (Schuber *et al.,* 1983; Tassoni *et al.,* 1996). PAs are involved in diverse plant processes like growth, development, senescence and stress response (Walters 2003). It is known that they regulate gene expression, cell proliferation and modulate the activity of some ion channels (Alcázar *et al.,* 2010; Kusano *et al.,* 2008; Martin-Tanguy 2001; Smith 1985). This chapter will summarize briefly the bioactivity of PAs in plants.

PAs, especially spermidine are necessary for *A. thaliana* viability. Double knock out mutants for both spermidine synthases, both arginine decarboxylases genes, or of *SAMDC1* and *SAMDC4* show defective seed development and they are embryo lethal (Ge *et al.,* 2006; Imai *et al.,* 2004; Urano *et al.,* 2005). Putrescine and spermine are also known to be involved in abiotic stress response, for instance acting as protective compounds against drought and salinity (Alcázar *et al.,* 2010; Yamaguchi *et al.,* 2007), whereas the spermine structural isomer thermospermine plays a critical role in stem elongation in *A. thaliana* (Kakehi *et al.,* 2008).

Exogenous treatment of plants with PAs or treatment with inhibitors of biosynthetic enzymes, as well as overexpression and knock out of genes involved in polyamine biosynthesis in transgenic plants have demonstrated that PAs play important roles in diverse processes. However, a precise common function or general mode of action of PAs *in planta* is far from being understood.

Unequivocal functional roles have been established in mammals. Spermidine is the substrate for a two-step posttranslational modification of the eukaryotic translation initiation factor 5A (eIF5A). Within this modification a specific lysine residue of the inactive eIF5A is converted into the unusual amino acid hypusine, by transferring the 4-aminobutyl moiety of spermidine to the ε-amino group, which is then further hydroxylated (Park 2006; Wolff *et al.,* 2007). This modification of eIF5A enables the protein to be confined to the cytoplasm where it is involved in protein biosynthesis (Lee *et al.,* 2009). The plant eIF5A has been implicated in development and senescence (Wang *et al.,* 2003). Cellular spermidine is therefore involved in the activation of eIF5A, which can then interact with both RNA and proteins and is presumed to play an important role in the translation machinery (Park 2006).

PAs have also been reported to block various ion channels. At physiological pH, PAs are positively charged and can therefore interact electrostatically with negatively charged proteins, including ion channels. Intracellular PAs block a fast-activating vacuolar (FV) cation channel from barley (Brüggemann *et al.*, 1998) and slow-activating channels in red beet (Dobrovinskaya *et al.*, 1999) in a charge dependent manner (spermine 4^+ > spermidine 3^+ > putrescine 2^+). Cytoplasmic PAs block the inward potassium currents across the plasma membrane of guard-cells and thus modulate stomatal movement, providing a link between stress conditions, PA levels and stomatal regulation (Liu *et al.*, 2000). In barley root epidermal and cortical cells, PAs inhibit the inward Na^+ and K^+ currents. By repressing Na^+ influx into roots and preventing K^+ loss from shoots, PAs help maintain K^+/Na^+ homeostasis and improve tolerance to high salinity (Zhao *et al.*, 2007).

1.2 The general phenylpropanoid pathway and branched compounds

Phenylpropanoids are abundant, structurally diverse natural compounds. These compounds consist of an aromatic C-6 ring and an aliphatic C-3 side chain. The origin of phenylpropanoids is the plastid localized shikimate pathway resulting in the synthesis of aromatic amino acids (Herrmann 1995). Its product *L*-phenylalanine is deaminated to cinnamic acid by *L*-phenylalanine ammonia lyase (PAL), the first enzyme of the phenylpropanoid pathway (Vogt 2010) (Figure 1-3). Cinnamic acid is subsequently hydroxylated by a cytochrome P450 monooxygenase, the cinnamate-4-hydroxylase (C4H) to form *p*-coumaric acid (Bell-Lelong *et al.*, 1997). Activated coenzyme A (CoA) esters of *p*-coumaric acid are the products of 4-coumarate:CoA ligase (4CL) and are central for the biosynthesis of several other metabolites (Lee *et al.*, 1995). In the large array of plant natural products, compounds issuing from the phenylpropanoid pathway fulfill important functions, as they are involved in development and multiple interactions of the plant with its environment. For example, isoflavonoids, coumarins and stilbenes are antimicrobial metabolites produced by plants for defending themselves against pathogens (Besseau *et al.*, 2007). Flavonoids, anthocyanins and aurones are plant pigments, for instance principal color determinants in most flowers (Ono *et al.*, 2006) and therefore important for plant-pollinator interactions. Aside from this, flavonoids are involved in an array of other processes, including plant pathogen interaction, seed development, protection against UV irradiation and function as antioxidants (Hernández *et al.*, 2009). The flavonoid biosynthetic pathway starts with the condensation of one molecule coumaroyl CoA and three of malonoyl CoA, yielding naringenin chalcone. This reaction is carried out by the enzyme chalcone synthase. Chalcones are precursors of structurally diverse flavonoids which can be divided in subgroups like flavones, flavonols, flavandiols, anthocyanins, condensed tannins and aurones (Winkel-Shirley 2001).

Phenylpropanoids are also important constituents of plant polymers like lignin and suberin. Lignin is a complex phenolic polymer that imparts strength, rigidity, and hydrophobicity to plant secondary cell walls (Bonawitz and Chapple 2010). This complex biopolymer is derived from monolignols such as *p*-hydroxyphenyl (H), guaiacyl (G) and syringyl (S) units, which are phenylpropanoid alcohols. Monolignols are formed from the universal precursor *p*-coumaroyl CoA (Figure 1-3) via conjugation to shikimate or quinate and subsequent cytochrome P450-catalyzed hydroxylations as well as SAM-dependent methylations (Humphreys and Chapple 2002). In suberin, ferulic acid moieties are conjugated to fatty acids by specific BAHD-transferases (D'Auria 2006; Molina *et al.,* 2009). Besides being incorporated into complex polymers like suberin and lignin, HCAs can be linked to PAs by the same type of BAHD-like transferases described in more detail in the next chapter.

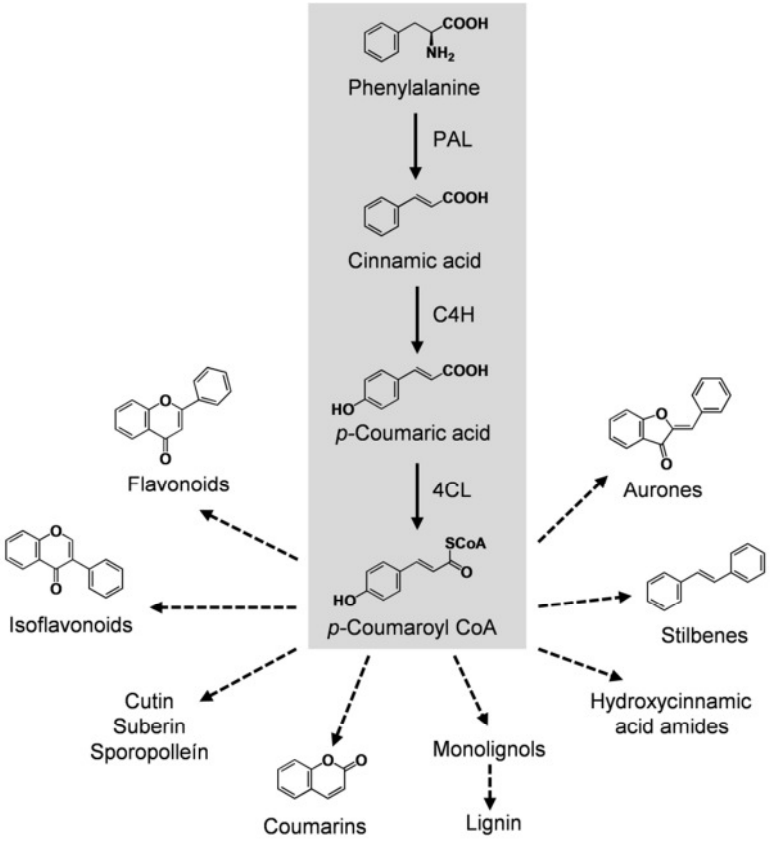

Figure 1-3: Phenylpropanoid pathway and branched secondary metabolites (modified from Vogt (2010)). The general phenylpropanoid pathway is shaded in grey. PAL, L-phenylalanine ammonia lyase; C4H, cinnamate 4-hydroxylase; 4CL, 4-coumarate CoA ligase.

1.3 HCAAs: occurrence and biosynthesis

It has been known for decades that plants can conjugate PAs with various HCAs. The resulting HCAAs form a huge class of secondary metabolites abundant in many plant species investigated and frequently associated with flowers and reproductive tissues (Edreva *et al.,* 2007). Carboxy groups of HCAs and amino groups of aliphatic or aromatic amines are joined

in an amide linkage, the characteristic feature of HCAAs. In plants, the most widely distributed amine components are putrescine, spermidine, spermine, agmatine, tyramine and tryptamine (Edreva *et al.*, 2007; Martin-Tanguy 1985). HCAAs occur as water-soluble or as water-insoluble forms. In the water-soluble forms, one single amine group of an aliphatic amine is bound to a HCA. The water-insoluble forms can be divided into two classes depending on the aliphatic or aromatic nature of the amine, which are linked to cinnamic acids.

In a historical survey, feruloylputrescine was first found in citrus leaves and fruits in 1965 by Wheaton and Stewart. Caffeoylputrescine was detected in seeds of the legume *Pentaclethra macrophylla* (Mbadiwe 1978) and also in several organs of the solanaceous *Nicotiana tabacum* (Buta and Izac 1972). HCAAs have been found in reproductive organs of flowers in a wide range of plants (Martin-Tanguy *et al.*, 1978; Ponchet *et al.*, 1980; Werner *et al.*, 1995) and are often linked to flower development and sexual reproduction. The reproductive organs are characterized by high titers of HCAAs, which are their main phenolic constituents, while those compounds are absent from sepals and petals (Martin-Tanguy *et al.*, 1978). Specifically bis- and tris-conjugated spermidine derivatives have been described in pollen for several plant species belonging to diverse families like Asteraceae, Fagaceae, Betulaceae, Juglandaceae and Brassicaceae (Bokern *et al.*, 1995; Fellenberg *et al.*, 2008; Lin and Mullin 1999; Meurer *et al.*, 1986; 1988a; 1988b; Nimtz *et al.*, 1996).

HCAAs are thought to play multiple, so far indistinct roles in plant development and resistance to biotic and abiotic stressors (Evans and Malmberg 1989; Walters 2003). Changes in HCAA levels and activities of HCAA biosynthetic enzymes have been implicated in a variety of plant growth processes and flower development. Correlations between HCAA titers and flowering have been observed in several plant species (Cabanne *et al.*, 1981; Tarenghi and Martin-Tanguy 1995; Wyss-Benz *et al.*, 1988). They accumulate to high levels in male and female floral organs and their lack can be linked to male sterility (Martin-Tanguy 1985; 1997).

HCAAs, mainly putrescine, spermidine and tyramine conjugated to feruloyl moieties, accumulate also in seeds of maize and rice, but decrease dramatically during germination suggesting that HCAAs function as storage compounds. After hydrolysis, these could provide additional amines necessary for cell division and expansion during germination (Bonneau *et al.*, 1994; Martin-Tanguy 1985). In addition, HCAAs could also be used as biochemical markers for seed dormancy of petunia (Jassey and Monin 1987).

Beside the poorly understood role of HCAAs during developmental processes, several studies support the implication of certain amides as plant defense compounds. The involvement of HCAAs in wound-healing tuber-tissue of potato is well established. For example, feruloyl amides of tyramine are covalently linked to the cell wall in natural and wound periderms of

potato and are putative constituents of potato suberin (Negrel *et al.,* 1996) which then can act as a barrier against potential pathogens. HCAA biosynthetic enzymes are wound inducible in potato tubers and leaves (Guillet and De Luca 2005; Negrel *et al.,* 1993). Wounding was also shown to induce feruloyl- and *p*-coumaroyltyramine accumulation in tomato (Pearce *et al.,* 1998).

The accumulation of HCAAs in plant response to pathogen infection has been reported extensively. A clear evidence for defense-related functions of HCAAs after herbivore attack was found in *Nicotiana attenuate* plants. A R2R3-type MYB (NaMYB8) transcription factor was shown to specifically target the HCAA biosynthesis, mainly of caffeoylputrescine and dicaffeoylspermidine, both increasing after herbivore attack. Silencing of NaMYB8, results in complete loss of both HCAAs and a higher susceptibility to insect herbivores relative to wild type. This effect could be partially abolished by spraying plants with caffeoylputrescine (Gális *et al.,* 2006; 2010; Kaur *et al.,* 2010). HCAAs are also proposed to have antifungal properties (Lee *et al.,* 2004; Walters *et al.,* 2001). In leaves of the model plant *A. thaliana*, HCAAs of agmatine and putrescine are involved in defense against *Alternaria brassicicola* (Muroi *et al.,* 2009).

Furthermore, HCAAs are shown to have antioxidant properties (Rajan *et al.,* 2001) and they might serve as UV protectants and natural insecticides, since N^1 coumaroyl spermidine was shown to block arthropod glutamate receptors (Gális *et al.,* 2010; Klose *et al.,* 2002). All these putative functions of plant HCAAs in growth, development and stress interaction are predominantly based on correlative evidence, but the exact mechanism of the molecular mode of action is still unknown.

The entry point in HCAA biosynthesis is catalyzed by BAHD-like *N*-hydroxycinnamoyl transferases. Agmatine coumaroyltransferase (ACT) was the first enzyme characterized in barley (Bird and Smith 1983), followed by putrescine and spermidine hydroxycinnamoyl transferases from tobacco callus and cell suspension (Meurer-Grimes *et al.,* 1989; Negrel 1989; Negrel *et al.,* 1991). Several orthologous enzymes with different donor and acceptor specificities are well summarized in Bassard *et al.*, (2010). These types of enzymes are described as catalyzing the condensation of various amines with HCAs, using CoA esters for the provision of activated carboxyl groups. This coupling reaction is catalyzed by *N*-hydroxycinnamoyl transferases mostly belonging to the BAHD acyltransferase family named according to the first letter of the initially characterized members of this family (St-Pierre and Luca 2000). For a long time it was accepted that HCA transferases catalyze the transfer of HCAs such as *p*-coumaroyl-, caffeoyl, feruloyl- or sinapoyl-moieties towards several amines to form the final conjugates and were considered as end products. Recently it was shown that the resulting HCAAs can be further derivatized. Metabolic flux analyses in potato tuber

suggested that feruloyltyramine and feruloyloctopamine could result from hydroxylation and methylation of the corresponding *p*-coumaroyl conjugates (Matsuda *et al.,* 2005). Recent evidence, including the hydroxylation of *p*-coumaroyltyramine by wheat, sweet basil and *A. thaliana* cytochrome P450 enzymes belonging to the CYP98 clade (Morant *et al.,* 2007) and the methylation of 5-hydroxyferuloyl moieties of spermidine conjugates by a cation-dependent *O*-methyltransferase from *A. thaliana* (Fellenberg *et al.,* 2008) suggest that this may be more common than anticipated. Furthermore, glycosylated HCAAs have also been identified for example in *A. thaliana* seeds (Böttcher *et al.,* 2008) but the responsible glycosyl transferases are not yet known.

1.4 Anther and pollen development

In flowering plants, development of the haploid male gametophytes (pollen grains) takes place in a specialized structure called the anther, where the future pollen grains are surrounded by sporophytic cells. At the mature stage the male gametophyte, the pollen grain, is a three-cell organism consisting of a vegetative cell and two sperm cells. Pollen development requires expression of both sporophytic and gametophytic genes and occurs in a series of distinct steps (McCormick 1993; McCormick 2004) starting with the formation of the pollen mother cell. First the pollen mother cell undergoes meiosis to form a tetrad of cells which are enclosed within the wall of the mother cell and physically connected to each other. Each tetrad is enclosed in a thick wall of callose, a β-1,3-glucan. Tetrad separation and release from the mother cell are accomplished by the action of callase, a mixture of enzymes containing endoglucanases and exoglucanases secreted by the tapetum. Next, the unicellular microspore undergoes an asymmetric mitosis, giving rise to a large vegetative cell, which eventually forms the pollen tube and the smaller generative cell. The generative nucleus undergoes a second round of mitosis and divides into two sperm nuclei. During pollination, one sperm nucleus will fertilize the egg cell while the other will fertilize the endosperm, a process termed "double fertilization". The timing of this second mitosis varies in different plant families, sometimes occurring within the anther (as in grasses and crucifers like *A. thaliana*), although more commonly it occurs during pollen tube growth. The final stages in pollen development are dehydration of the pollen grain and release from anthers.

Formation of viable pollen is a complex process that depends on accurate execution of developmental programs in both the sporophytic and gametophytic anther tissues. It occurs in the anther in the pollen sac, which is surrounded by a one-cell layer tissue, the tapetum. The tapetum cells are essential for the development of the pollen grain. Physiological functions attributed to the tapetal cells include contribution to microspore release by callase production, supply of metabolites, nutrition, and production of sporopollenin precursors (Piffanelli *et al.,* 1998). After release of microspores from callose-encased tetrads, the

sporopollenin based exine wall forms by the deposition of sporopollenin precursors generated by the tapetum, producing the sculptured baculae and tecta of the exine. During the maturation of microspores into pollen grains, cellulose- and pectin-rich intine forms between the exine and microspore plasma membrane (Blackmore *et al.,* 2007). In the last stages of pollen development, the final component of the pollen wall, the pollenkitt or tryphine is deposited on the mature pollen grain exine, accompanied by simultaneous programmed cell death of the tapetum (Parish and Li 2010).

The exine consists of a robust material called sporopollenin, which is biochemically an extremely resistant compound and contains derivatives of aliphatics, such as fatty acids, and phenolic compounds (Ariizumi and Toriyama 2011; Domínguez *et al.,* 1999). The exact chemical composition of the exine is a topic of continuing research. This resistant layer could function as a mechanical and chemical protector of pollen from various environmental stresses like microbial attack when pollen moves from anther to stigma or when it is released into the atmosphere, specifically in wind pollinated plants. Furthermore it preserves the characteristic rigid form of the pollen grain.

The outermost layer, the pollen coat, results upon disintegration of the tapetal layer in the late stage of pollen development. These coatings can be divided into tryphine, known from *Brassicaceae*, pollenkitt produced in most monocot and dicot pollen and elastoviscin, which is only found in Orchidaceae (Pacini 2000). This extracellular matrix of the pollen grains has been connected to diverse functions between anther opening and pollen hydration on the stigma, as a barrier to protect the pollen from water loss, UV radiation, fungi and bacteria. The pollen coat may facilitate pollen dispersal by enabling adhesion of pollen grains to each other, to pollinators or to the stigma and may be required for initiation of pollination including recognition by the stigma and rehydration of pollen (Murphy 2006; Pacini and Hesse 2005). The composition of this material is highly heterogeneous and includes waxes, lipids, carotenoids, phenolics, carbohydrates and proteins (Pacini and Hesse 2005). The occurrence of HCAAs as pollen components is a well-established feature in the Rosaceae and the Fagales (Meurer *et al.,* 1988a; Strack *et al.,* 1990). Their identification in *A. thaliana* pollen grains has now opened the door for detailed molecular and biochemical investigation as well as the elucidation of their molecular function.

1.5 HCAAs in *A. thaliana*

HCAAs have been recently identified in flower buds of *A. thaliana* simultaneously and indenpendently by several research groups (Fellenberg *et al.,* 2008; Grienenberger *et al.,* 2009; Matsuno *et al.,* 2009). Molecular and biochemical characterization of a cation-dependent *O*-methyltransferase (CCoAOMT)-like protein regarding localization, enzymatic activity and *in*

vivo function presented strong evidence for the involvement of this enzyme in HCAA biosynthesis in flowers in *A. thaliana*. This CCoAOMT-like enzyme, encoded by the locus *At1g67990*, is predominantly expressed in young flower buds. Immunolocalization studies, using a specific polyclonal antibody confirmed its unique presence in the tapetum of developing anthers before anthesis. Therefore, the enzyme was named *A. thaliana* tapetum-specific methyltransferase 1 AtTSM1 (Figure 1-4). The absence of AtTSM1 from vascular bundle cells simultaneously provides evidence that AtTSM1 is not involved in lignin monomer formation, a role usually associated with CCoAOMTs (Humphreys and Chapple 2002).

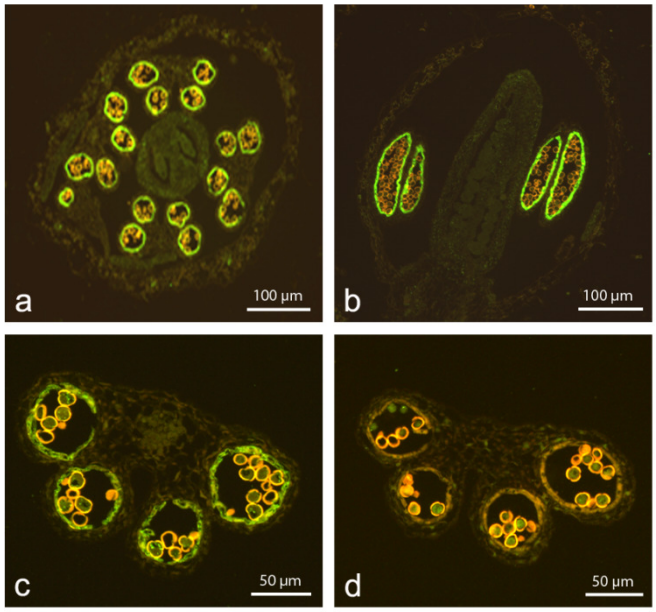

Figure 1-4: Localization of AtTSM1 in flower buds of *A. thaliana* (Fellenberg *et al.,* 2008). (a) Cross section; (b) longitudinal section; (c) cross section and close-up of a single stamen; (d) pre-immune control of (c). Green fluorescence signal indicates the specific presence of AtTSM1 in tapetal tissue.

RNAi mediated suppression of *AtTSM1* expression resulted in partly misshapen siliques with reduced seed set. When methanolic extracts were analyzed for changes of flower metabolites of the corresponding *AtTSM1* RNAi plants compared to wild type, a drastic shift in the ratio of two aromatic compounds was observed (also see Figure 1-5). The amount of N^1,N^5,N^{10}-tris-(5-hydroxyferuloyl)spermidine was increased while the amount of N^1,N^{10}-bis-(5-hydroxyferuloyl)-N^5sinapoylspermidine was decreased compared to control plants. These HCAAs differ only in a single methyl group attached to one of the three 5-hydroxyferuloyl moieties, resulting in a sinapoyl moiety. The suppression of AtTSM1 leads to a reduction of methylated HCAA and an accumulation of the precursor. This scenario is consistent with the *in vitro* substrate specificity of recombinant AtTSM1, with a preference for aromatic phenylpropanoid esters including N^1,N^5,N^{10}-tris-(5-hydroxyferuloyl)spermidine. Hence,

AtTSM1 is supposed to catalyze the methylation of the newly discovered HCAAs in the tapetum of the anther (Figure 1-5) which are later found on mature pollen grains.

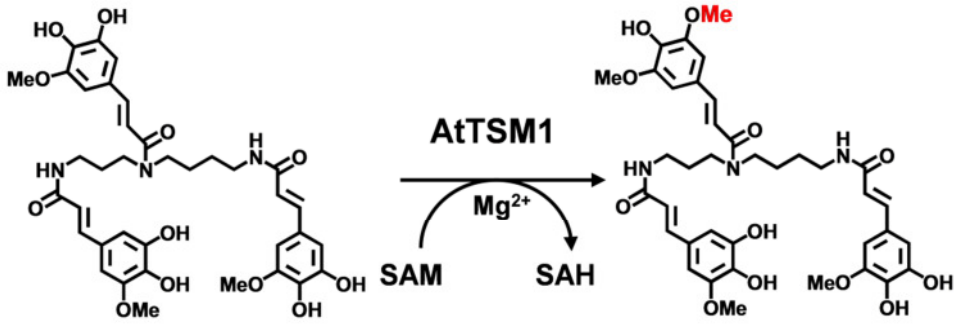

Figure 1-5: Proposed *in vivo* reaction catalyzed by AtTSM1 in the tapetum of developing anthers. N^1,N^5,N^{10}-tris-(5-hydroxyferuloyl)spermidine is converted in the presence of *S*-adenosyl-L-methionine (SAM) and magnesium ions into N^1,N^{10}-bis-(5-hydroxyferuloyl)-N^5sinapoylspermidine and *S*-adenosyl-L-homocysteine (SAH). The transferred methyl group is highlighted in red.

1.6 Questions addressed in this thesis

HCAAs are found throughout the plant kingdom. For the model organism *A. thaliana* these phenolic compounds were first described in flower buds and seeds (Böttcher *et al.,* 2008; Fellenberg *et al.,* 2008). The characterization of a tapetum specific cation dependent *O*-methyltransferase AtTSM1 facilitated the identification of N^1,N^5,N^{10}-Tris-(5-hydroxyferuloyl)-spermidine and N^1,N^{10}-Bis-(5-hydroxyferuloyl)-N^5-sinapoylspermidine in anthers and pollen grains. The subsequent research initiated the systematic investigation of the biosynthesis and the function of HCCAs in anthers of *A. thaliana*.

The first part of this thesis asks the question: *How are these phenolic compounds synthesized and what kind of HCAAs are present on pollen grains?* To address this question several genes were selected which are proposed to encode proteins involved in HCAA biosynthesis, like PA biosynthetic enzymes, acyl transferases, cytochrome P450-dependent hydroxylases and *O*-methyltransferases. Expression analysis was performed and the corresponding knock out mutants were analyzed in respect to HCAA accumulation. Furthermore, a search for additional HCAAs in *A. thaliana* flowers and pollen grains was conducted.

The main aim of the second part was to understand regulatory aspects of this biosynthetic pathway. For instance, what happens to the levels of biosynthetic transcripts and the precursors of these compounds once the key step, the transfer of HCAs to spermidine, is blocked? *Does spermidine accumulate if the conjugation does not take place?* It could be possible that the free PA spermidine is enriched in such mutants or the levels are maintained by unknown regulatory mechanisms. To answer this question, determination of the amounts of free PAs and HCAAs in anthers of wild type *A. thaliana* and HCAA biosynthetic mutants is of critical importance.

The last part of this work is focused on the most interesting yet unresolved question: *What is the biological function of (mainly tris-conjugated) HCAAs on A. thaliana pollen grains*? *Why has such a complex pathway evolved in this plant during the last few million years?* Once the corresponding genes and mutants are identified, pollen can be analyzed with regard to viability and fertility. The fitness of the pollen grains with respect to stress tolerance and under a variety of biotic and abiotic conditions can be systematically investigated.

2 Results

2.1 Contribution to publications

Chapter 2.2.2: Fellenberg, C., Böttcher, C. and Vogt, T. (2009) Phenylpropanoid conjugate biosynthesis in flower buds of *Arabidopsis thaliana*. *Phytochemistry* **70:** 1392-1400

Own contributions:

Experimentation: design and performance of expression analysis and mutant characterization (90 %)

Data analysis: data analysis of the above data (80 %)

Writing: design and preparation of figures (80 %); writing of the manuscript (30 %)

Other contributions: C. Böttcher performed the non-targeted metabolite profiling

Chapter 2.2.3: Fellenberg, C.*, van Ohlen, M.*, Handrick, V. and Vogt, T. (2012) The role of CCoAOMT1 and COMT1 in *Arabidopsis* anthers. *Planta* **236:** 51-61 * these authors contributed equally to this work

Own contributions:

Experimentation: design of all experiments, performance of HPLC analysis of pollen metabolites (70%)

Data analysis: data analysis of the above data and support of remaining analysis (60 %)

Writing: design and preparation of figures (50 %); writing of the manuscript (60 %)

Other contributions: M. van Ohlen performed the localization and expression studies and mutant characterization as part of a diploma thesis under my supervision; MS-data collection and interpretation of flavonoids was performed in cooperation with V. Handrick

Chapter 2.2.4: Fellenberg, C., Ziegler, J., Handrick, V. and Vogt, T. (2012) Polyamine homeostasis in wild type and phenolamide deficient *Arabidopsis thaliana* stamens. *Frontiers in Plant Science* **3:** 180

Own contributions:

Experimentation: design and performance of all experiments (90 %)

Data analysis: complete data analysis (100 %)

Writing: design and preparation of figures (90 %); writing of the manuscript (80 %)

Other contributions: J. Ziegler performed the HPLC measurements

2.2 How are pollen HCAAs synthesized in *A. thaliana*?

2.2.1 Aims and summary

HCAAs are a class of secondary metabolites, occurring in a wide range of plant species. They accumulate in reproductive organs of higher plants and their presence is apparently linked to plant fertility.

The biosynthesis and exact biological role of such conjugates during flower development is not well understood. The presence of tris-conjugated HCAAs in *A. thaliana* has only been recently established in flowers (Fellenberg *et al.*, 2008; Grienenberger *et al.*, 2009; Matsuno *et al.*, 2009) while mono- and bis-acylated compounds have been known from other sources like *A. thaliana* seeds and *Nicotiana attenuata* leaves (Böttcher *et al.*, 2008; Onkokesung *et al.*, 2012). Annotation and localization of a cation-dependent *O*-methyltransferase AtTSM1, methylating a single 5-hydroxyferulic acid moiety, in tris-substituted HCAAs in the tapetum of young flower buds enabled the subsequent identification of several genes with a putative role in HCAA biosynthesis.

Based on corresponding *A. thaliana* knockout mutants and real time PCR data, a biosynthetic pathway specifically for the major tris-substituted HCAAs is proposed. This pathway involves two methylation steps, catalyzed by two different cation-dependent *O*-methyltransferases (AtTSM1 and CCoAOMT1), a cytochrome P450 enzyme (Cyp98A8) dependent hydroxylation step, and a conjugating acyl transfer performed by a BAHD-like hydroxycinnamate (HCA)-acyltransferase (SHT).

LC/MS-based HCAA profiling of *cyp98A8* knockout mutants identified new feruloyl- and 4-coumarolylspermidine conjugates in flowers of *A. thaliana*, consistent with a role of this enzyme in formation of the hydroxylation pattern of these conjugates, whereas knockouts of CCoAOMT1 and the HCA-transferase lead to a drastic decrease or a complete loss of the major HCAAs identified in wild type flower buds. A complex pattern of minor bis- and trisacylspermidine compounds, likely the products of additional HCA-transferases, were identified in wild type as well as in knockout lines.

Based on expression analysis in *A. thaliana* wild type flower buds, RNAi mediated knock down and homozygous knock out lines of the corresponding biosynthetic genes, we propose a negative feedback regulatory mechanism where the knock out or suppression of the proposed final hydroxylation and methylation steps performed by AtTSM1 and CYP98A8 apparently leads to a transcriptional down-regulation of upstream genes of the HCAA biosynthetic pathway.

2.2.2 Publication

Phenylpropanoid polyamine conjugate biosynthesis in *Arabidopsis thaliana* flower buds

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Abstract

Phenylpropanoid polyamine conjugates have been identified in flowers of many plant species. Their presence in Arabidopsis thaliana has only been recently established in flower buds and pollen grains. Annotation and location of a cation-dependent O-methyltransferase AtTSM1 specifically to the tapetum of young flower buds enabled the subsequent identification of several genes with a putative role in phenylpropanoid polyamine conjugate biosynthesis. Based on the analysis of several A. thaliana knockout mutants, a biosynthetic pathway of these conjugates is proposed, which involves two methylation steps catalyzed by different cation-dependent O-methyltransferases, a cytochrome P450 (CYP98A8) catalyzed hydroxylation, and a conjugating acyl transfer performed by a BAHD-like, hydroxycinnamoyl (HC)-transferase. LC/MS based metabolite profiling of the cyp98A8 knockout line identified new feruloyl- and 4-coumaroylspermidine conjugates in the corresponding flowers consistent with a role of this gene in the hydroxylation of these conjugates. A pattern of minor amounts of bis- and tris-acylspermidine conjugates, likely the products of additional HC-transferases were identified in wild type as well as in the mutant lines. Transcript suppression of the genes early in the pathway was observed in knockout or RNAi-lines of the genes encoding late enzymatic steps. The implication of these findings for spermidine conjugate biosynthesis in flower buds of A. thaliana is discussed.

Keywords: *Arabidopsis thaliana*, knockout mutants, flower buds, polyamine conjugates, phenylpropanoids, metabolite profiling, Real-time PCR

Phytochemistry 2009, 70: 1392-1400. (DOI. 10.1016/j-phytochem.2009.08.010)

2.3 Do CCoAOMT1 and COMT1 have redundant functions in HCAA biosynthesis?

2.3.1 Aims and summary

A. thaliana caffeoyl coenzyme A *O*-methyltransferase1 (CCoAOMT1) and caffeic acid *O*-methyltransferase 1 (COMT1) are necessary for the biosynthesis of monolignols, the lignin precursors. Beside their established role in lignin formation, CCoAOMT1 and COMT1 also methylate other phenolic compounds. For example CCoAOMT1 was proposed to be involved in an early methylation steps leading towards the formation of scopoletin in *A. thaliana* roots. In our work we could show that it also plays an essential role for the accumulation of HCAAs in *A. thaliana* anthers and pollen grains, most likely by methylation of the proposed precursor caffeoyl coenzyme A. CCoAOMT1 knock out mutant plants show a severe, but not total, reduction of flower-specific HCAAs. Thus another *O*-methyltransferase must partially compensate the loss of CCoAOMT1. This could be COMT1. The aim of this work was to further support the suggested role of CCoAOMT1 in HCAA biosynthesis, by localization studies in *A. thaliana* flower buds and to investigate if COMT1 is responsible for the remaining HCAAs in *ccoaomt1* plants.

Using immunofluorescence microscopy and specific antibodies raised against recombinant CCoAOMT1 and COMT1 the distribution of both enzymes in *A. thaliana* stamens was determined. CCoAOMT1, as expected, is localized in the vascular systems of all flower organs but also in the tapetum of developing anthers, the site of HCAA biosynthesis. By contrast COMT1 shows no signal in tapetal cells but in directly adjacent cell layers of the endothecium and the epidermis. Methanolic extracts of *ccoaomt1* pollen grains show a strong reduction in HCAAs, whereas the phenylpropanoid pattern of wild type and *comt1* pollen shows no differences, consistent with the exclusion of COMT1 from the tapetum.

This cell type specific distribution of both OMTs and the phenolic profile of pollen grains of corresponding knock out plants indicate two distinct roles of these enzymes within *A. thaliana* anthers. CCoAOMT1 contributes to the HCAA profile in the tapetum and on pollen grains, probably methylating the precursor caffeoyl CoA. However COMT1 neither contributes to the accumulation of HCAAs nor to the flavonol pattern of pollen grains, but methylates the flavonol quercetin in the biosynthesis of various isorhamnetin glycosides in floral epidermal tissues.

2.3.2 Publication

The role of CCoAOMT1 and COMT1 in *Arabidopsis* anthers

Christin Fellenberg, Maike van Ohlen, Vinzenz Handrick and Thomas Vogt

C. Fellenberg and M. van Ohlen contributed equally to this work.
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Abstract

Arabidopsis caffeoyl coenzyme A dependent *O*-methyltransferase 1 (CCoAOMT1) and caffeic acid *O*-methyltransferase 1 (COMT1) display a similar substrate profile although with distinct substrate preferences and are considered the key methyltransferases (OMTs) in the biosynthesis of lignin monomers, coniferyl and sinapoylalcohol. Whereas CCoAOMT1 displays a strong preference for caffeoyl coenzyme A, COMT1 preferentially methylates 5-hydroxyferuloyl CoA derivatives and also performs methylation of flavonols with vicinal aromatic dihydroxy groups, such as quercetin. Based on different knockout lines, phenolic profiling, and immunohistochemistry, we present evidence that both enzymes fulfil distinct, yet different tasks in *Arabidopsis* anthers. CCoAOMT1 besides its role in vascular tissues can be localized to the tapetum of young stamens, contributing to the biosynthesis of spermidine phenylpropanoid conjugates. COMT1, although present in the same organ, is not localized in the tapetum, but in two directly adjacent cells layers, the endothecium and the epidermal layer of stamens. In vivo localization and phenolic profiling of comt1 plants provide evidence that COMT1 neither contributes to the accumulation of spermidine phenylpropanoid conjugates nor to the flavonol glycoside pattern of pollen grains.

Keywords: *Arabidopsis*, *O*-methyltransferase, phenylpropanoids, pollen, spermidine, tapetum

Planta 2012, 70: 51-61. (DOI. 10.1007/s00425-011-1586-6)

2.4 Does the HCAA precursor spermidine increase in *sht* plants?

2.4.1 Aims and summary

PAs like putrescine, spermidine and spermine are small organic compounds found in all living organisms. They occur as free molecular basses, but also conjugated to other molecules like proteins or HCAs. Those HCAAs are one of the main *A. thaliana* pollen tryphine constituents as describes in the two previous chapters. Now the question arises whether the knock out of the HCA conjugating step leads to a change, presumably a rise, in the levels of free PAs within the organ of HCAA biosynthesis, the stamen.

To analyze the levels of free PAs in wild type stamens compared to *sht* plants a new technique was required to establish PA levels in very small samples, as in case of *A. thaliana* stamen. The third chapter of this thesis is focused on the development of this new and sensitive PA quantification method. 9-fluorenylmethyl chloroformate (FMOC-Cl) was chosen as a PA derivatization reagent coupled with subsequently fluorescence detection by HPLC. With this advanced PA quantification method an accurate PA determination is possible with very limited amounts of material like in case of *A. thaliana* stamens. Hence, this improved, rapid and simple quantification method can be widely used in plant science and enables a high throughput determination of PAs.

The ratio of HCA-bound to free spermidine in *A. thaliana* wild type stamen and pollen grains was very high. Therefore an increase in PA content of *sht* deficient stamens compared to wild type could be expected. However, quantification of the three major PAs showed no significant influence on PA amounts, specifically spermidine, even though the conjugation of HCAs is drastically reduced indicating a highly controlled homeostasis of PAs in *A. thaliana* stamen and pollen grains. To complete this study, transcript levels of relevant PA and HCAA-genes, encoding biosynthetic and degradative enzymes in wild type and *sht* lines were included to reveal potential important regulatory mechanisms at this level.

2.4.2 Publication

Polyamine homeostasis in wild type and phenolamide deficient *Arabidopsis thaliana* stamens

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Abstract

Polyamines (PAs) like putrescine, spermidine, and spermine are ubiquitous polycationic molecules that occur in all living cells and have a role in a wide variety of biological processes. High amounts of spermidine conjugated to hydroxycinnamic acids are detected in the tryphine of *Arabidopsis thaliana* pollen grains. Tapetum localized spermidine hydroxycinnamic acid transferase (SHT) is essential for the biosynthesis of these anther specific tris-conjugated spermidine derivatives. *Sht* knockout lines show a strong reduction of hydroxycinnamic acid amides (HCAAs). The effect of HCAA-deficient anthers on the level of free PAs was measured by a new sensitive and reproducible method using 9-fluorenylmethyl chloroformate (FMOC) and fluorescence detection by HPLC. PA concentrations can be accurately determined even when very limited amounts of plant material, as in the case of *A. thaliana* stamens, are available. Analysis of free PAs in wild type stamens compared to *sht* deficient mutants and transcript levels of key PA biosynthetic genes revealed a highly controlled regulation of PA homeostasis in *A. thaliana* anthers.

Keywords: Arabidopsis, FMOC-derivatization, hydroxycinnamic acid, phenolamides, polyamine, spermidine, stamen

Frontiers in Plant Science 2012, 3: 180. (DOI. 10.3389/fpls.2012.00180)

2.5 Additional results

2.5.1 Which other transferase is involved in HCAA biosynthesis beside SHT?

The key step in HCAA biosynthesis is the linkage of HCA to the amino groups of PAs (Bassard *et al.*, 2010). SHT, a BAHD-like acyl transferase, has been described to catalyze this reaction in the tapetum of developing anthers in *A. thaliana* (Fellenberg *et al.*, 2009; Grienenberger *et al.*, 2009). *sht* mutant plants show a drastic reduction of HCAAs but there are still HCAAs present in flower buds and pollen grains, mainly bis- and monoacylated spermidine conjugates (Fellenberg *et al.*, 2009; Handrick *et al.*, 2010). This suggests the existence of at least one additional HCA transferase beside SHT. Two candidates were selected which had recently been described as HCA spermidine transferases in *A. thaliana*; a spermidine disinapoyl transferase (SDT) and a spermidine coumaroyl transferase (SCT) (Luo *et al.*, 2009). Both BAHD enzymes were hypothesized to contribute to the HCAA pattern of *A. thaliana* pollen grains.

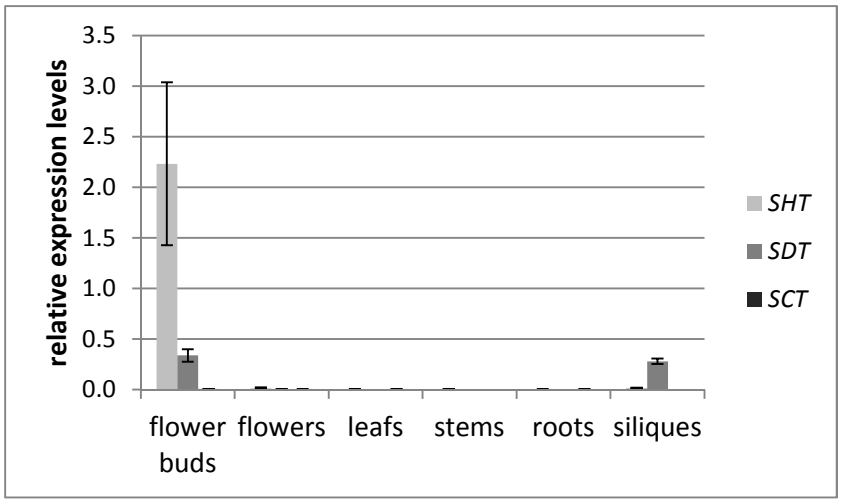

Figure 2-1: Organ specific transcript profile of three acyl transferases proposed to be associated with HCAA biosynthesis in *A. thaliana*; *At2g19070* encoding SHT; *At2g2351*0 encoding SDT and *At2g25150* encoding SCT. The small subunit of PP2A was used as a reference gene. Means ± SD determined from three independent experiments are shown.

To investigate if SDT and SCT are expressed in the same organs as SHT, transcript profiles of both genes were determined by quantitative RT-PCR (Figure 2-1). In comparison to SHT, SDT is also expressed in flower buds but to a much lower extent and additionally in siliques. The transcripts of SHT and SDT are barely detectable in other organs (flowers, leafs, stems and roots). The expression of SCT was nominal in all organs analyzed.

To determine if SDT and/or SCT are responsible for the remaining HCAAs in *sht* pollen, double knock out mutants of *sht* and either *sdt* or *sct* were generated and the pollen HCAA pattern was analyzed. Three pollen samples, obtained independently by harvesting from different plants of either wild type or mutants, were extracted and analyzed as described

previously by using a targeted profiling LC-MS/MS method (Handrick *et al.*, 2010). Extracts were analyzed regarding the presence of 38 individual HCAAs and relatively quantified compared to wild type (shown as fold change). Comparison of wild type, *sht*, *sdt* and *sht sdt* pollen is presented in Table 2-1. Thirty-seven out of 38 known HCAAs (Handrick *et al.*, 2010) have been detected in wild type pollen except N,N'-Bis-(5-hydroxyferuloyl)spermdine (compound 20). Almost all 15 tris-HCAAs were absent in *sht* pollen, only two were still detectable (compound 26 and 38) but their reduction is drastic. All five unknown HCAAs were not detected in *sht* pollen preparations, as well as seven bis-conjugated compounds (compound 2, 9-12, 16 and 20), while remaining HCAAs were clearly detectable, though their abundance was reduced. Only one bis-HCAA-*O*-hexoside (compound 3) is twofold increased compared to wild type, while the same compound is decreased in *sdt* mutants. Furthermore, *sdt* single mutants show only minor effects regarding their pollen HCAA pattern. Bis-sinapoylspermidine (compound 14) is reduced; all other compounds could be detected with the same intensity as in wild type.

The additional knock out of SDT in *sht* plants affected the HCAA pattern significantly. Ten HCAAs in *sht sdt* double knock out pollen (highlighted in grey in Table 2-1) exhibited a further reduction, relative to *sht*, all of them bis-conjugated HCAAs. The remaining 28 HCAAs showed comparable abundance as in *sht* pollen.

The distribution of HCAAs in *sct* and *sht sct* pollen (table 5-1) displayed no significant changes compared to wild type or *sht* single mutants. The single knock out of SCT revealed no changes of any HCAA, whereas the *sht sct* mutant pollen showed no significant further reduction compared to the *sht* single mutant pollen. In combination with the lack of significant transcript accumulation, this virtually excludes any role of SCT in HCAA formation in stamens.

Table 2-1 Relative quantification of spermidine HCAAs in pollen methanolic extracts obtained from wild type, *sht*, *sdt* and *sht sdt A. thaliana* plants, n=3. Reduction is given in fold change compared to wild type, grey indicates compounds which show decreased amounts compared to *sht*.

ID	HCAA	Wt	*sht*		*sdt*		*sht sdt*	
		Peak area ± SD (counts)	Peak area ± SD (counts)	Reduction (fold)	Peak area ± SD (counts)	Reduction (fold)	Peak area ± SD (counts)	Reduction (fold)
1	N-Feruloyl-N'-(5-hydroxyferuloyl) spermidine-*O*-hexoside	1.19E+04 ± 1.0E+03	6.28E+03 ± 2.0E+02	1.9 [c]	1.03E+04 ± 2.1E+03	1.2	b.d.l.	missing [a]
2	N,N'-Bis-(*p*-coumaroyl) spermidine-*O*-hexoside	9.46E+03 ± 1.5E+02	b.d.l.	missing [a]	1.03E+04 ± 1.9E+03	0.9	b.d.l.	missing [a]
3	N-Feruloyl-N'-sinapoyl spermidine-*O*-hexodise	4.52E+03 ± 1.2E+02	9.68E+03 ± 1.1E+03	0.5 [c]	9.95E+02 ± 3.8E+02	4.5 [c]	b.d.l.	missing [a]
4	N-(5-hydroxyferuloyl)-N'-sinapoylspermidine-*O*-hexoside	2.15E+03 ± 1.9E+02	1.25E+03 ± 2.4E+02	1.7 [c]	2.26E+03 ± 4.4E+02	1.0	b.d.l.	missing [a]
5	N,N'-Bis-feruloylspermidine-*O*-hexoside	2.76E+04 ± 9.8E+02	5.28E+02 ± 6.9E+02	52.3 [c]	3.00E+04 ± 6.0E+03	0.9	b.d.l.	missing [a]
6	Unknown	2.92E+03 ± 5.2E+02	b.d.l.	missing [a]	2.58E+03 ± 2.9E+02	1.1	b.d.l.	missing [a]
7	N-Feruloyl-N'-(5-hydroxyferuloyl) spermidine	6.86E+04 ± 1.7E+03	4.57E+03 ± 1.0E+03	15.0 [c]	5.66E+04 ± 9.0E+03	1.2	b.q.l.	trace [b]
8	N-(5-hydroxyferuloyl)-N'-sinapoylspermidine	5.53E+04 ± 5.2E+03	3.07E+03 ± 2.7E+02	18.0 [c]	4.70E+04 ± 1.1E+04	1.2	1.56E+02 ± 1.6E+02	354.5 [c,d]
9	N-(5-hydroxyferuloyl)spermidine	7.45E+02 ± 3.4E+02	b.d.l.	missing [a]	7.21E+02 ± 8.4E+01	1.0	b.d.l.	missing [a]
10	N-Caffeoyl-N'-feruloylspermidine	8.04E+03 ± 9.5E+02	b.d.l.	missing [a]	7.99E+03 ± 5.1E+02	1.0	b.d.l.	missing [a]
11	N-(*p*-Coumaroyl)spermidine	9.76E+03 ± 7.7E+02	b.d.l.	missing [a]	9.76E+03 ± 1.2E+03	1.0	b.d.l.	missing [a]
12	N-(*p*-Coumaroyl)-N'-feruloyl spermidine	7.38E+04 ± 1.0E+04	b.d.l.	missing [a]	6.59E+04 ± 8.2E+03	1.1	b.q.l.	trace [b]

13	N,N′-Bis-(*p*-coumaroyl) spermidine	1.54E+05 ± 5.4E+03	b.q.l.	trace [b]	1.59E+05 ± 1.5E+04	1.0	4.15E+02 ± 7.7E+01	371.7 [c]
14	N,N′-Bis-sinapoylspermidine	1.54E+04 ± 5.8E+02	4.00E+03 ± 2.3E+02	3.9 [c]	5.62E+03 ± 1.8E+03	2.7 [c]	b.d.l.	missing [a]
15	N-Feruloylspermidine	1.79E+04 ± 5.1E+02	b.d.l.	missing [a]	1.54E+04 ± 1.8E+03	1.2	b.d.l.	missing [a]
16	N,N′-Bis-feruloylspermidine	3.84E+05 ± 4.9E+03	1.79E+03 ± 8.0E+02	214.9 [c]	3.61E+05 ± 5.1E+04	1.1	1.18E+03 ± 3.3E+02	326.3 [c,d]
17	N-Feruloyl-N′-sinapoyl spermidine	4.96E+04 ± 2.2E+03	7.32E+03 ± 1.2E+03	6.8 [c]	9.70E+03 ± 2.7E+03	5.1 [c]	b.q.l.	trace [b]
18	Unknown	3.25E+02 ± 9.6E+01	b.d.l.	missing [a]	3.64E+02 ± 1.5E+02	0.9	b.d.l.	missing [a]
19	N-Caffeoyl-N′-(5-hydroxyferuloyl) spermidine	1.67E+04 ± 2.2E+03	b.q.l.	trace [b]	1.52E+04 ± 9.6E+02	1.1	b.d.l.	missing [a]
20	N,N′-Bis-(5-hydroxyferuloyl) spermidine	b.d.l.	b.d.l.	missing [a]	b.d.l.	missing [a]	b.d.l.	missing [a]
21	N-(*p*-Coumaroyl)-N′,N′′-bis-(5-hydroxyferuloyl)spermidine	1.55E+04 ± 2.1E+03	b.d.l.	missing [a]	1.41E+04 ± 2.5E+03	1.1	b.d.l.	missing [a]
22	N,N′,N′′-Tris-(5-hydroxyferuloyl) spermidine	2.31E+05 ± 2.2E+04	b.d.l.	missing [a]	2.26E+05 ± 2.9E+04	1.0	6.76E+02 ± 2.7E+02	342.4 [c]
23	N-Caffeoyl-N′-(*p*-coumaroyl)-N′′-(5-hydroxyferuloyl)spermidine	9.86E+02 ± 2.1E+02	b.d.l.	missing [a]	9.69E+02 ± 4.1E+02	1.0	b.d.l.	missing [a]
24	N-Caffeoyl-N′-feruloyl-N′′-(5-hydroxyferuloyl)spermidine	7.22E+03 ± 7.6E+02	b.d.l.	missing [a]	7.55E+03 ± 5.0E+02	1.0	b.d.l.	missing [a]
25	N-(*p*-Coumaroyl)-N′-(5-hydroxy feruloyl)-N′′sinapoylspermidine	5.56E+04 ± 8.2E+03	b.q.l.	trace [b]	4.57E+04 ± 1.1E+04	1.2	4.98E+02 ± 2.0E+02	111.5 [c]
26	N^1,N^{10}-Bis-(5-hydroxyferuloyl)-N^5-sinapoylspermidine	4.98E+05 ± 5.4E+04	1.15E+03 ± 3.6E+02	435.0 [c]	4.03E+05 ± 1.0E+05	1.2	4.89E+03 ± 2.0E+03	101.8 [c]

27	Unknown	1.48E+03 ± 6.3E+02	b.d.l.	missing [a]	2.02E+03 ± 4.5E+02	0.7	b.d.l.	missing [a]
28	Unknown	8.16E+02 ± 1.1E+02	b.d.l.	missing [a]	9.15E+02 ± 2.8E+02	0.9	b.d.l.	missing [a]
29	N,N′,N′′-Tris-(*p*-coumaroyl) spermidine	2.62E+03 ± 3.2E+02	b.d.l.	missing [a]	2.34E+03 ± 2.6E+02	1.1	b.d.l.	missing [a]
30	N-(*p*-Coumaroyl)-N′-feruloyl-N′′-(5-hydroxyferuloyl)spermidine	7.34E+03 ± 8.7E+02	b.d.l.	missing [a]	6.78E+03 ± 1.3E+03	1.1	b.d.l.	missing [a]
31	N-(5-hydroxyferuloyl)-N′,N′′-bis-sinapoylspermidine	4.50E+04 ± 3.5E+03	b.d.l.	missing [a]	4.07E+04 ± 8.9E+03	1.1	b.d.l.	missing [a]
32	N,N′-Bis-feruloyl-N′′-(5-hydroxyferuloyl)spermidine	1.30E+05 ± 6.6E+03	b.d.l.	missing [a]	1.22E+05 ± 1.7E+04	1.1	1.73E+02 ± 3.0E+02	750.9 [c]
33	Unknown	9.15E+03 ± 5.4E+02	b.d.l	missing [a]	8.59E+03 ± 1.5E+03	1.1	b.d.l.	missing [a]
34	N,N′,N′′-Tris-(*p*-coumaroyl) spermidine	3.15E+03 ± 9.2E+01	b.d.l	missing [a]	2.55E+03 ± 3.0E+02	1.2	b.d.l.	missing [a]
35	N,N′-Bis-(*p*-coumaroyl)-N′′-feruloylspermidine	1.82E+03 ± 1.5E+02	b.d.l	missing [a]	1.61E+03 ± 2.3E+02	1.1	b.d.l.	missing [a]
36	N-Feruloyl-N′-(5-hydroxyferuloyl) -N′′-sinapoylspermidine	1.61E+04 ± 5.6E+02	b.d.l	missing [a]	1.32E+04 ± 2.3E+03	1.2	b.d.l.	missing [a]
37	N-(*p*-Coumaroyl)-N′,N′′-bis-feruloylspermidine	8.55E+03 ± 4.6E+02	b.d.l	missing [a]	8.44E+03 ± 9.3E+02	1.0	b.d.l.	missing [a]
38	N,N′,N′′-Tris-feruloylspermidine	1.12E+05 ± 2.3E+03	4.58E+01 ± 7.9E+01	2442.0 [c]	1.07E+05 ± 1.4E+04	1.0	2.41E+02 ± 1.2E+02	464.1 [c]

[a] The substance is missing in mutant plants; [b] The substance is detectable in mutant plants, but present in concentration below its quantification limit; [c] The reduction is statistically significant compared to wild type ($p < 0.05$); [d] The reduction is statistically significant compared to sht ($p < 0.05$)
b.d.l., below detection limit (signal to noise ratio is less than 3); b.q.l., below quantification limit (signal to noise ratio is less than 10)

2.5.2 What is the biological relevance of HCAAs on *A. thaliana* pollen?

HCAAs are a class of secondary metabolites distributed widely in plants and have been described in reproductive organs of many plant species. They are implicated in a variety of developmental processes but their exact biological function is far from clear. The identification of several biosynthetic genes and the characterization of the corresponding mutants provide a good basis for further investigating physiological roles of these compounds. As described in chapter 2.5.1, both SHT and SDT are involved in the conjugation of HCAs with spermidine, resulting in a progressive decrease of pollen HCAAs in *sht* and *sht sdt* mutant plants. This section will describe potential biological roles of HCAAs on the pollen surface by comparing wild type, *sht* and *sht sdt* plants regarding plant and pollen phenotype and pollen fecundity under normal and various stress conditions.

To examine whether the reduced HCAA content on the pollen surface is accompanied by a sterile plant phenotype, as described for the AtTSM1 mutant (Fellenberg *et al.*, 2008) plants were grown under greenhouse conditions and phenotypically analyzed. No differences could be observed in silique development as shown in Figure 2-2. This is also displayed in comparable seed development, mutant plants yielded normal seeds in numbers similar to wild type plants (WT: 7987±2311; sht: 8575±1769; sht sdt: 8140±1902). Furthermore there were no observable differences in the vegetative parts of *sht*, *sht sdt* and wild type plants. They had comparable growth rates and were similar in size.

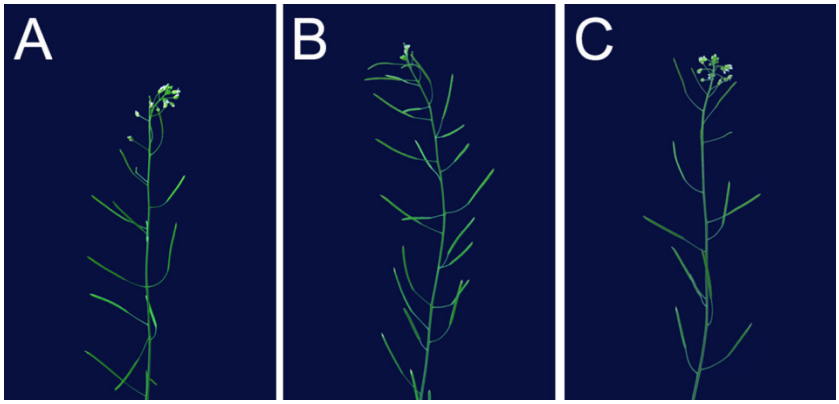

Figure 2-2: Phenotypes of *A. thaliana* inflorescences of WT (A), *sht* (B) and *sht sdt* (C) plants. Typical inflorescences of 7 weeks old plants show no differences in silique development.

When mature pollen grains were harvested from adult *A. thaliana* plants, a difference in coloration was observed (Figure 2-3). Wild type pollen displays a yellow greenish color whereas both mutant pollen are bright yellow. Apparently, the difference in coloration of the pollen is due to the reduction of HCAAs, which do not overlay the accompanying flavonoids anymore as they do in wild type. Whether other pigments like carotenoids accumulate in wild type and mutant pollen is not clear yet.

Figure 2-3: Comparison of pollen grains of WT (A), *sht* (B) and *sht sdt* (C) plants. WT pollen exhibit a greenish coloration whereas mutant pollen is colored yellow.

To measure the cell viability, pollen grains were harvested and stained with fluorescein diacetate (FDA) and propidium iodide (PI). This staining is based on the conversion of FDA by nonspecific esterases to a fluorescent analog (Heslop-Harrison and Heslop-Harrison 1970) whereas PI, which is excluded from living cells but stains the DNA and cytoplasm of dead cells, was used as a counter-stain (Huang *et al.*, 1986). The results of the viability staining are shown in Figure 2-4. Numerous replications of this staining procedure indicated that the numbers of vital pollen in the wild type and the mutants were equal. Mature pollen grains show a viability of around 50 percent.

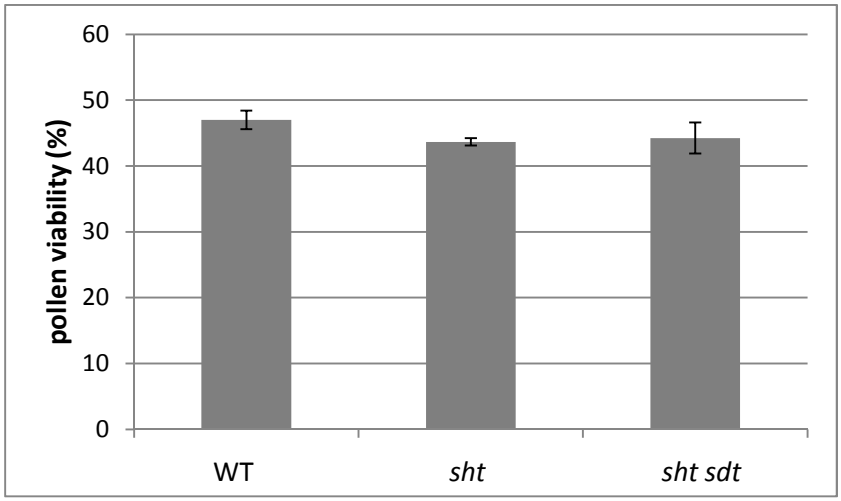

Figure 2-4: Viability of pollen grains assayed with fluorescein diacetate and propidium iodide. Means ± SD determined from three independent experiments are shown. Eight hundred pollen grains were counted to determine pollen viability for each replicate

While mutant plants appeared wild type in overall appearance, and these plants had full siliques, indicating that the loss of HCAAs does not prevent fertilization, it remained a possibility that *sht* and *sht sdt* pollen might display defects in pollen tube development. To determine if the morphology or dynamics of mutant pollen tubes were affected, the growth of wild type and mutant pollen tubes was analyzed *in vitro*, using a standard pollen growth medium (see Appendix) and the germination frequency was determined 12 h after transfer of pollen grains to germination medium. Under these conditions, the germination and growth of

the *sht* and *sht sdt* pollen could not be distinguished from that of the wild type pollen. Each type of pollen grain germinated at a rate that varied from approximately 20 to 50 % as shown in figure 2-5 A. The length of the pollen tubes generated by each type of pollen varied from 1100 to 1400 µm. In addition, there was no visible difference in the morphology of the pollen tubes.

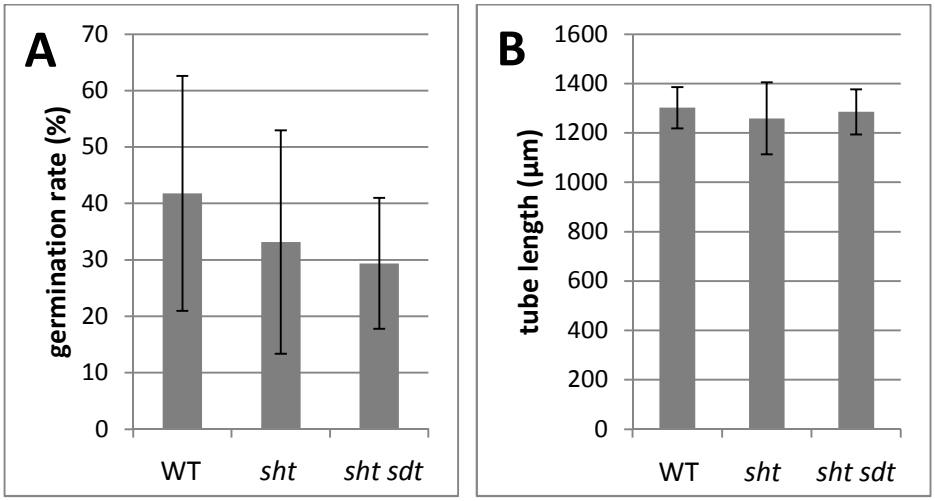

Figure 2-5: Pollen germination rate (A) and tube length (B) assayed *in vitro*. Means ± SD determined from three independent experiments are shown. Seven hundred pollen grains were counted for germination and 40 pollen tubes were measured for pollen tube length for each replicate

Since these *in vitro* germination assays presumably do not fully mimic the *in planta* pollen-pistil interaction, pollen germination was also investigated *in vivo*. Therefore emasculated wild type flower buds were hand pollinated with either mutant or wild type pollen, and after 2 and 8 h of incubation the pollen-tube length was examined by staining with aniline blue to detect formed callose plugs. The results (Figure 2-6) showed that the growth of the mutant and wild type pollen tubes through the transmitting tissue is comparable. For example after 2 h the length of the pollen tubes was about 300 µm in every sample whereas after 8 h the tubes reached a length of around 500 µm.

Because HCAAs can be easily extracted with methanol they are considered to be components of the extracellular matrix of pollen grains, known as tryphine or pollen coat. Those pollen coatings have diverse functions and are for example involved in pollen-pistil interaction. Thus the question arises whether the loss of HCAAs on pollen surface influences this interaction. To address this question a liquid assay for monitoring adhesion of pollen grains was used (Zinkl *et al.*, 1999). Unpollinated mature wild type pistils were saturated with either freshly harvested wild type or mutant pollen. Pollinated pistils were washed vigorously (see Materials and Methods) and the adhering pollen grains were counted. The change of pollen stigma adhesion was analyzed with 15 pistils combined with the three different pollen samples in three replicates.

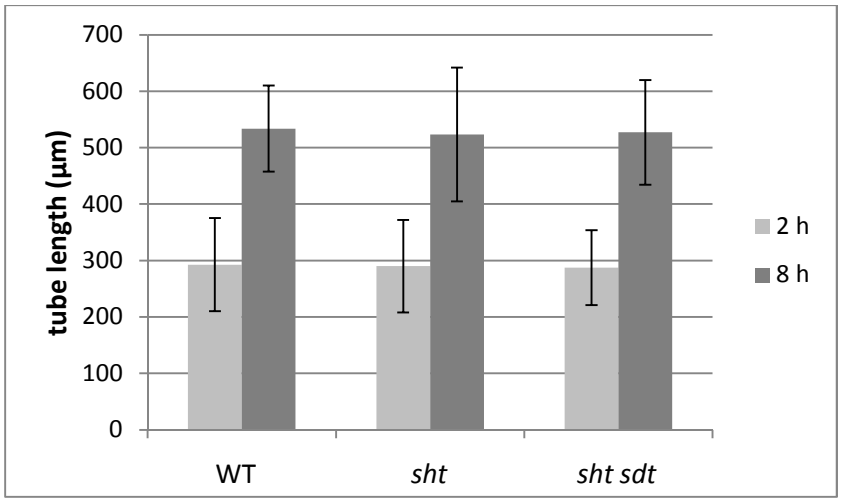

Figure 2-6: *In vivo* germination of wild type and mutant pollen. Germination was monitored using aniline blue staining after pollination of wild type stigmas with either wild type or mutant pollen. Pollen tube length was measured 2 and 8 hours after pollination.

The quantity of pollen adhering to the stigmatic surface after the washing procedure is summarized in Figure 2-7. On average 25 pollen grains were observed on all stigmas, indicating that the adhesion potential of wild type, *sht* and *sht sdt* pollen is not affected. The reduction of HCAAs on the pollen surface does not affect the adhesive function of pollen to the stigma. Taken together these results strongly suggested that the reduction of HCAAs on the pollen surface of *A. thaliana* does not affect pollen fertilization ability.

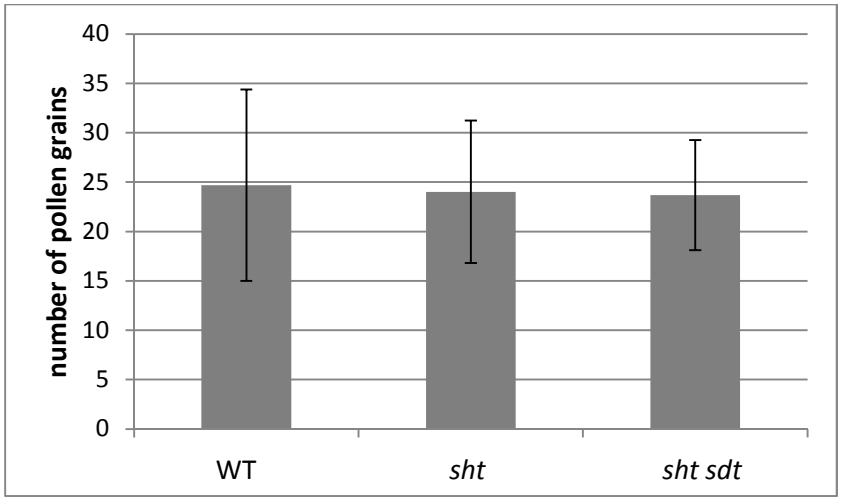

Figure 2-7: Assessment of pollen adhesion using a liquid assay. Means ± SD determined from two independent experiments are shown. Fifteen stigmata per sample and experiment were analyzed.

HCAAs have been suggested to function as storage compounds, which after hydrolysis supply additional amines required to facilitate cell division and expansion (Bonneau *et al.*, 1994). To test whether this suggestion is true for the pollen germination process, the stability of HCAAs during pollen germination was investigated. From *in vivo* germination assays described before it was known that after 2 hours pollen germination is already initiated and

after 4 hours pollen tubes reach the first ovules. Accordingly, these time points were assumed to represent a good timeframe for analysis of the pollen tube growth process. To follow the stability or a possible degradation of HCAAs during pollen tube growth, unpollinated wild type pistils were prepared (10 per sample) and either directly extracted with methanol or pollinated followed by extraction after mentioned time points. Methanolic extracts were then analyzed by HPLC and the corresponding chromatograms are shown in Figure 2-8.

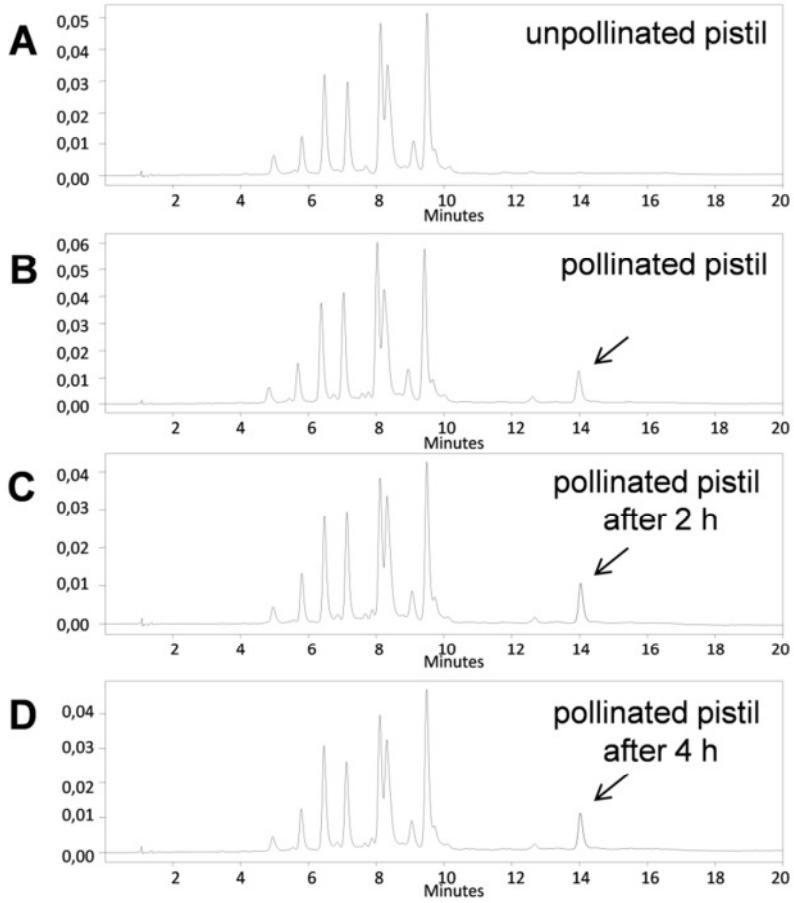

Figure 2-8: HPLC chromatograms of *A. thaliana* pistils with or without pollen grains at 318 nm. Unpollinated wild type pistils and pollinated pistils 0, 2 and 4 hours after pollination were extracted with methanol and analyzed by HPLC. (A) pistil without WT pollen; (B) pistil with WT pollen; (C) pistil with WT pollen 2 hours after pollination; (D) pistil with WT pollen 4 hours after pollination. N^1,N^{10}-bis-(5-hydroxyferuloyl)-N^5-sinapoylspermidine is indicated by an arrow.

The HPLC-profile of pollinated pistils (B) shows an additional peak compared to unpollinated pistils (A), identified as the most abundant pollen HCAA N^1,N^{10}-bis-(5-hydroxyferuloyl)-N^5-sinapoylspermidine. The profiles of pollinated pistils after two (C) and four (D) hours are virtually identical, N^1,N^{10}-bis-(5-hydroxyferuloyl)-N^5-sinapoylspermidine is still detectable in similar amounts. An accurate quantification of N^1,N^{10}-bis-(5-hydroxyferuloyl)-N^5-sinapoylspermidine was impossible since the number of pollen grains per pistil is not comparable between the different samples. Nevertheless, the level of N^1,N^{10}-bis-(5-

hydroxyferuloyl)-N[5]-sinapoylspermidine seems not to be substantially changed during the analyzed timeframe.

Contribution of HCAAs to abiotic stress tolerance

UV-light: HCAAs have been described in pollen grains of many plant species (see Introduction) including wind pollinators. In those cases pollen has to face harsh environmental conditions before reaching their target, like UV radiation and temperature stress which can minimize reproductive success. Thus another focus of this work was to evaluate the pollen vigor of *A. thaliana* wild type, *sht* and *sht sdt* mutant plants under abiotic stress conditions to determine if HCAAs on the pollen surface can improve pollen fitness under those conditions.

Since HCAAs absorb UV light it could be possible that they can protect the gametophyte against UV radiation during pollen dispersal. For UV-B stress treatment plants were grown in a sun simulator three week after sowing. Wild type and *sht* mutant plants were compared with or without increased UV-B radiation. *sht sdt* plants have not been tested because of their unavailability at that time. Plants have been compared regarding reproduction efficiency. Wild type and SHT-deficient plants show no difference in seed numbers (Figure 2-9 A) indicating that a reduction in HCAAs on the pollen surface has no influence on reproduction efficiency under UV-B light. Induction of genes involved in biosynthesis of UV protective compounds is well studied in plants. For example chalcone synthase, a key flavonoid biosynthetic gene, is induced after UV treatment (Fuglevand *et al.*, 1996). Hence, expression of *SHT* and *AtTSM1* mRNA was analyzed in wild type flower buds with or without supplemental UV-B light. Figure 2-9 B shows no UV-dependent induction of either HCAA biosynthetic gene.

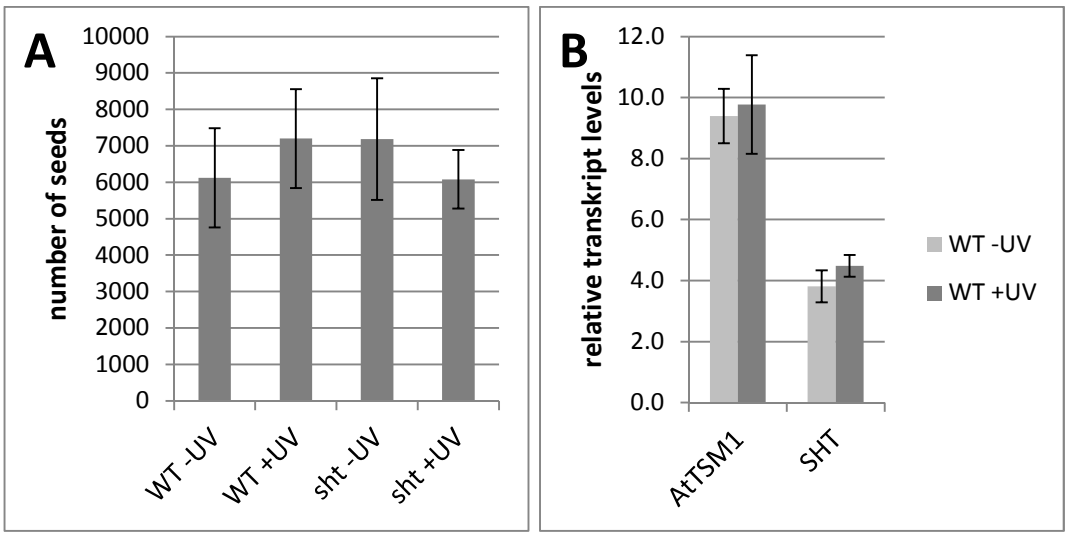

Figure 2-9: (A) Number of seeds per plant grown with or without supplemental UV-B. Means ± SD determined from 16 plants per treatment are shown. (B) Transcript accumulation of *AtTSM1* and *SHT* in flower buds after UV treatment. Means ± SD determined from 4 plants per treatment are shown.

Heat/Freezing: The predominant *A. thaliana* pollen HCAAs are water insoluble, therefore a hydrophobic protection of the pollen grain provided by HCAA accumulation on the surface to protect the gametophyte from desiccation during pollen propagation could be possible. Hence the pollen fertility from wild type and mutant plants was tested under increased and freezing temperatures. As a measure of pollen vigor the *in vitro* germination rate was determined after temperature treatment.

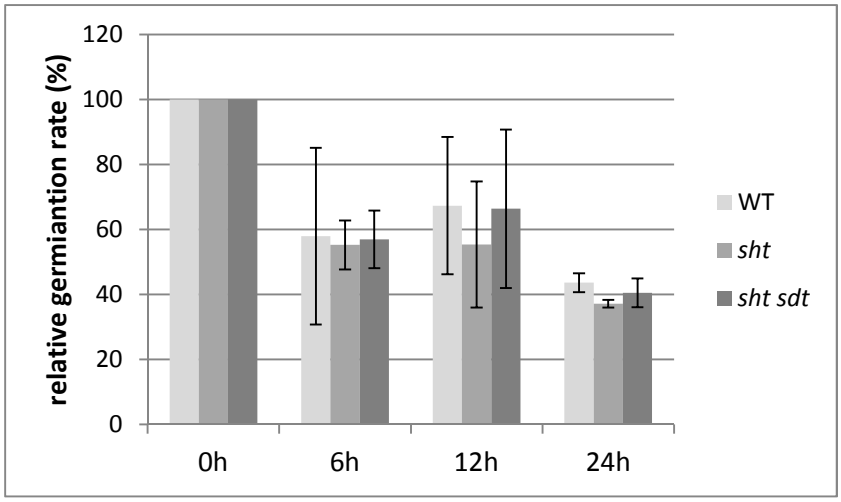

Figure 2-10: Relative pollen germination rate after incubation at 37 °C. Means ± SD determined from three independent experiments are shown. On average 2000 pollen grains were counted per sample and treatment.

Collected pollen was either incubated at 37°C or subjected to freeze-thaw cycles including 1 hour incubation at –20°C followed by 1 hour thawing at room temperature. After temperature treatment or the respective thawing period, pollen of each sample was transferred to germination medium (as described before) and incubated 12 hours in a humid chamber. High variance in germination percentage between the distinct experiments could be observed; therefore relative germination rate is depicted to make the different experiments more comparable.

The relative germination percentage after 0, 6, 12 and 24 hours at 37°C is summarized in Figure 2-9. 6. Twelve hours incubation of pollen grains at 37°C resulted in a germination rate that was decreased to around 60 %, while after 24 hours about 40 % of the pollen grains developed normal tubes. However, no differences were observed in germination frequency between wild type and mutant pollen. Similar results were observed when pollen was incubated at 45°C (data not shown).

Collected pollen was exposed to repeated freeze-thaw cycles up to 8 times (Figure 2-10). A decreased pollen germination rate could be observed over time, but wild type, *sht* and *sht sdt* pollen show no differences under the conditions used.

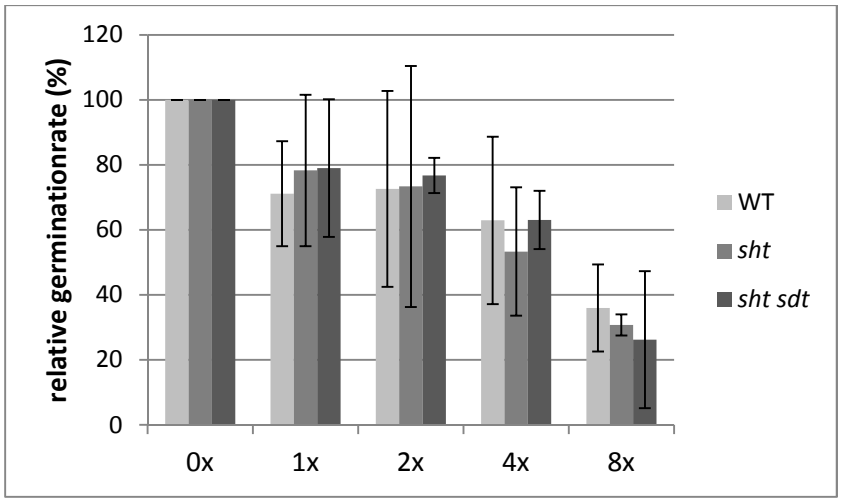

Figure 2-11: Relative pollen germination rate during freeze-thaw stress. Means ± SD determined from three independent experiments are shown. On average 2000 pollen grains were counted per sample and treatment.

In conclusion, HCAA deficient pollen grains did not show any restriction in fertility and reproduction efficiency, indicating that these compounds are not connected to plant reproduction, at least in *A. thaliana*. Furthermore, HCAA occurrence does not influence pollen germination frequency under UV-light exposure and temperature stress. Other possible biological function of HCAAs on the pollen grain surface of *A. thaliana* are referred in the discussion below.

3 Discussion and perspectives

Initial localization and characterization of a tapetum specific *O*-methyltransferase (AtTSM1) led to the identification of HCAAs in anthers and pollen grains of *A. thaliana* for the first time. This enzyme catalyzes the final methylation of N^1,N^5,N^{10}-tris-5-hydroxyferuloyl spermidine resulting in N^1,N^{10}-bis-(5-hydroxyferuloyl)-N^5sinapoylspermidine (Fellenberg *et al.,* 2008). Both compounds are synthesized in tapetal cells and are later found on the pollen grain surface. Similar HCAAs have been described for several plant species but their biosynthesis and physiological relevance was far from clear. The identification of these compounds in the model organism *A. thaliana* has now facilitated a detailed analysis of the biosynthesis and biological function of plant HCAAs, which was the goal of this thesis.

Based on their co-expression with AtTSM1, possible HCAA biosynthetic genes have been selected and analyzed regarding their localization and participation in HCAA accumulation. With this we elucidated the pathway leading to HCAAs in *A. thaliana* anthers and pollen. In parallel studies, corresponding genes and enzymes for HCA transfer to spermidine and HCAA hydroxylation were identified and characterized (Grienenberger *et al.,* 2009; Matsuno *et al.,* 2009). The combined description of HCAA biosynthesis, identification of biosynthetic enzymes and characterization of corresponding knock out mutants enabled a functional analysis of these compounds *in planta*.

3.1 HCAA biosynthesis in anthers of *A. thaliana*

The results presented here generated substantial progress towards the understanding of HCAA biosynthesis in anthers of *A. thaliana*. Besides AtTSM1, four additional genes encoding HCCA biosynthetic enzymes were identified in this work. Two of them, a BAHD acyl transferase (SHT) and cytochrome P450 dependent hydroxylase (CYP98A8) have been characterized simultaneously whereas CYP98A9, a paralog of CYP98A8, was also connected to HCAA biosynthesis (Grienenberger *et al.,* 2009; Matsuno *et al.,* 2009). The current knowledge of this anther specific pathway is schematically summarized in Figure 3-1.

3.1.1 BAHD acyltransfeases

The linkage of PA biosynthesis with the phenylpropanoid pathway and the coupling of hydroxycinnamoyl and amine moieties can be considered the key step in HCAA biosynthesis. This critical step is catalyzed by at least two BAHD-like acyltransferases, SHT and SDT in the tapetum of *A. thaliana*. SHT mutants show a drastic reduction of pollen HCAAs, mainly in the tris-substituted compounds. This observation is consistent with the published data of Grienenberger *et al.,* (2009), who also showed a correlation between *SHT* expression and HCAA occurrence on the pollen surface.

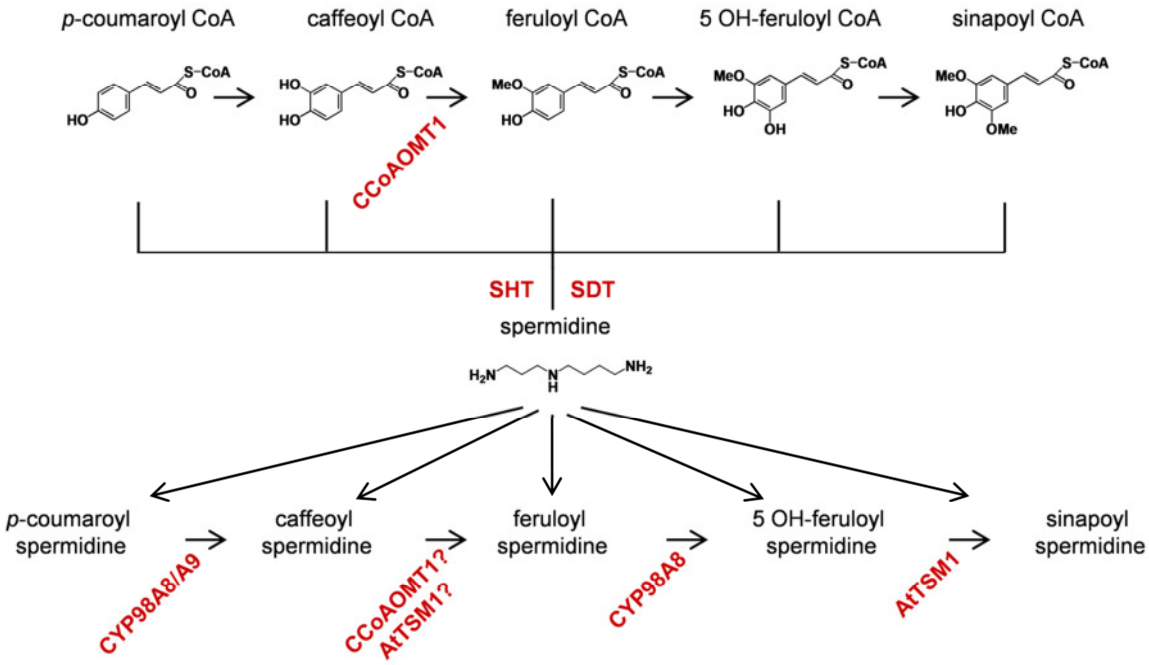

Figure 3-1: Proposed biosynthetic pathway leading to HCAA formation in *A. thaliana* anthers. Identified HCAA-biosynthetic enzymes include: CCoAOMT1, caffeoyl CoA *O*-methyltransferase 1; SHT, spermidine hydroxycinnamoyl transferase; SDT, spermidine disinapoyl transferase; CYP98A8/A9, cytochrome P450 dependent hydroxylases; AtTSM1, tapetum specific *O*-methyltransferase 1.

SDT, initially identified as a spermidine disinapoyl transferase required for the production of disinapoyl spermidine and its glycoside in *A. thaliana* seeds (Luo *et al.*, 2009), also contributes to HCAA production in tapetal cells. Single knock out mutants of SDT show only marginal effects on pollen HCAA accumulation, indicating that SHT may largely compensate for the loss of SDT. Double knock out mutants of SHT and SDT display a more dreastic HCAA-reduction compared to *sht*, strongly suggesting that SDT is an additional enzyme required for HCAA accumulation in pollen grains. From the HCAA profiling data, SCT does not seem to participate in HCAA accumulation on the pollen grains: *sct* mutants show no effect, while *sht sct* pollen revealed the same phenolic profiling pattern as *sht* pollen (Appendix Table 5-1). *In vitro* data from Luo *et al.*, (2009) suggest that SCT is a spermidine coumaroyl transferase expressed in roots, but a connection to naturally occurring HCAAs in *A. thaliana* is not yet known. It is possible that SDT can substitute for the loss of SCT in *sht sct* lines. To test whether this is true, HCAA profiling of *sht sdt sct* triple mutants, to see if these pollen show a further reduction or complete absence of HCAAs, could be informative.

The HCAA profiling data of *sht* and *sht sdt* pollen presented here are in agreement with *in vitro* enzymatic activity of the corresponding recombinant enzymes. Spermidine is the only PA that appeared to be efficiently acylated by both enzymes *in vitro* and spermidine containing HCAAs are the only compounds of this class known so far in *A. thaliana* flowers. This feature seems to be common for *N*-hydroxycinnamoyl transferases, usually show a higher specificity for the PA acyl acceptor than for the acyl donors (Bassard *et al.*, 2010;

Kristensen *et al.,* 2004). SHT accepts a broad range of hydroxycinnamoyl-CoA esters as acyl donors, but shows a preference for feruloyl CoA. Enzyme assays performed with SHT revealed triacylated spermidine conjugates as *in vitro* products (Grienenberger *et al.,* 2009). This favored transfer activity of SHT is supported by the strong reduction of tris acylated spermidine conjugates in *sht* pollen. Bis-conjugates are also affected in *sht* pollen, but are almost completely absent in the case of the additional knock out of SDT, which is reported to add two acyl groups to its acyl acceptor (Luo *et al.,* 2009). SDT was shown to preferentially act as a spermidine sinapoyl CoA transferase either *in vitro* or when overexpressed in *A. thaliana* leaves (Luo *et al.,* 2009). This finding is contradicted by the diversity of bis-acylated spermidine conjugates found on the pollen surface (Handrick *et al.,* 2010). Bis conjugates carrying feruloyl, 5-hydroxyferuloyl and sinapoyl moieties are reduced by the additional loss of SDT, indicating that this enzyme has broader selectivity with respect to the acyl donor in the tapetum of the developing anther. Another BAHD acyltransferase responsible for HCAA formation in *A. thaliana* leaves has been reported recently and was identified as an agmatine coumaroyltransferase (ACT). ACT has a preference for agmatine but also accepts putrescine as an acyl acceptor and is responsible for the biosynthesis of coumaroyl- and feruloyl-HCAAs in leaves as defense compounds after pathogen infection (Muroi *et al.,* 2009).

Decoration of HCAAs

Mutant fower buds of CYP98A8 knock out show a lack of HCAAs containing 5-hydroxyferuloyl moieties, compensated by coumaroyl and feruloyl HCAAs, indicating that CYP98A8 catalyzes the *meta* hydroxylation of HCAAs. This finding was confirmed by expression of this enzyme in yeast microsomes catalyzing the hydroxylation of triferuloyl and tricoumaroyl spermidine (Matsuno *et al.,* 2009). Its paralog CYP98A9 has also been shown to contribute to HCAA biosynthesis in anthers but can only hydroxylate tris-coumaroyl spermdine *in vitro* (Matsuno *et al.,* 2009). Hence three enzymes, an *O*-methyltransferase (AtTSM1) and two cytochrome P450 dependent hydroxylases (CYP98A8/9), have been identified, which could act as decorating enzymes on HCAAs in the tapetum (Fellenberg *et al.,* 2008; Matsuno *et al.,* 2009). These enzymes clearly modify HCAA structures *in vitro*, but it is not known whether this is the only mode of action or if they also act on free HCAs prior conjugation to PAs. The enzymatic properties *in vitro*, at least for AtTSM1, show that this enzyme acts on a variety of methyl group acceptors, from flavonoids to hydroxycinnamic esters. The elucidation of HCAA biosynthesis in the tapetum of *A. thaliana* clearly shows for the first time that HCAAs can further be modified after conjugation of HCAs to PAs.

3.1.2 Transport from the tapetum to pollen grains

HCAAs are synthesized in the tapetum. For the complete set of analyzed biosynthetic enzymes it is known that they are localized or expressed in tapetal cells (Figure 3-2), except

for SDT where it has not been shown yet, though it is known to be expressed in anthers (Fellenberg *et al.*, 2012b). AtTSM1 is the only enzyme which is restricted exclusively to the tapetum (Fellenberg *et al.*, 2008). SHT, SDT and CYP98A8 are additionally expressed in seeds, SCT and CYP98A9 are also localized in root tips, whereas CCoAOMT1 is found in all organs (Fellenberg *et al.*, 2009; Luo *et al.*, 2009; Matsuno *et al.*, 2009). Anthers and seeds are both known for HCAA production in *A. thaliana*. However, no HCAAs have been detected in root tips so far. Possibly the quantities are below detection limits or these compounds are synthesized only under specific conditions (Luo *et al.*, 2009).

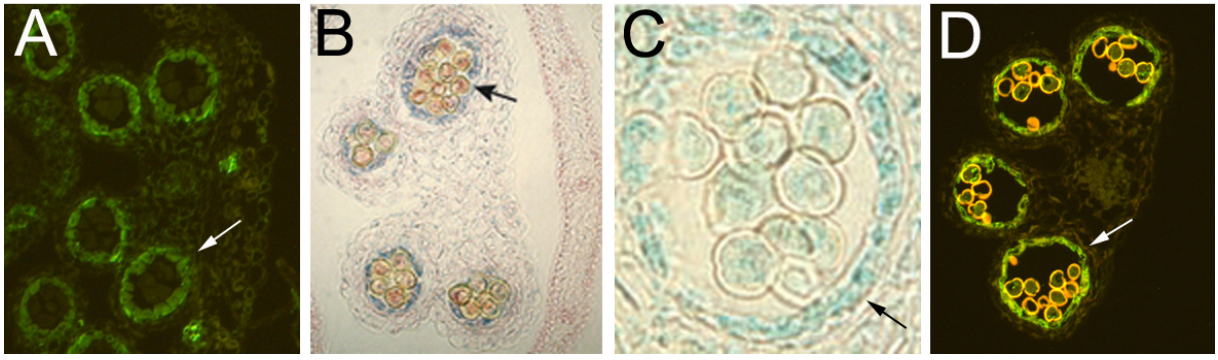

Figure 3-2: HCAA biosynthesis occurs in tapetal cells. (A) immunlocalization of CCoAOMT1 (Fellenberg *et al.*, 2012a); (B) SHT and (C) CYP98A8 expression analyzed by GUS staining (Grienenberger *et al.*, 2009; Matsuno *et al.*, 2009); (D) immunlocalization of AtTSM1 (Fellenberg *et al.*, 2008). Arrows indicate the localization of respective biosynthetic enzymes in the tapetum.

The tapetum cells synthesize proteins, lipids and secondary metabolites that are deposited on the microspores and comprise the outer strata of the pollen wall, the exine and tryphine layers. Alteration in tapetal cell function is often accompanied by male sterility, indicating the important role of this tissue in pollen development (Wilson and Zhang 2009). Enzymes catalyzing formation of pollen coat components are localized in tapetal cells, but their products are found on the surface of the pollen, as is the case for HCAAs. Thus, these compounds must be transported from the tapetum to the anther locule and then onto the pollen surface. It is widely accepted that sporopollenin-precursors are transported through the tapetum plasma membrane, because exine assembly takes place before tapetum degeneration. An ABC transporter in *A. thaliana* has recently been identified to be involved in this process (Choi *et al.*, 2011). In contrast, tryphine or pollenkitt constituents are released after tapetal cells undergo programmed cell death.

Tapetal cells of *Brassicaceae* contain two classes of specific storage organelles, elaioplasts and tapetosomes. The elaioplasts are plastids that accumulate sterol esters (Wu *et al.*, 1999), whereas the tapetosomes are derived from endoplasmic reticulum (ER) and show a structural similarity to seed oil bodies, which contain vast amounts of triglycerides, alkanes and flavonoids (Hsieh and Huang 2007). These organelles are partially degraded immediately

after the rupture of tapetal cells and become components of pollen coats. Whether HCAAs are also stored in these metabolic sinks has not been reported so far. It could be that HCAA biosynthesis is associated with ER membranes mediated by CYPs, as described for other branches of phenylpropanoid metabolism (Jørgensen *et al.,* 2005), and later transported to the ER-derived tapetosomes. This has been shown for flavonoids in *Brassica* (Hsieh and Huang 2007), the second abundant phenolic compounds in pollen tryphine beside HCAAs. A more detailed analysis of subcellular localization of HCAA biosynthetic enzymes and resulting HCAAs could allow the testing of these hypotheses.

3.2 Regulatory aspects of HCAA biosynthesis

The biosynthesis of HCAAs requires the action of two separate pathways, the phenylpropanoid and the PA pathway. Signaling compounds such as jasmonic acid and abcisic acid induce accumulation of HCAAs in barley leaves (Lee *et al.,* 1997; Ogura *et al.,* 2001). This known induction by oxylipins enabled the identification of transcription factors associated with HCAA biosynthesis. Using methyl jasmonate treated tobacco cell cultures, Gális *et al.,* (2006) identified an inducible MYB- transcription factor (NtMYBJS1) that positively regulates several phenylpropanoid-related genes and thereby the biosynthesis of feruloyl- and caffeoylputrescine. Both HCAAs possess defense-related functions against herbivores (Kaur *et al.,* 2010) and the responsible acyltranferases, whose expression is controlled by this MYB-transcription factor, have been characterized recently in *Nicotiana attenuata* (Onkokesung *et al.,* 2012). Similar studies by Shinya *et al.,* (2007) led to the identification of another MYB-transcription factor (NtMYBGR1), which upon overexpression in tobacco cell cultures, induces phenylpropanoid gene expression and HCAA formation. Both studies indicate a transcriptional regulation of HCAA biosynthesis mediated by MYB-transcription factors. MYB99 (*At5g62320*) is co-expressed with HCAA biosynthetic genes (SHT, CYP98A8 and AtTSM1) in *A. thaliana* anthers (Alves-Ferreira *et al.,* 2007) and was therefore investigated as a likely candidate to control HCAA biosynthesis in tapetal cells. However, corresponding knock out mutants showed no effect on HCCA formation (data not shown).

The present study indicates a tight regulation of HCAA parent compounds. Elimination of SHT leads to a dramatic decrease in HCAA biosynthesis. Nevertheless, blocking the acyl transfer to spermidine is not accompanied by increased PA levels in *A. thaliana* anthers (Fellenberg *et al.,* 2012b). Additionally, flavonoids, derived from the phenylpropanoid pathway, of pollen grains are unchanged in HCAA biosynthetic mutants (Fellenberg *et al.,* 2009). Hence, both pathways, HCAA and flavonoid biosynthesis, seem to act independently. The SHT knock out does not lead to repression of phenylpropanoid biosynthesis-associated genes. The expression of both phenylammonia lyases (PAL) and 4-coumarate coenzyme A ligases (4CL) are not significantly changed in *sht* anthers compared to wild type (Bachelor

thesis J. Grosche). Furthermore, no evidence has been found so far that HCA accumulation is influenced in HCAA deficient anthers. However, the mechanism regulating constant levels of the parent compounds in HCAA-deficient anthers is so far unknown. A transcriptomic approach, comparing gene expression of wild type and *sht* anthers or tapetal cells could give insight into regulatory networks of HCAA biosynthesis and could help to identify new genes involved in HCAA metabolism, catabolism or regulation.

3.3 HCAAs: widespread pollen constituents with currently unknown function

The presence of HCAAs in male floral organs has been reported for decades. Particularly bis- and tris-substituted putrescine and spermidine conjugates have been detected in anthers and pollen grains of several plant species (see Introduction), including wind-, insect- and self-pollinating plants. Remarkably, all those HCAAs show structural variability among the species with different substitution levels. Putrescine and more frequently spermidine are found conjugated to various HCAs like p-coumaroyl, caffeoyl, feruloyl, 5-hydroxyferuloyl and sinapoyl moieties. While the occurrence of HCAAs is intensively studied, their biological function in male reproductive organs is still obscure. The identification of HCAAs in anthers and pollen grains of the model plant *A. thaliana* and the isolation of knock out mutants showing strong reduction of HCAAs provide important tools to investigate their role in pollen development and reproduction.

3.3.1 HCAAs for pollen fertility and functionality

Since HCAAs were found in male and female floral organs, they are primary assumed to be linked to plant reproduction (Martin-Tanguy 1985). For instance, in maize, inhibition of HCAA accumulation causes male sterility due to formation of non-viable pollen (Martin-Tanguy 1997). Additionally, tobacco plants transformed with the Ri TL-DNA of *Agrobacterium rhizogenes* exhibit an inhibited HCAA formation which is correlated with male sterility (Sun *et al.*, 1991). First evidence for a similar phenotype in *A. thaliana* was described for AtTSM1 RNAi suppressed plants (Fellenberg *et al.*, 2008). These transgenic lines show a strong reduction of N^1,N^{10}-bis-(5-hydroxyferuloyl)-N^5sinapoylspermidine, the major pollen HCAA, which is accompanied by partially impaired seed production.

However, detailed phenotypic analysis of *sht* and *sht sdt* pollen grains presented here did not further confirm this presumed function of HCAAs. The progressive reduction of pollen HCAAs induced by the knock out of both key biosynthetic transferases SHT and SDT did not affect plant or pollen fertility. *Sht* and *sht sdt* mutant plants showed no decline in seed generation, and pollen grains are able to develop pollen tubes both *in vitro* and *in vivo* with the same frequency and efficiency as wild type pollen. Thus HCAAs does not seem to be essential

for sexual reproduction in *A. thaliana*. The impaired seed production in AtTSM1 knock down plants could be attributed to abnormal pollen development due to unspecific RNA interference effects, which occurs in up to 10% of transgenic *A. thaliana* plants (Xing and Zachgo 2007) and may not be due to changed HCAA accumulation.

Moreover, an indirect impact of HCAAs on pollen tube growth by regulating the pool of parent compounds to provide a reservoir of spermidine is not obvious. PAs, especially spermidine, participate in pollen tube elongation in *A. thaliana* by increasing cytosolic Ca^{2+} concentration, the central second messenger modulating pollen tube growth (Wu *et al.*, 2010). Hence, pollen HCAAs could serve as a spermidine reservoir which is released during pollen tube growth. Similar storage functions have been proposed for HCAAs in rice seeds (Bonneau *et al.*, 1994). Nevertheless, degradation of HCAAs during pollen germination could not be observed in *A. thaliana*. HCAAs seem to be stable during the process of pollen germination. Four hours after pollination, the amount of the major HCAAs is comparable to the amount directly after pollination. Thus, pollen HCAAs are probably not responsible for regulating the pool of parent compounds or providing a reservoir of spermidine. Constant PA levels in *A. thaliana* wild type and *sht* anthers also indicate that production of HCAAs is not the only mechanism for PA homeostasis if it participates at all. In summary, there is no evidence so far that HCAAs on the pollen surface of *A. thaliana* influence pollen fertility or plant fecundity and reproduction, at least under standardized greenhouse conditions.

What other function might HCCAs on the pollen surface serve? HCAAs are components of the tryphine, which fulfills diverse functions. One is to mediate pollen adhesion to the anther until dispersal, to keep pollen grains together during transport and to facilitate adhesion to the stigma or to insect bodies (Pacini and Hesse 2005). Lipophilic molecules found in the pollen exine mediate pollen-stigma adhesion and therefore pollen capture (Zinkl *et al.*, 1999). Whether HCAAs can influence pollen adhesiveness is unknown so far. Results presented here showed no significant changes in pollen adhesion to the sigma using a liquid assay and also did not show any improved or reduced adhesive properties compared to mutant lines using atomic force microscopy studies (Matthias Menzel, Frauenhofer Institute, Mechanics of Material, Halle, pers. Communication). In both cases, high variability of this material and experimental design may mask minor differences. On average, wild type and HCAA deficient pollen grains revealed similar adhesion capabilities (page 57).

3.3.2 HCAAs in abiotic/biotic stresses

Environmental stress arises from conditions that are unfavorable for optimal growth and development. Plant sexual reproduction has long been recognized as being highly stress-sensitive (Zinn *et al.*, 2010). From the time after anthesis, releasing the pollen grains, until pollination, the gametophyte needs protection against various stresses. Plants deploy various

protection strategies for the pollen like accumulation of polysaccharides to reduce desiccation (Nepi *et al.,* 2001), UV protectants against radiation and rigid pollen walls to avoid pathogen invasion.

Young *et al.,* (2004) were able to show that high temperature stress negatively effects reproductive development in *Brassica napus*. In this model, temperature stress causes decreased pollen viability as well as reduced *in vitro* pollen germination and therefore disrupts seed production. The same holds true for maize where *in vitro* fertilization is sensitive to heat shock with a threshold temperature between 36 and 40°C (Dupuis and Dumas 1990). Stress conditions, like chilling, freezing and heat have in common that they cause a secondary stress, which is water stress. How can pollen grains protect themselves from these stressors? The lipid pollen coat protects the mature pollen grains from desiccation after anther dehiscence (Dickinson *et al.,* 2000). However, the presence or absence of hydrophobic HCAAs on the pollen coat of *A. thaliana* showed no effect on *in vitro* pollen germination efficiency upon heat or freezing stress (page 60).

HCCAs are often discussed as UV protectants based on the UV-absorbing properties of the cinnamic acid moieties (Bienz *et al.,* 2005; Liu 2010). Analysis of photochemical properties of HCAAs revealed a high sensitivity to light exposure and a UV-induced (E/Z) photoisomerisation of C=C bonds (Youhnovski *et al.,* 1998). Due to their UV-absorbing properties, HCAAs may protect the pollen grain and its genetic material from damage by UV light. However, no direct evidence for participation of HCAAs in protection of *A. thaliana* pollen grains was found in this work (page 59). Reproduction efficiency of HCAA deficient plants was unaffected compared to wild type under elevated UV light. Both wild type and *sht* plants developed normal seed sets after UV-B exposure. Additionally, the expression of HCAA biosynthetic genes was not changed in either genotype. For *A. thaliana*, it was shown that one of the molecular responses to UV exposure is the induction of genes encoding metabolic functions of flavonoid related biosynthetic enzymes (Wolf *et al.,* 2010) and that these UV-protectants increase after UV exposure (Lois 1994). This is not the case for pollen HCAAs in *A. thaliana*. However this assay was performed in growth chambers where plants were allowed to self-pollinate. This process, from dehiscence of the anther to pollination is relatively short in *A. thaliana*, thus it might be possible that UV-doses were too low to damage the gametophyte. Nevertheless, an induction of DNA repair pathways, particularly including photolyases, was also not observed after exposing collected pollen to UV-B radiation *in vitro* (data not shown). Taking together, the described examples suggest that HCAAs do not influence pollen germination ability upon temperature or UV stress. However, unchanged germination of pollen grains is not necessarily associated with successful fertilization and fruit production, although often a correlation with fruit set has been observed (Dafni and Firmage

2000). Pollen germination is controlled by the vegetative nucleus and may occur in the absence of a viable generative nucleus (Sedgley and Griffin 1989).

Naturally occurring PAs and HCAs possess chelating ability for transition metal ions (Korkina 2007; Nezbedova *et al.*, 2001). Thus it might be possible that HCAAs also have similar properties. Pollen germination, fertilization and therefore plant reproduction is a vulnerable process, which is also effected by heavy metals. These have inhibitory effects on pollen germination (Sabrine *et al.*, 2010; Sawidis and Reiss 1995) and can cause ultrastructure changes like swelling of mitochondria and cytoplasmic vacuolization (Speranza *et al.*, 2007). Hence, HCAAs could combine chelating properties of parent compounds to protect the pollen grain from heavy metal stress. To test this assumption wild type and mutant pollen germination could be studied *in vitro* with medium containing various metal ions. However, considering widespread pollen HCAAs as protectants against heavy metals, caused by air pollution, which arose during the last decades, seems to be unlikely.

The often described antimicrobial activities of plant HCAAs were not studied extensively during this work. The main pollen HCAAs of *A. thaliana* pollen grains are water insoluble which makes liquid growth inhibition assays difficult. Overcoming this insolubility problem, possible fungicidal activity of HCAAs were assayed by the method of Gottstein *et al.*, (1984) and performed in collaboration with Norbert Arnold (IPB). Extracts of wild type and *sht* pollen were spotted on a thin layer silica plate and sprayed with a suspension of the phytopathogen *Cladosporium cucumerinum*. After incubation in a humid chamber the plate was overgrown with a dark colored mycelium. However, neither extract showed white spots (inhibition zones), indicating that methanolic extracts of *A. thaliana* pollen grains do not contain antifungal activity against this pathogen (data not shown).

3.3.3 HCAAs: New components of the pollen wall

When plants colonized the terrestrial environment, they had to protect themselves against various stresses. One strategy to adapt to environmental influences is the biosynthesis of polymers like lignin, suberin, cutin or sporopollenin to provide physical strength, barriers limiting water or nutrient loss and invasion by pathogens. Lignin is a complex heteropolymer consisting of phenylpropane subunits (Bonawitz and Chapple 2010) while hydrophobic cutin and suberin are polymers of fatty acids derivatives , glycerol and phenolics linked by ether and carbon-carbon bonds (Beisson *et al.*, 2012). The chemically resistant and rigid outer pollen wall, the exine, is mainly composed of sporopollenin, an extremely stable polymer containing derivatives of aliphatic compounds, such as fatty acids and phenolics (Ariizumi and Toriyama 2011).

It was shown recently that some of the 38 different HCAAs found in the tryphine of *A. thaliana* are incorporated to the pollen wall polymer by ester bonds (Handrick *et al*., manuscript in preparation). After alkaline hydrolysis of the cell wall, N^1,N^5-dicaffeoyl- and N^1,N^5- diferuloyl spermidine were identified only in wild type pollen. Those covalently linked HCAAs are virtually absent in *sht* pollen suggesting that the BAHD-like acyltransferase SHT could provide precursors for pollen wall linked HCAAs. The first evidence for the presence of HCAAs in the pollen exine surface was found in *Corylus* pollen (Gubatz *et al.,* 1986). The occurrence of nitrogen containing compounds as a possible component of the sporopollenin is rather unexpected, since nitrogen is assumed to be absent from the pollen wall (Bubert *et al.,* 2002). The hydroxyl groups of HCAAs could be covalently linked to hydroxy groups of fatty acids, forming ether bonds, in agreement with the description of sporopollenin as a complex polyether with a high degree of cross-linking (Bubert *et al.,* 2002; Domínguez *et al.,* 1999). Hence, HCAAs as components of the pollen wall may increase the cross-linkage of sporopollenin subunits, by providing hydroxygroups as potential linkers.

Several genes have been identified as being involved in the biosynthesis of exine components, such as MS2, CYP704B1, NEF1 and RPG1 (Aarts *et al.,* 1997; Ariizumi *et al.,* 2004; Dobritsa *et al.,* 2009; Guan *et al.,* 2008) elimination of which results in disrupted exine formation. Changes in or inhibition of exine formation is usually accompanied by male sterility which is not the case in *A. thaliana* HCAA-deficient pollen grains, suggesting that HCAAs are not absolutely required for functional exine formation but might support the rigidity of the pollen wall. As shown in Figure 3-3, s*ht* pollen display irregularities and depressions relative to wild type by scanning electron microscopy (Grienenberger *et al.,* 2009). If these morphological changes of the pollen grains reflect the *in planta* situation or if the collapse is due to vacuum applied during the preparation of the pollen grains for microscopy is inconclusive, but it clearly indicates some instability in *sht* deficient pollen grains. Atomic force microscopic analysis is currently in progress to further confirm the relation of covalently linked HCAAs to the pollen wall and its stiffness.

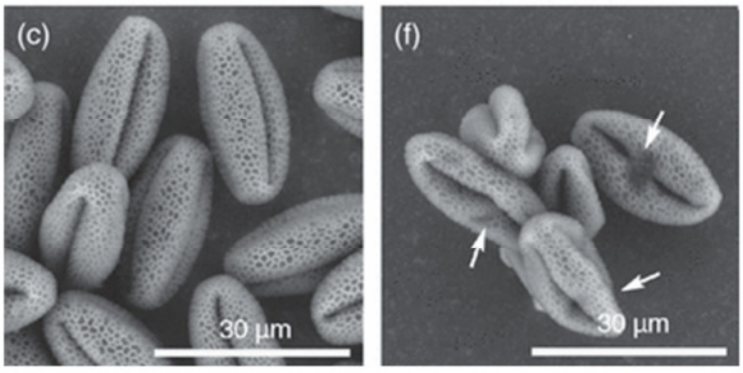

Figure 3-3: Scanning electron micrograph of *A. thaliana* wild type (c) and *sht* (f) pollen (Grienenberger *et al.,* 2009). Arrows indicate irregularities of the pollen wall only visible in mutant pollen.

How sporopollenin precursors polymerize and how HCAAs are incorporated into the cell wall are poorly understood processes. Peroxidases are suggested to be involved in the cross-linking of HCAAs with cell wall components (Kristensen *et al.,* 2004). Bernards *et al.,* (1999) identified a suberization-associated anionic peroxidase of potato with strong preference for HCAAs of tyramine, suggesting that this enzyme mediates polymerization of HCAAs in the phenolic domain of suberin. In lignin formation, a comparable polymerization process occurs, in which peroxidases and/or laccases have been considered as likely candidates for the activation of monolignols (Bonawitz and Chapple 2010). Laccases comprise a multi-gene family in plants which has seventeen members in *A. thaliana* (Turlapati *et al.,* 2011). Recently two laccases have been implicated in lignification. Knock out mutation of LAC15 leads to decreased lignin content in seeds (Liang *et al.,* 2006) while LAC4 has been shown to be a target of MYB58, a transcriptional activator of lignin biosynthesis (Zhou *et al.,* 2009). Additionally, a laccase-like polyphenol oxidase, transparent testa 10, has been shown to be involved in oxidative polymerization of flavonoids in the seed coat of *A. thaliana* (Pourcel *et al.,* 2005). Laccases possibly involved in sporopollenin formation or HCAA incorporation into this polymer could include LAC8, since this gene is strongly expressed in pollen grains (Turlapati *et al.,* 2011), as well as LAC1 and LAC5, whose expression is changed in male sterility 1 plants (MS1). MS1 is a transcriptional regulator critical for exine formation (Alves-Ferreira *et al.,* 2007; Yang *et al.,* 2007). Analysis and characterization of the corresponding proteins and knock out mutants could provide further insight into the sporopollenin polymerization process, the requirement of HCAAs for pollen wall stability and shed some light on the significance and impact of HCAA insertion into the pollen wall of *A. thaliana*. This could provide conclusive evidence for an anticipated, yet not sufficiently characterized function of these universal pollen metabolites.

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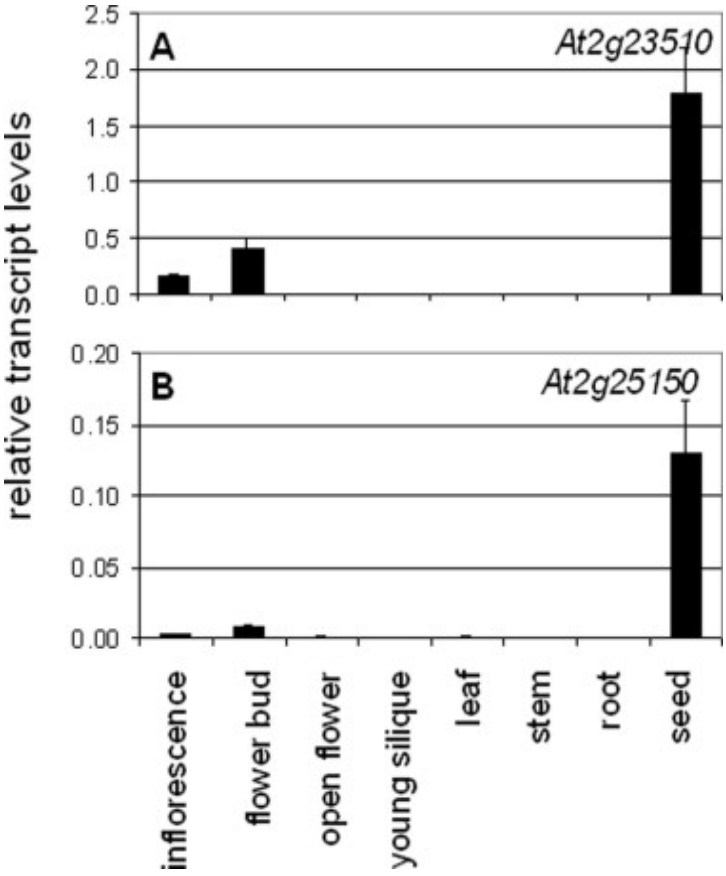
5 Appendix

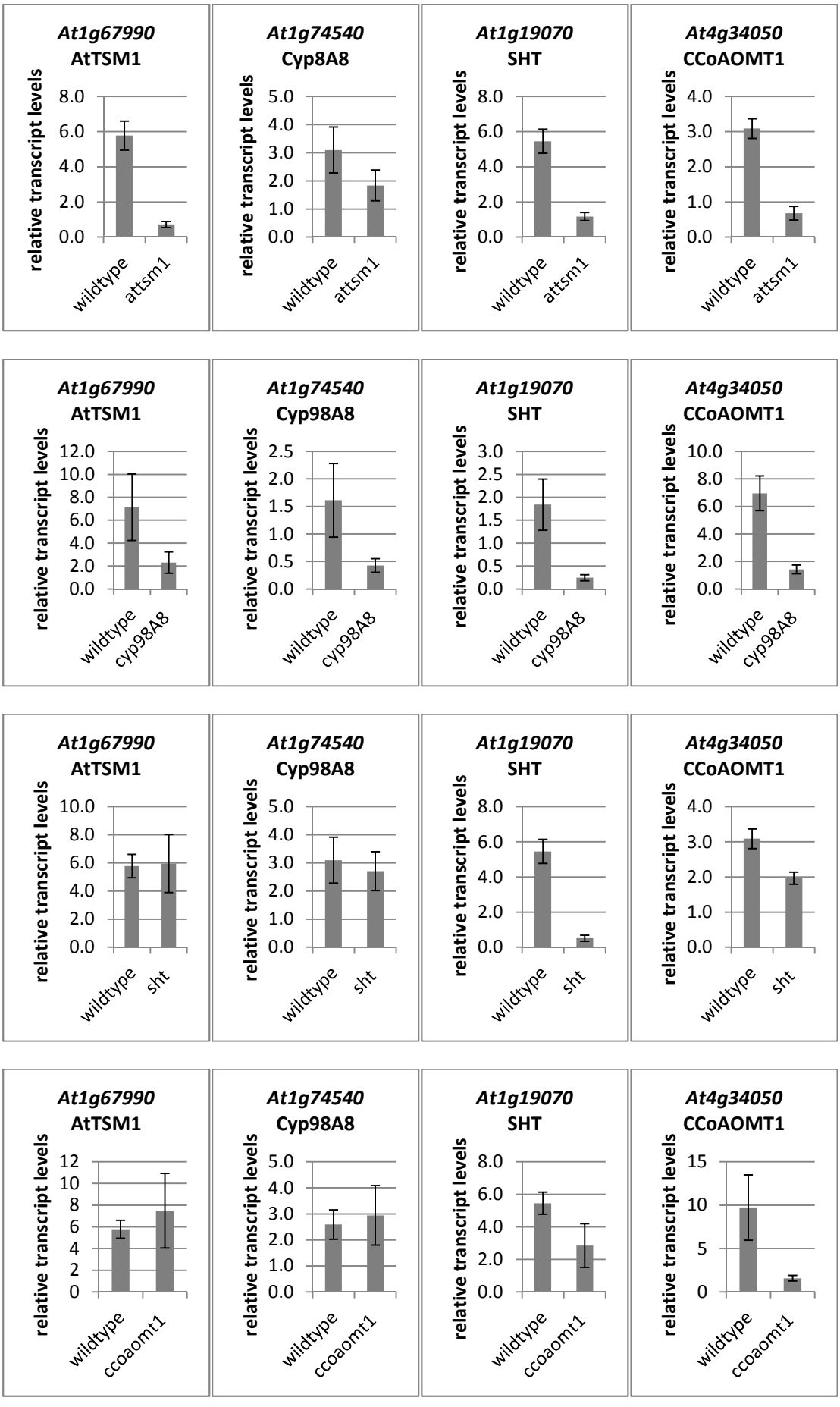
5.1 Supporting information to 2.2.2

Suppl. Data 1: Relative quantification of compounds 1-28 in flowers of *HCA-transferase* KO, *cyp98A8* KO and corresponding wildtype lines using UPLC/ESI(+)-QTOF-MS. Red and purple indicate compounds which accumulate preferentially in wildtype plants whereas green denotes spermidine conjugates or phenylpropanoids which show enhanced amounts in the knockout lines.

no.	ret. time	quantifier	mean peak area ± sd [cps] (N=6)				fold change				*P* (Student´s t-test)	
	[s]	*m/z*	*sht* KO	wt (*SHT*)	*cyp98A8* KO	wt (CYP98A8)	wt / *sht*	*sht* / wt	wt / *cyp98A8*	*cyp98A8*/wt	wt - *sht*	wt - *cyp98A8*
1	288	438.24	n.d.	174500 ± 24000	159100 ± 23300	249900 ± 50900	-	-	1.57	0.64	1E-17	1E-05
2	284/294	468.25	n.d.	109500 ± 19100	170500 ± 29400	97500 ± 15000	-	-	0.57	1.75	1E-15	2E-07
3	290/300	498.26	n.d.	429700 ± 92700	408900 ± 78100	407900 ± 65000	-	-	1.00	1.00	1E-13	9E-01
4	270	514.25	33700 ± 6100	85000 ± 17500	n.d.	85700 ± 15400	2.52	0.40	-	-	3E-09	1E-14
5	293	528.27	63900 ± 7090	42960 ± 5648	56500 ± 7700	41200 ± 5800	0.67	1.49	1.37	0.73	6E-08	4E-07
6	271	544.27	15400 ± 3300	44300 ± 9000	n.d.	69100 ± 14500	2.88	0.35	-	-	6E-10	1E-14
7	241	600.29	n.d.	8800 ± 2517	20787 ± 5400	12100 ± 2300	-	-	0.58	1.72	4E-11	9E-05
8	246	630.30	n.d.	3300 ± 1500	6400 ± 1800	2900 ± 500	-	-	0.45	2.21	8E-08	6E-06
9	240/250	660.31	n.d.	20500± 8200	17500 ± 5000	18900 ± 3900	-	-	1.08	0.93	2E-08	4E-01
10	398	584.27	n.d.	n.d.	26900 ± 3800	n.d.	-	-	-	-	-	2E-16
11	404	614.29	n.d.	n.d.	67800 ± 8000	n.d.	-	-	-	-	-	5.E-18
12	410	644.30	n.d.	2200 ± 280	272400 ± 32800	1800 ± 300	-	-	0.01	151	2E-18	7E-18
13	406/416	674.31	n.d.	38400 ± 7800	2686100 ± 348300	34200 ± 4022	-	-	0.01	78.5	4E-14	4E-17
14	390	690.30	n.d.	43400 ± 8700	n.d.	37900 ± 5100	-	-	-	-	3E-14	6E-17
15	365	706.30	2280 ± 600	139900 ± 21500	2400 ± 370	123400 ± 11500	61.4	0.02	51.4	0.02	2E-16	2E-20
16	387	720.3	n.d.	11000 ± 2200	n.d.	11800 ± 1300	-	-	-	-	3E-14	5E-19
17	343	722.29	2800 ± 650	119300 ± 35700	2600 ± 400	86400 ± 16700	42.6	0.02	33.2	-	1E-10	1E-13
18	365	736.31	16300 ± 3800	1337600 ± 230000	20200 ± 1900	1377300 ± 145600	82.1	0.01	68.2	0.01	1E-15	3E-19
19	386/399	750.32	n.d.	26600 ± 5900	n.d.	301300 ± 3200	-	-	-	-	2E-13	4E-20
20	354	836.36	n.d.	n.d.	4500 ± 1100	n.d.	-	-	-	-	-	9E-12
21	308	998.40	n.d.	n.d.	2100 ± 600	n.d.	-	-	-	-	-	1E-10
22	73	515.13	14400 ± 2000	2700 ± 800	3300 ± 400	3600 ± 600	0.19	5.33	1.09	0.92	7E-15	5E-2
23	109	353.09	34600 ± 3300	13100 ± 2800	17900 ± 1200	18300 ± 3000	0.38	2.64	1.02	0.98	3E-14	2E-01
24	202	377.08	27400 ± 4500	13600 ± 3200	10700 ± 2000	12600 ± 2600	0.50	2.01	1.18	0.85	2E-08	9E-02
25	218	379.10	22200 ± 4800	7300 ± 700	6100 ± 800	7000 ± 800	0.33	3.04	1.15	0.87	3E-10	6E-02
26	284	333.06	7800 ± 1700	n.d.	n.d.	n.d.	-	-	-	-	3E-13	-
27	181	465.10	11200 ± 2100	3000 ± 600	3600 ± 400	4000 ± 700	0.27	3.73	1.11	0.91	5E-12	6E-2
28	191	449.10	27600 ± 1100	7500 ± 2000	8300 ± 1000	9700 ± 1200	0.27	3.68	1.17	0.86	2E-06	1E-03



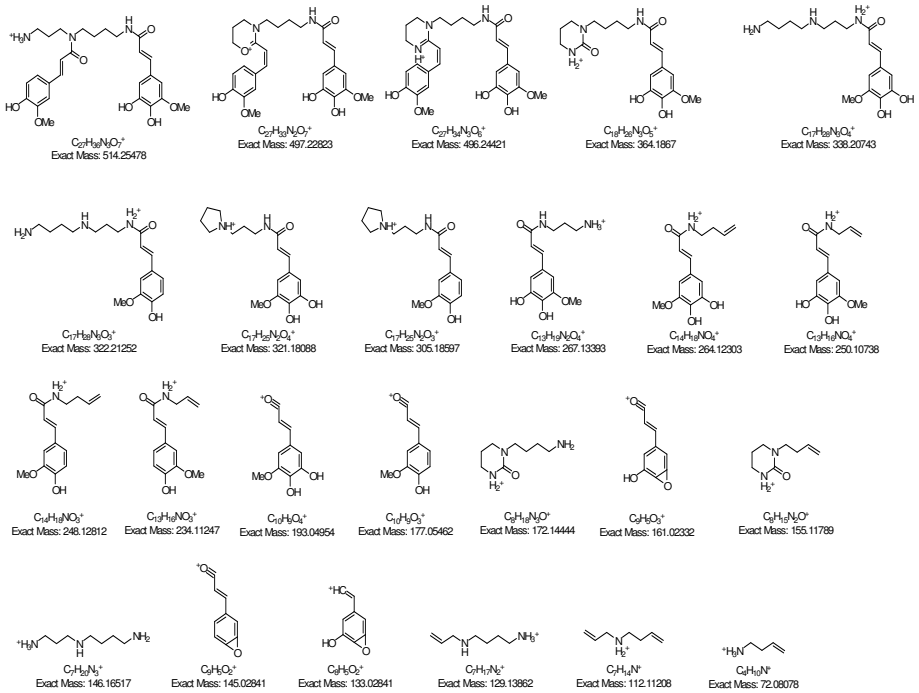
Suppl. Data 2: Organ specific transcription profile of two BAHD acyltransferases in *A. thaliana*. A, *At2g23510* encoding SDT and B, *At2g25150* encoding SCT.


At1g67990
AtTSM1
relative transcript levels
wildtype
attsm1
At1g74540
Cyp8A8
At1g19070
SHT
At4g34050
CCoAOMT1
At1g67990
AtTSM1
wildtype
cyp98A8
At1g74540
Cyp98A8
At1g19070
SHT
At4g34050
CCoAOMT1
At1g67990
AtTSM1
wildtype
sht
At1g74540
Cyp98A8
At1g19070
SHT
At4g34050
CCoAOMT1
At1g67990
AtTSM1
wildtype
ccoaomt1
At1g74540
Cyp98A8
At1g19070
SHT
At4g34050
CCoAOMT1

Suppl. Data 3: Transcriptional pattern of selective genes proposed to be involved in polyamine conjugate biosynthesis in *A. thaliana* RNAi and knockout lines. Effects of these knockouts on transcript levels in flower buds are shown.

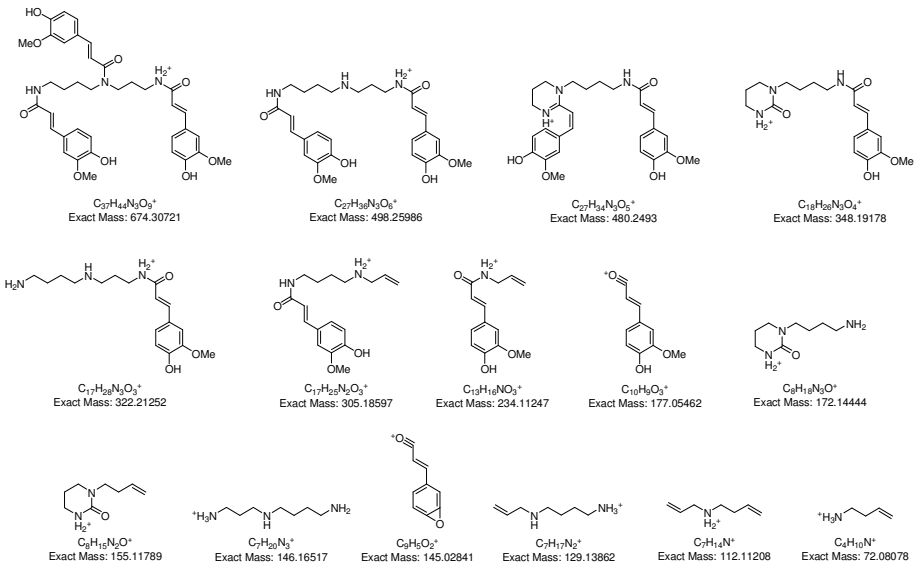
Suppl. Data 4:

***N*-(Feruloyl)-*N´*-(5-hydroxyferuloyl)spermidine (4).** *UPLC/ESI(+)-QTOF-MS*, t_r = 271 s, *m/z* (rel. int. (%)) = 514.2544 $[M+H]^+$. *UPLC/ESI(+)-CID-MS* of *m/z* 514, CE = 40 eV, *m/z* (rel. int. (%)) = 514.252 (9), 497.220 (2), 496.248 (2), 364.190 (1), 338.106 (15), 322.213 (30), 321.178 (9), 305.182 (8), 267.130 (5), 264.120 (1), 250.105 (100), 248.125 (12), 234.111 (19), 193.050 (48), 177.055 (62), 172.143 (3), 161.024 (10), 155.119 (4), 146.164 (5), 145.028 (13), 133.027 (3), 129.140 (3), 112.111 (2), 72.081 (2).



Elemental compositions, mass-to-charge ratios and putative molecular structures of fragment ions generated upon CID of [**4**+H]$^+$.

***N*,*N´*,*N´´*-Trisferuloylspermidine (13)**. *UPLC/ESI(+)-QTOF-MS*, t_r = 406/416 min, *m/z* (rel. int. (%)) = 712.262 (1) $[M+K]^+$, 696.290 (18) $[M+Na]^+$, 693.284 (6) $[2M+H+K]^{2+}$, 674.306 (100) $[M+H]^+$, 498.257 (17), 356.633 (14) $[M+H+K]^{2+}$, 348.645 (3) $[M+H+Na]^{2+}$, 480.249 (7), 322.212 (3), 305.185 (1), 234.112 (1), 177.055 (6), 145.028 (2). *UPLC/ESI(+)-CID-MS* of *m/z* 674, CE = 40 eV, *m/z* (rel. int. (%)) = 674.305 (1), 498.257 (100), 480.251 (13), 348.193 (1), 322.215 (8), 305.187 (11), 234.111 (14), 177.055 (22), 172.144 (1), 146.166 (1), 145.028 (2), 129.140 (1), 112.112 (1), 72.081 (1).



Elemental compositions, mass-to-charge ratios and putative molecular structures of fragment ions generated upon CID of [**13**+H]$^+$.

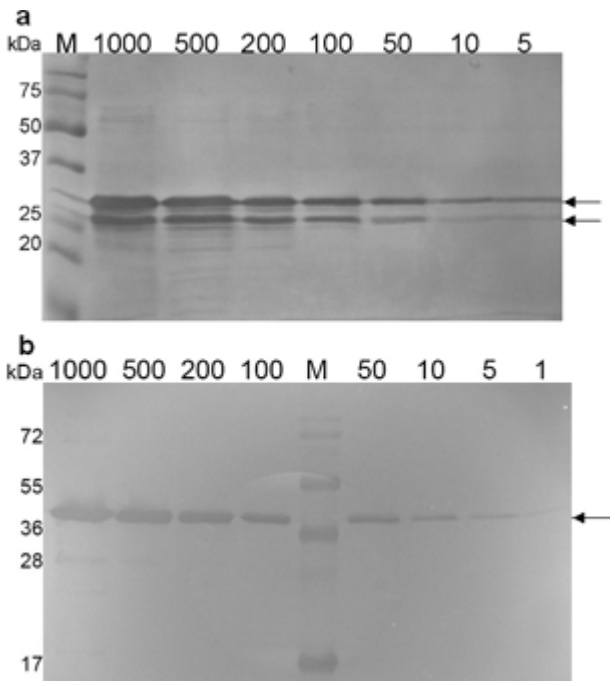
5.2 Supporting information to 2.3.2

Suppl. Data 1: Gene specific primers used in RT-qPCR analysis of *Arabidopsis* genes

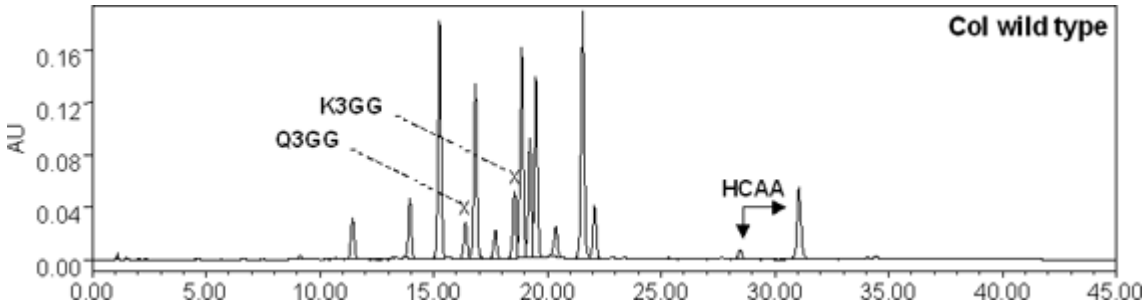
Gen	Primer
At4g34050 for At4g34050 rev	5'-CAACGAAGACATCATCGAC-3' 5'-TGCTGTCACTTCCCTGAG-3'
At4g54160 for At4g54160 rev	5'-TGGTGGCATTGGTGCTACACTC-3' 5'-GAGAAGGAGCATCTTCGATGAC-3'
At1g67990 for At1g67990 rev	5'-GATGGTCGATTACCTGAC-3' 5'-CTTTCATCAACCGGAACTC-3'
At1g67980 for At1g67980 rev	5'-CGAGGTCTCTCAGATTTCCATTG-3' 5'-TCCGACTTTGGTTCTGCTACG-3'
At1g61990 for At1g61990 rev	5'-AGACAAGGTTCACTCAATCCGTG-3' 5'-CATTCAGGACCAAACTCTTCAGC-3'
At1g62000 for At1g62000 rev	5'-GACTCATCTACTTGGGATGGTAAC-3' 5'-CAAAACTCTATTTCCACTAATCAGA-3'
At4g26220 for At4g26220 rev	5'-AGGTTGGTGGGATCATAGTG-3' 5'-GACTTCTATCCTCCACTCGG-3'
At1g24735 for At1g24735 rev	5'-ATGGTTTGTGCTTGTATG-3' 5'-CTATTCCAGTAACATGTC-3'

Suppl. Data 2: Annotation of flavonoid-glycosides in *Arabidopsis* wild type and *comt1* flowers and pollen. *M/z* data obtained by ESI-Q Trap MS/MS.

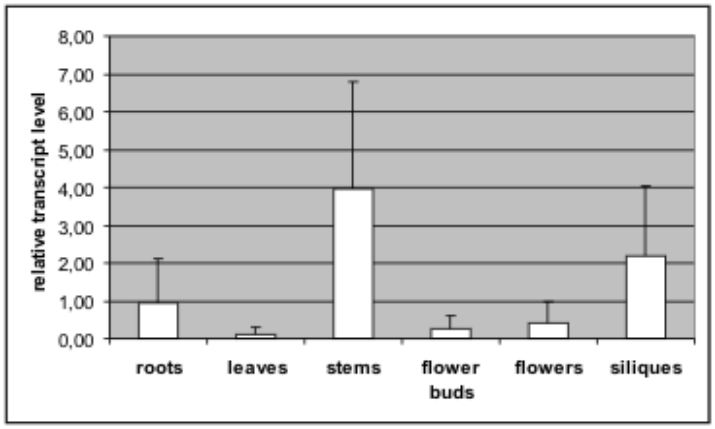
Annotation	Flavonoid-glycoside	$[M+H]^+$ *m/z*	significant fragments *m/z*
standard	Quercetin	303.1	-
standard	Isorhamnetin	317.0	302.0
comt1 - up	Que-hex-rham	611.2	449.0 303.1
comt1 - up	Que-rham-rham	595.2	449.0 303.2
comt1 – missing	Isorham-hex-rham	625.2	478.9 463.1 317.0
comt1 - missing	Isorham-rham-rham	609.1	463.1 317.3
pollen	Que-3-O-diglucoside	627.1	449.0 303.1
pollen	Kae-3-O-diglucoside	611.1	423.1 287.1



Suppl. Data 3: Sensitivity of polyclonal anti-CCoAOMT1 and anti-COMT1 antibodies based on serial dilutions of purified recombinant proteins from 1000 to 1 ng. CCoAOMT1 (a) from *E. coli*; COMT1 (b) from *E. coli*. Please note that CCoAOMT1 (~ 30 kDa) is partially cleaved in *E. coli* by an unknown protease (2nd band around 27 kDa).



Suppl. Data 4: HPLC chromatogram of *Arabidopsis* whole flower bud methanolic extracts at 350 nm. Both HCAAs and the two pollen specific flavonol diglycosides are marked.



Suppl. Data 5: Quantitative real time PCR-data of relative transcript levels of the *At4g26220* gene in different *Arabidopsis* organs of flowering eight week old plants.

5.3 Supporting information to 2.4.2

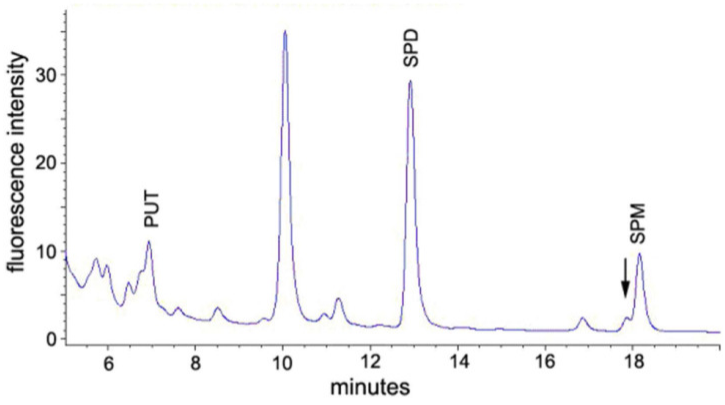


FIGURE A1 | *A. thaliana* stem extract derivatized with FMOC-Cl. The pattern of elution is identical to the flower bud extract (**Figure 2**). The large unmarked peak is an derivatization artifact. Put, putrescine; spd, spermidine; spm, spermine. Thermospermine most likely elutes as a left shoulder of spermidine. Notice the different scale compared to **Figure 2**.

5.4 Supporting information to 2.5

Material and Methods

Plant material

Wild type *A. thaliana* (Columbia 1092), and all knock out mutants (SALK_055511C for the *At2g19070* gene encoding SHT; SM3.38374 for the *At2g23510* gene encoding SDT and SALK_120466 for the *At2g25150* gene encoding SCT) were obtained and propagated from the European *A. thaliana* Stock Center (Alonso *et al.,* 2003; Sessions *et al.,* 2002). T-DNA insertions were confirmed by DNA amplification using the left T-DNA border specific primer LBa1 (5′-TGGTTCACGTAGTGGGCCATCG-3′) for SALK lines and SMsp (5′-TACGAATAAGAGCGTCCATTTTAGAGT-3′) for SM lines and the gene specific primer as follows: SDT-for (5′-GAAACTACAATGTCAAACCGT-3′) for the SM3.38374 line and SCT-rev (5′-CGCTGGGTCAATGGTCG-3′) for the SALK_120466 line; SALK_055511C was isolated and published previously (Fellenberg *et al.,* 2009). The *sht sdt* and *sht sct* double mutant lines were isolated from a F2 population of a *sht* and *sdt* or *sht* and *sct* cross.

Real time PCR

For transcript analysis *A. thaliana* Columbia 1092 wild type organs were harvested and analyzed according to Fellenberg et al. (2012). Briefly, RNA was extracted by a standard phenol/chloroform protocol, reverse transcribed using Superscript® reverse transcriptase (Invitrogen), and qPCR was performed using SYBR®-green qPCR mastermix (Applied Biosystems). The small subunit of phosphatase 2A served as a reference gene. Expression levels were recorded for the genes AtTSM1, SHT as described previously (Fellenberg *et al.,* 2009) SDT (forward 5′-TATTGGGATTTTCGGATCG-3′; reverse 5′-CCATATCCGATTCCAGCCTAGA-3′) and SCT (forward 5′-CTTGAAAAGAAACCAGTTGAGC-3′; reverse 5′-TTGCCCGAAAGAGGGTAGTAA-3′).

Pollen harvest and storage

Pollen was collected from 5 to 6 weeks old plants using a modified vacuum cleaner as described by Johnson-Brousseau and McCormick 2004. Two different nylon meshes with a pore size of 40 μm and 11 μm (neoLab, Heidelberg, Germany) were fixed in sequence on a plastic pipe and connected to a vacuum cleaner (Bosch, Gerlingen, Germany). Pollen was harvested by passing the pipe across the flowers. Pollen was removed from the 11 μm mesh and used directly or stored in 1.5 ml tubes at -80°C.

LC-MS/MS analyses of pollen grains

Pollen grains were extracted in 25 μl 80% (v/v) methanol and analysed as described in Handrick *et al*., (2010).

Pollen viability test

Pollen viability was evaluated using fluorescein diacetate (FDA) which was dissolved in acetone at 5 mg ml^{-1} stored at -20°C and propidium iodide (PI) which was dissolved in water at 1 mg ml^{-1} stored at 4°C. Freshly harvested pollen was suspended in 10% sucrose and dyes were diluted to a final concentration of 0.1 mg ml^{-1} (FDA) and 10 μg mL^{-1} (PI). Observations were made with an epifluorescence microscope AxioImager (Zeiss, Jena, Germany).

***In vitro* and *in vivo* pollen germination**

Vacuum cleaned pollen was germinated on medium according to Li et al. (Li *et al.,* 1999) containing 18% sucrose, 0.01% boric acid, 1mM $MgSO_4$, 1mM $CaCl_2$, 1 mM $Ca(NO_3)_2$ and 0.8 % low melt agar, pH 7.0. The total and germinated pollen grains were counted and pollen tube length was measured under a microscope after incubation for 12 hours in a humid chamber at room temperature.

Unpollinated pistils were prepared from *A. thaliana* flower buds at stage 10 or 11 (Smyth *et al.,* 1990) and grown over night in a phyto chamber for completely development of sticmatic papillae. Pistils were pollinated by either brushing wild type or mutant dehiscent anthers over the papillae. Callose staining of the pollen tubes was performed according to (Preuss *et al.,* 1993).

Pollen adhesion assay

Unpollinated pistils were prepared from *A. thaliana* flower buds at stage 10 or 11 (Smyth *et al.,* 1990) and grown over night in a phyto chamber for completely development of stigmatic papillae. Stigma was saturated with vacuum cleaned pollen and pollinated for 15 minutes. Pollinated pistils were than washed in phosphate buffer [50 mM KPi, pH7.4, 1 % (v/v) Tween 20], mixed for 5 seconds and centrifuged for 5 min at 7200 g. Afterwards pistils were fixed for 2h in 90 % (v/v) ethanol and 10 % (v/v) acetic acid and furthermore stained with 5 mg ml-1 3, 3'-diethyloxacarbocyanine iodide ($DiOC_2$). Adhered pollen grains were counted with bright field illumination using an AxioImager (Zeiss).

HCAA stability

Unpollinated pistils were prepared from Arabidopsis thaliana flower buds at stage 10 or 11 (Smyth *et al.,* 1990) and grown over night in a phyto chamber for completely development of stigmatic papillae. Pistils were pollinated by either brushing wild type or mutant dehiscent anthers over the papillae. 10 unpollinated pistils or 10 pollinated pistils 0, 2 and 4 hours after pollination were prepared and pooled for further extraction. Plant material was homogenized using a CryoMill (Retsch, Haan, Germany) and extracted with 100 μl 80 % (v/v) methanol),

incubated for 10 min in a Sonorex Super RK 510 ultrasonic bath (Bandelin Electronic, Berlin, Germany) and centrifuged for 15 min at 4°C and 18,000g. Supernatant was analyzed by RP-HPLC on a 12.5 cm, 4 mm i.d., 5 µM Nucleosil C18-column (Macherey-Nagel, Düren, Germany) at a flow rate of 1 ml/min with a gradient from 5% (v/v) B (acetonitrile, 0.1% trifluoroacetic acid) in A (water, 0.1% trifluoroacetic acid) to 35% (v/v) B in A within 30 min.

UV-B exposure

A. thaliana plants were grown in the greenhouse for 3 weeks and then transferred to the sun simulator of the Research Unit Environmental Simulation at the Helmholtz Center (Munich, Germany). This sun simulator provides a natural photobiological environment using a combination of four lamp types (metal halide lamps, quartz halogen lamps, blue fluorescent tubes and UV-B fluorescent tubes) to obtain a natural balance of simulated global radiation throughout the ultraviolet to infrared spectrum. The cut-off of short-wave ultraviolet radiation was pbtained by appropriate soda-lime and acrylic glass filters (Döhring *et al.,* 1996; Thiel *et al.,* 1996). The climatic parameters were adjusted to a relative humidity of 70%, a temperature of 22°C during 16 hours of daylight, and a temperature of 18°C during night. The light intensity in the sun simulator was stepwise increased during a 7-day-period of acclimatization up to a mean value of PAR (photosynthetically active radiation, 400-700 nm) of 700 µmol m^{-2} s^{-1}. After this acclimatization in the absence of UV-B radiation (280-315 nm), the plants were treated by adding UV-B radiation of 1.5 W m^{-2}. This corresponds to a biologically effective UV-B radiation of 600 mW m^{-2} (calculated after Caldwell (1971) normalized at 300 nm). Control plants were grown under the same conditions in the absence of UV-B radiation. Plants were harvested after 19 days of UV-B treatment.

Tabel 5-1: Relative quantification of spermidine HCAAs in pollen methanolic extracts obtained from wild type, *sht*, *sct* and *sht sct A. thaliana* plants, n=3. Reduction is given in fold change compared to wild type.

ID	HCAA	Wt	*sht*		*sct*		*sht sct*	
		Peak area ± SD (counts)	Peak area ± SD (counts)	Reduction (fold)	Peak area ± SD (counts)	Reduction (fold)	Peak area ± SD (counts)	Reduction (fold)
1	N-Feruloyl-N′-(5-hydroxyferuloyl) spermidine-*O*-hexoside	1.19E+04 ± 1.0E+03	6.28E+03 ± 2.0E+02	1.9 [c]	1.13E+04 ± 7.3E+02	1.0	5.81E+03 ± 2.2E+02	2.0 [c]
2	N,N′-Bis-(*p*-coumaroyl) spermidine-*O*-hexoside	9.46E+03 ± 1.5E+02	b.d.l.	missing [a]	9.51E+03 ± 3.8E+02	1.0	b.d.l.	missing [a]
3	N-Feruloyl-N′-sinapoyl spermidine-*O*-hexodise	4.52E+03 ± 1.2E+02	9.68E+03 ± 1.1E+03	0.5 [c]	4.65E+03 ± 3.3E+02	1.0	8.87E+03 ± 5.3E+02	0.5 [c]
4	N-(5-hydroxyferuloyl)-N′-sinapoylspermidine-*O*-hexoside	2.15E+03 ± 1.9E+02	1.25E+03 ± 2.4E+02	1.7 [c]	2.85E+03 ± 3.5E+02	0.8	1.01E+03 ± 3.8E+01	2.1 [c]
5	N,N′-Bis-feruloylspermidine-*O*-hexoside	2.76E+04 ± 9.8E+02	5.28E+02 ± 6.9E+02	52.3 [c]	2.72E+04 ± 4.2E+03	1.0	6.59E+01 ± 5.0E+01	418.5 [c]
6	Unknown	2.92E+03 ± 5.2E+02	b.d.l.	missing [a]	2.97E+03 ± 5.4E+02	1.0	b.d.l.	missing [a]
7	N-Feruloyl-N′-(5-hydroxyferuloyl) spermidine	6.86E+04 ± 1.7E+03	4.57E+03 ± 1.0E+03	15.0 [c]	5.85E+04 ± 1.2E+04	1.2	3.64E+03 ± 5.4E+02	18.9 [c]
8	N-(5-hydroxyferuloyl)-N′-sinapoylspermidine	5.53E+04 ± 5.2E+03	3.07E+03 ± 2.7E+02	18.0 [c]	5.30E+04 ± 6.8E+03	1.0	2.47E+03 ± 1.8E+02	22.4 [c]
9	N-(5-hydroxyferuloyl)spermidine	7.45E+02 ± 3.4E+02	b.d.l.	missing [a]	8.60E+02 ± 2.6E+02	0.9	b.d.l.	missing [a]
10	N-Caffeoyl-N′-feruloylspermidine	8.04E+03 ± 9.5E+02	b.d.l.	missing [a]	8.83E+03 ± 9.2E+02	0.9	b.d.l.	missing [a]
11	N-(*p*-Coumaroyl)spermidine	9.76E+03 ± 7.7E+02	b.d.l.	missing [a]	9.98E+03 ± 1.2E+03	1.0	b.d.l.	missing [a]
12	N-(*p*-Coumaroyl)-N′-feruloyl spermidine	7.38E+04 ± 1.0E+04	b.d.l.	missing [a]	6.28E+04 ± 3.3E+03	1.2	b.d.l.	missing [a]

13	N,N′-Bis-(*p*-coumaroyl) spermidine	1.54E+05 ± 5.4E+03	b.q.l.	trace [b]	1.72E+05 ± 2.9E+04	0.9	b.q.l.	trace [b]
14	N,N′-Bis-sinapoylspermidine	1.54E+04 ± 5.8E+02	4.00E+03 ± 2.3E+02	3.9 [c]	1.71E+04 ± 5.4E+02	0.9	4.69E+03 ± 8.2E+02	3.3 [c]
15	N-Feruloylspermidine	1.79E+04 ± 5.1E+02	b.d.l.	missing [a]	1.50E+04 ± 9.2E+02	1.2	b.d.l.	missing [a]
16	N,N′-Bis-feruloylspermidine	3.84E+05 ± 4.9E+03	1.79E+03 ± 8.0E+02	214.9 [c]	3.46E+05 ± 2.6E+04	1.1	2.53E+03 ± 3.2E+02	151.7 [c]
17	N-Feruloyl-N′-sinapoyl spermidine	4.96E+04 ± 2.2E+03	7.32E+03 ± 1.2E+03	6.8 [c]	5.30E+04 ± 3.7E+03	0.9	1.03E+04 ± 1.3E+03	4.8 [c]
18	Unknown	3.25E+02 ± 9.6E+01	b.d.l.	missing [a]	2.88E+02 ± 1.6E+02	1.1	b.d.l.	missing [a]
19	N-Caffeoyl-N′-(5-hydroxyferuloyl) spermidine	1.67E+04 ± 2.2E+03	b.q.l.	trace [b]	1.45E+04 ± 1.3E+03	1.2	b.d.l.	missing [a]
20	N,N′-Bis-(5-hydroxyferuloyl) spermidine	b.d.l.	b.d.l.	missing [a]	b.d.l	missing [a]	b.d.l.	missing [a]
21	N-(*p*-Coumaroyl)-N′,N′′-bis-(5-hydroxyferuloyl)spermidine	1.55E+04 ± 2.1E+03	b.d.l.	missing [a]	1.36E+04 ± 1.9E+03	1.1	b.d.l.	missing [a]
22	N,N′,N′′-Tris-(5-hydroxyferuloyl) spermidine	2.31E+05 ± 2.2E+04	b.d.l.	missing [a]	2.20E+05 ± 2.7E+04	1.1	b.q.l.	trace [b]
23	N-Caffeoyl-N′-(*p*-coumaroyl)-N′′-(5-hydroxyferuloyl)spermidine	9.86E+02 ± 2.1E+02	b.d.l.	missing [a]	8.45E+02 ± 3.0E+02	1.2	b.d.l.	missing [a]
24	N-Caffeoyl-N′-feruloyl-N′′-(5-hydroxyferuloyl)spermidine	7.22E+03 ± 7.6E+02	b.d.l.	missing [a]	7.00E+03 ± 1.2E+03	1.0	b.d.l.	missing [a]
25	N-(*p*-Coumaroyl)-N′-(5-hydroxy feruloyl)-N′′sinapoylspermidine	5.56E+04 ± 8.2E+03	b.q.l.	trace [b]	4.50E+04 ± 5.9E+03	1.2	b.d.l.	missing [a]
26	N^{1},N^{10}-Bis-(5-hydroxyferuloyl)-N^{5}-sinapoylspermidine	4.98E+05 ± 5.4E+04	1.15E+03 ± 3.6E+02	435.0 [c]	4.04E+05 ± 5.9E+04	1.2	6.97E+02 ± 1.1E+02	715.0 [c]

27	Unknown	1.48E+03 ± 6.3E+02	b.d.l.	missing [a]	2.16E+03 ± 2.9E+02	0.7	b.d.l.	missing [a]
28	Unknown	8.16E+02 ± 1.1E+02	b.d.l.	missing [a]	7.84E+02 ± 5.0E+01	1.0	b.d.l.	missing [a]
29	N,N′,N′′-Tris-(*p*-coumaroyl) spermidine	2.62E+03 ± 3.2E+02	b.d.l.	missing [a]	2.91E+03 ± 1.8E+02	0.9	b.d.l.	missing [a]
30	N-(*p*-Coumaroyl)-N′-feruloyl-N′′-(5-hydroxyferuloyl)spermidine	7.34E+03 ± 8.7E+02	b.d.l.	missing [a]	5.87E+03 ± 1.0E+03	1.3	b.d.l.	missing [a]
31	N-(5-hydroxyferuloyl)-N′,N′′-bis-sinapoylspermidine	4.50E+04 ± 3.5E+03	b.d.l.	missing [a]	3.27E+04 ± 2.1E+03	1.4	b.d.l.	missing [a]
32	N,N′-Bis-feruloyl-N′′-(5-hydroxyferuloyl)spermidine	1.30E+05 ± 6.6E+03	b.d.l.	missing [a]	1.14E+05 ± 1.5E+04	1.1	b.d.l.	missing [a]
33	Unknown	9.15E+03 ± 5.4E+02	b.d.l	missing [a]	8.59E+03 ± 9.5E+02	1.1	b.d.l.	missing [a]
34	N,N′,N′′-Tris-(*p*-coumaroyl) spermidine	3.15E+03 ± 9.2E+01	b.d.l	missing [a]	2.52E+03 ± 2.3E+02	1.3	b.d.l.	missing [a]
35	N,N′-Bis-(*p*-coumaroyl)-N′′-feruloylspermidine	1.82E+03 ± 1.5E+02	b.d.l	missing [a]	1.57E+03 ± 1.3E+02	1.2	b.d.l.	missing [a]
36	N-Feruloyl-N′-(5-hydroxyferuloyl) -N′′-sinapoylspermidine	1.61E+04 ± 5.6E+02	b.d.l	missing [a]	1.26E+04 ± 6.6E+02	1.3	b.d.l.	missing [a]
37	N-(*p*-Coumaroyl)-N′,N′′-bis-feruloylspermidine	8.55E+03 ± 4.6E+02	b.d.l	missing [a]	7.78E+03 ± 9.3E+02	1.1	b.d.l.	missing [a]
38	N,N′,N′′-Tris-feruloylspermidine	1.12E+05 ± 2.3E+03	4.58E+01 ± 7.9E+01	2442.0 [c]	1.04E+05 ± 4.9E+03	1.1	b.d.l.	missing [a]

[a] The substance is missing in mutant plants; [b] The substance is detectable in mutant plants, but present in concentration below its quantification limit; [c] The reduction is statistically significant compared to wild type ($p < 0.05$); [d] The reduction is statistically significant compared to sht ($p < 0.05$)
b.d.l., below detection limit (signal to noise ratio is less than 3); b.q.l., below quantification limit (signal to noise ratio is less than 10)

5.5 List of Publications

Fellenberg, C., Ziegler, J., Handrick, V. and Vogt, T. (2012) Polyamine homeostasis in wild type and phenolamide deficient Arabidopsis thaliana stamens. *Front Plant Sci* **3:** 180

Bektas, I., Fellenberg, C. and Paulsen, H. (2012) Water-soluble chlorophyll protein (WSCP) of Arabidopsis in the gynoecium and developing silique. *Planta* **236:** 251-259

Fellenberg, C., van Ohlen, M., Handrick, V. and Vogt, T. (2012) The role of CCoAOMT1 and COMT1 in *Arabidopsis* anthers. *Planta* **236:** 51-61

Fellenberg, C., Böttcher, C. and Vogt, T. (2009) Phenylpropanoid conjugate biosynthesis in flower buds of *Arabidopsis thaliana*. *Phytochemistry* **70:** 1392-1400

Fellenberg, C., Milkowski, C., Hause, B., Lange, P.R., Böttcher, C. and Vogt, T. (2008) Tapetum specific location of a cation-dependent *O*-methyltransferase in *A. thaliana*. *Plant Journal* **56:** 132-145

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Eidesstattliche Erklärung

Hiermit erkläre ich, dass ich die vorliegende wissenschaftliche Arbeit selbstständig und ohne fremde Hilfe angefertigt habe. Ich erkläre weiterhin, dass ich keine anderen als die von mir angegebenen Quellen und Hilfsmittel benutzt habe und die den Werken wörtlich und inhaltlich entnommenen Stellen als solche kenntlich gemacht habe.

Mit dieser Arbeit bewerbe ich mich erstmals um die Erlangung des Doktorgrades. Diese Arbeit wurde an keiner anderen Fakultät oder Universität zur Begutachtung eingereicht.

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