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Molecules of Interest

Phenolamides: Bridging polyamines to the phenolic metabolism

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ABSTRACT

Phenolamides constitute a diverse and quantitatively major group of secondary metabolites resulting from the conjugation of a phenolic moiety with polyamines or with deaminated aromatic aminoacids. This review summarizes their bioactivities and their reported roles in plant development, adaptation and defence compared to those of their polyamine precursors. The most conclusive recent developments point to their contribution to cell-wall reinforcement and to direct toxicity for predators and pathogens, either as built-in or inducible defence. Phenolamides were often considered as accumulated end-chain products. Recent data bring a light on their biosynthesis and suggests their possible contribution in the branching of the phenylpropanoid metabolism.

1. Introduction

Phenolamides are frequently referred to as hydroxycinnamic acid amides (HCAA) or phenylamides. They have been reported throughout the plant kingdom, usually as main phenolic constituents of reproductive organs and seeds. They are regarded either as products of polyamine catabolism or as polyamines or phenolics storage forms. However, they seem to have specific functions in plant development and defence, as metabolic intermediates and final products. This review sums-up HCAAs occurence, biosynthesis and potential functions in plants in connection with polyamines and phenolic metabolism.

2. Polyamines

The name "polyamines" refers to aliphatic organic compounds with more than one amino group. Putrescine, spermidine and spermine are the most widespread in all living organisms especially in actively proliferating tissues. They are also the most common in plants, while cadaverine was also reported in legumes. Recent data suggest that the spermine isomer thermospermine might be also widespread and was present before spermine in aerial plants (Kakehi et al., 2008; Minguet et al., 2008). Norspermidine, norspermine and homospermine on the other hand were described as taxonomic markers of Bryophytes, Pteridophytes, Gymnosperms and Fungi (Hamana and Matsuzaki, 1985). The positive charge of polyamines at physiological pH confers them the property to bind negatively charged macromolecules or to modulate the activity of some ion channels. In plants, polyamines are found not only in the cytoplasm but also in vacuoles, plastids and mitochondria (Kumar et al., 1997). Several recent reviews provide a good overview on the current knowledge on polyamine biosynthesis, catabolism and bioactivity, including their roles in plant development and adaptative responses. Those will thus be just summarized briefly.

2.1. Biosynthesis and catabolism

Intracellular concentrations of polyamines are quite high and range from several hundreds of micromolar to a few millimolar. Due to their important biological functions, polyamine concentrations are very tightly controlled. While biosynthesis, catabolism, conjugation and transport contribute to polyamines homeostasis, catabolism also contributes to their bioactivity.

Polyamine biosynthesis involves similar pathways in bacteria, animals and plants (Kusano et al., 2008). Two alternative pathways starting from L-arginine have been confirmed in plants (Fig. 1). The ornithine decarboxylase pathway is favoured in meristematic and dividing cells, while the arginine decarboxylase pathway predominates in mature tissues and in response to environmental stress (Flores, 1991). Only one of these pathways was proposed to be operating in *Arabidopsis thaliana* since no ornithine decarboxylase has been predicted from its annotated genome (Hanfrey et al., 2001). However, Tassoni and coworkers (2003) reported ornithine decarboxylase activity associated with the plastid membranes in *Arabidopsis* leaves. In legumes, cadaverine is derived from lysine

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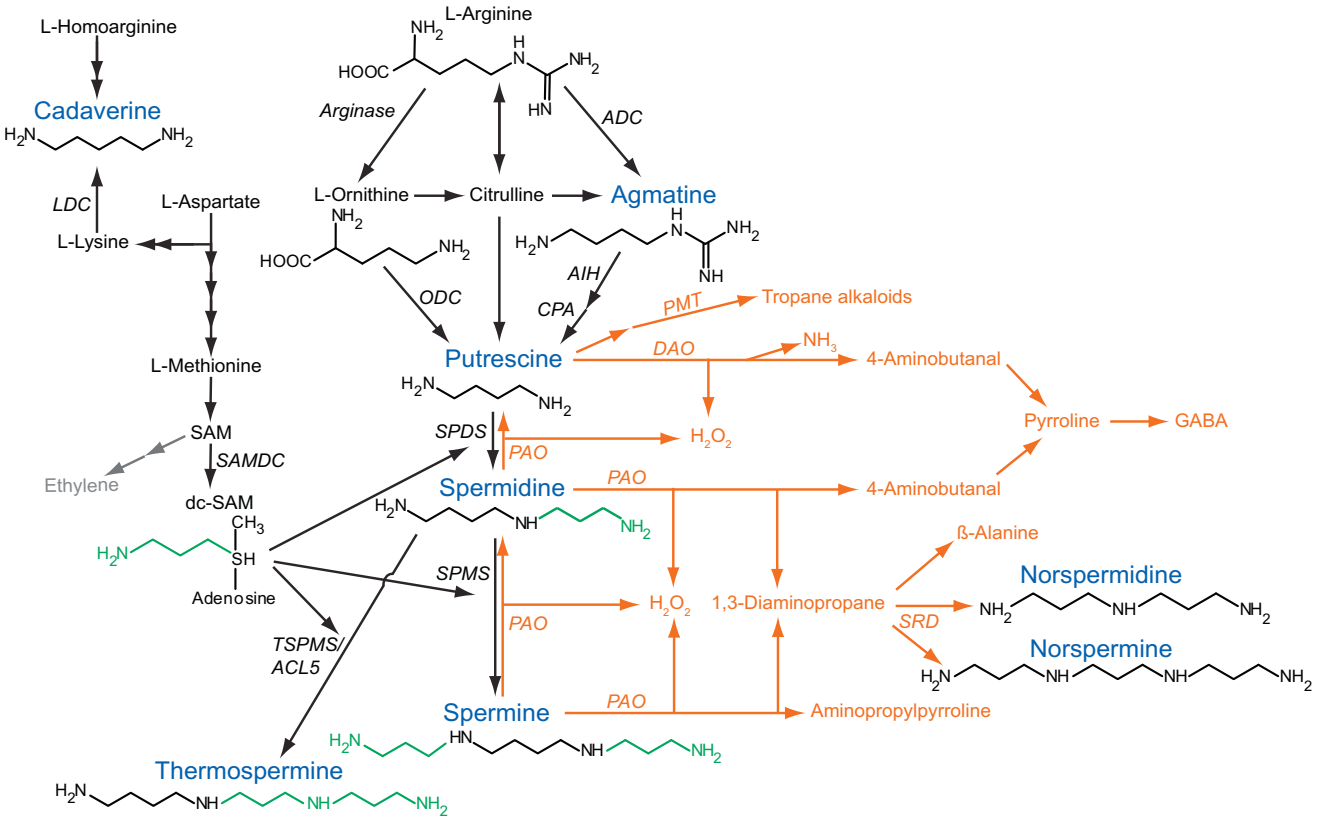

Fig. 1. Polyamine metabolism. Schematic biosynthetic pathways for common polyamines and related metabolites are indicated by black lines and catabolic processes in red. Common polyamines are in blue and enzymes in italics. Abbreviations: ACL5, ACAULIS5; ADC, arginine decarboxylase; AIH, agmatine iminohydrolase; CPA, *N*-carbamoylputrescine amidohydrolase; DAO, diamine oxidase; dc-SAM, decarboxylated *S*-adenosylmethionine; GABA, γ-aminobutyric acid; LDC, lysine decarboxylase; ODC, ornithine decarboxylase; PAO, polyamine oxidase; PMT, putrescine *N*-methyltransferase; SAM, *S*-adenosylmethionine; SAMDC, *S*-adenosylmethionine decarboxylase; SPDS, spermidine synthase; SPMS, spermine synthase; SRD, Schiff-base reductase/decarboxylase; TSPMS, thermospermine synthase.

via a lysine decarboxylase (EC 4.1.1.18). Most of the genes in the polyamine biosynthetic pathway are duplicated in plants. In *Arabidopsis*, two spermidine synthases and a spermine synthase associate to form a metabolon (Panicot et al., 2002). *ACL5*, one of the predicted *A. thaliana* spermine synthase genes was recently shown to encode a protein with thermospermine synthase activity (Knott et al., 2007). This suggested the presence of this spermidine isomer in plants and may explain the characteristic phenotypes in stem elongation and vascular development observed upon *ACL5* defect (Hanzawa et al., 1997; Clay and Nelson, 2005; Kakehi et al., 2008). Interestingly, a duplication of the genes encoding spermidine/spermine synthases has led to the evolution of putrescine *N*-methyltransferases, catalyzing the first step to the secondary metabolites nicotine and tropane alkaloids in Solanales (Minguet et al., 2008).

S-adenosylmethionine carboxylase (SAMDC) seems a critical point of regulation of polyamine homeostasis in all organisms (see e.g. Kumar et al., 1996; Martin-Tanguy, 1997) and might be responsible for antagonism between synthesis of higher polyamines and ethylene. Polyamine-controlled upstream ORF-mediated translational regulation of SAMDC was reported in mammals and plants. In *Arabidopsis*, two uORFs contribute to SAMDC regulation and translation repression at reduced levels of polyamines (Hanfrey et al., 2005).

Plant polyamine catabolism was recently reviewed by Moschou et al. (2008a) and is also summarized in Fig. 1. The main polyamine catabolic pathway, via both diamine and polyamine oxydases, generates H_2O_2. Catabolism of putrescine via diamine oxydase (DAO) also generates γ-aminobutyric acid, often considered as a mediator of plant defence. Plant polyamine oxydases (PAOs) catalyze the retroconversion from spermine and spermidine to spermidine and putrescine, respectively. They do not require acetylation of their substrates as shown for animal enzymes. Several DAO and PAO genes are found in plant genomes. Some of these enzymes were shown to be targeted to peroxisomes (Moschou et al., 2008a; Kamada-Nobusada et al., 2008) or the vacuole (Cervelli et al., 2004). This raises the question of their specific roles in the plant.

Polyamine homeostasis further relies on their conversion into secondary metabolites such as nicotine or tropane alkaloids in Solanaceae (Kusano et al., 2008) or in their conjugation with hydroxycinnamic acids (see below) or with proteins, hemicellulose or lignin (Creuss et al., 1991). Cross-linking mediated by transglutaminases might play a significant role in polyamine bioactivity for flower development and compatibility in reproduction (Serafini-Fracassini and Del Duca, 2008). Extracellular transglutaminases are expected to support organization of the cell-wall and pollen tube growth. For cytosolic enzymes, tubulin and actin have been identified as substrates (Del Duca et al., 1997), while chloroplastic forms would protect thylakoid proteins and Rubisco.

2.2. Role in plant growth and development

Polyamines, in particular spermidine, are essential for plant viability. Double insertional mutants of both arginine decarboxylase genes, both spermidine synthase genes, or of *SAMDC1* and *SAMDC4* *S*-adenosylmethionine carboxylases are embryo lethal in *A. thaliana* (Urano et al., 2005; Imai et al., 2004a; Ge et al., 2006). Mutants defective in spermine synthesis however grow normally (Imai

et al., 2004b). Spermidine is a precursor of deoxyhypusine, required for post-translational modification of the eukaryotic translational initiation factor eIF5A (Park, 2006) which may provide an explanation for the absolute putrescine/spermidine requirement for plant embryogenesis. In addition, single gene mutants in polyamine biosynthesis usually show strong phenotypes suggesting cross-talk with phytohormone control of plant development (Kumar et al., 1997; Kusano et al., 2007). One obvious reason for such a cross-talk is the share of the precursor *S*-adenosylmethionine for both ethylene and spermidine/spermine biosynthesis.

In bacteria and animals, polyamines are described as increasing cell growth and being essential for organ functionality (Kusano et al., 2008). This is however less clearly established in plants, except in the case of thermospermine. *A. thaliana* thermospermine synthase mutant *acl5* shows a very severe dwarf phenotype and xylem proliferation (Hanzawa et al., 2000) that is rescued by exogenous application of thermospermine but not spermine (Kakehi et al., 2008). *ACL5* is preferentially expressed in maturing xylem and is under negative feedback regulation by thermospermine (Clay and Nelson, 2005; Muñiz et al., 2008). Analysis of *acl5* suppressor mutants indicates that thermospermine controls stem elongation via the bHPLP-type transcription factor *SAC51* and one of its upstream open reading frames located in 5′ leader sequence (Takahashi and Kakehi, 2009). Plants seem to have acquired thermospermine biosynthesis ability at an early stage of evolution by horizontal gene transfer from prokaryotes (Minguet et al., 2008).

In addition to signalling, polyamines have other direct and indirect effects on plant development via mechanisms that also impact plant adaptation and defence. Those include electrostatic binding to macromolecules such as DNA, RNA and proteins, developmentally controlled cross-linking mediated by transglutaminases as mentioned above (Serafini-Fracassini and Del Duca, 2008), and interaction with ion channels and receptors, resulting in regulation of Ca^{++}, Na^{+} and K^{+} homeostasis (Kusano et al., 2008). The action of polyamines on cation channels depends on their net positive charge with spermine > spermidine >> putrescine. Production of H_2O_2 upon polyamine catabolism is another level of regulation of plant growth and development. Polyamine oxidases were shown to be developmentally regulated and associated to cell-wall strengthening, lignification and programmed cell death (Moschou et al., 2008a). In plants, senescence of different organs can be delayed by polyamines. Their role in developmental cell death is best documented in the case of *Nicotiana tabacum* flower corolla (Della Mea et al., 2007a,b).

2.3. Polyamines and abiotic stress

An extensive literature depicts the correlation between polyamines levels and physiological perturbations, as well as the protective effects of polyamines observed in response to environmental stress (Bouchereau et al., 1999; Kakkar and Sawhney, 2002; Urano et al., 2003; Alcázar et al., 2006a; Groppa and Benavides, 2008), including heavy metal stress (Groppa et al., 2001, 2008).

In grapevine, cultivars accumulating large amounts of free polyamines, exhibit a higher tolerance to osmotic stress than other cultivars (Paschalidis et al., 2009). In agreement with this observation, exogenous application of polyamines is reported to protect against abiotic stress (Chattopadhayay et al., 2002). Studies using loss-of-function mutants or transgenic plants overexpressing the genes for polyamine biosynthetic enzymes also support a role of polyamines in stress resistance. For instance, *Arabidopsis* or potato lines overexpressing the SPDS gene are tolerant to multiple environmental stresses (Kasukabe et al., 2004, 2006). Overexpression of the arginine decarboxylase (ADC2) gene in *Arabidopsis* results in increased putrescine level and drought tolerance (Alcázar et al., 2010). On the other hand, *ADC2* loss-of-function plants are more sensitive to salt stress (Urano et al., 2004). The *Arabidopsis acl5*/*spms* mutant is unable to produce spermine and is hypersensitive to salt and drought stresses (Yamaguchi et al., 2007). The symptoms are reversed by exogenous spermine. Remarkably, this mutant exhibits symptoms of Ca^{2+} deficiency (Yamaguchi et al., 2007), which points to an involvement of polyamines in Ca^{2+} regulation (Kusano et al., 2008).

The mode of action of polyamines during abiotic stress is still not well understood. It seems pleiotropic and depends on the stress and the plant species. A common feature of all types of abiotic stresses (salt, osmotic, drought, UV, heavy metals...) is oxidative stress promoted by the formation of reactive oxygen species (ROS). Polyamines induce antioxidative enzymes, increase amount of carotenoids and limit lipid peroxidation (Verma and Mishra, 2005). They are reported to enhance heavy metal tolerance by protecting gluthatione reductase and superoxide dismutase (Groppa et al., 2001). Spermine and spermidine also prevent leakage of amino acids or electrolytes (Chattopadhayay et al., 2002). According to Groppa and Benavides (2008) polyamines stabilize macromolecules, proteins and membranes. They were shown to modulate electrostatic protein–protein interactions (Berwanger et al., 2009), to affect DNA–protein interactions, translocation of protein kinases and gene expression by selective inhibition of cytosine dependent DNA methylases (Kuznetsov et al., 2006). Polyamines also indirectly inhibit plasma membrane and vacuolar H^{+}-ATPase antiporters and a long-term polyamine decrease contributes to maintain cation–anion equilibrium in the cytoplasm (Janicka-Russak et al., 2010). Putrescine is an efficient stimulator of ATP synthesis and causes depolarization of membranes. Conversely spermidine and spermine are described as uncouplers and prevent thylakoid membrane energization and reactive oxygen formation (Ioannidis and Kotzabasis, 2007). Polyamines have also been proposed to play a role in photosynthesis since they are capable of reversing stress-induced damages in photosynthetic apparatus (Sfakianaki et al., 2006). Polyamine conjugation by transglutaminases, especially to Rubisco seems to have an important role in protecting this protein from protease action, thus preserving its photosynthetic efficiency (Serafini-Fracassini et al., 1995).

Abiotic and biotic stresses seem to induce the export of spermidine/spermine into the apoplast for PAO/DAO-mediated catabolism resulting in H_2O_2 production. Accumulation of H_2O_2 results either in the tolerance response or plant cell death (PCD), depending on the levels of intracellular polyamines (Moschou et al., 2008b). When polyamine anabolism predominates, catabolism PCD fails to occur. Complex mechanisms for polyamine signalling and the subsequent responses to generate ROS upon abiotic stress have been described (Moschou et al., 2008a; Toumi et al., 2010). They involve synergetic or antagonist roles of hormones depending on the type of stress and on the responses. For example, putrescine seems to modulate ABA biosynthesis (Cuevas et al., 2008). Reciprocally, ABA modulates polyamine metabolism at transcriptional and metabolite levels (Alcázar et al., 2006b). ABA contributes to the conversion of the bound or conjugated forms to the free soluble form of polyamine (Ben Hassine et al., 2009). Increased putrescine content seems to repress GA biosynthesis (Alcázar et al., 2005). The calcium sensor calcineurin B-like 3 (CBL3) that mediates calcium signalling is described as another modulator of polyamine biosynthesis (Oh et al., 2008), while induction of NO production by spermine and spermidine has been reported in *Arabidopsis* (Tun et al., 2006). Under anoxic conditions, NO can react with polyamines to produce NONOates. Spermine NONOate has been favoured as a chemical NO donor (Yamasaki and Cohen, 2006). Several enzymes involved in polyamine biosynthesis are inhibited by NO-mediated *S*-nitrosylation (Wang et al., 2006). Putrescine,

spermidine and spermine exert different roles during abiotic stress response. The ratio of (spermidine + spermine)/putrescine seems to be fundamental for plant tolerance and survival (Groppa and Benavides, 2008) and modifications of this ratio seems a factor that controls plant response to different environmental cues.

2.4. Polyamines and disease resistance

Since the early 80s, a plethora of experiments have demonstrated implication of polyamines in plant disease resistance (Walters, 2003a,b). However, the molecular mechanisms involved are still unclear. The main problem for outlining clear rules is that total polyamine concentrations and ratios between individual polyamines markedly vary with plant species, with plant tissues and also with pathosystems investigated (Marina et al., 2008).

However, some aspects of this mode of action were recently elucidated (Fig. 2). A rapid biosynthesis and accumulation of polyamines was observed following fungal and viral infection or elicitor treatment. Spermidine and/or spermine levels were higher in the apoplast (Yamakawa et al., 1998; Marini et al., 2001; Cowley and Walters, 2002; Yoda et al., 2003, 2006; Marina et al., 2008; Moschou et al., 2008b). High apoplastic spermidine/spermine concentrations were shown to modulate three signals. One of them is Ca^{2+} influx. Spermine controlled Ca^{2+}/K^{+} channels and calcium-channel blockers attenuated this signal (Kusano et al., 2007). Another signal involved the action of spermidine and spermine as inhibitors of pectin dimerization. In the cell-wall, spermidine and spermine form complexes with pectins that prevent Ca^{2+}-induced pectin oligomerization and thus inhibits their biological activity as endogenous elicitors (Messiaen and Van Cutsem, 1999). Conversely, putrescine stabilizes pectic fragments. A third signal results from the production of H_2O_2 from spermine/spermidine oxidation by polyamine oxidases (Cona et al., 2006). This is a common defensive strategy in host- and non-host-hypersensitive response (Walters, 2003a,b; Mitsuya et al., 2009; Yoda et al., 2003, 2009). The use of DAO/PAO inhibitors before inoculation impaired host defence reactions (Mitsuya et al., 2009). H_2O_2 produced by PAO seems to contribute to the second phase of the oxidative burst (Angelini et al., 2008), and seems decisive for the fate of the cell. Polyamine catabolism-dependent H_2O_2 would trigger the hypersensitive response (HR) through a so-called "spermine-signalling pathway" (Kusano et al., 2007). The latter was suggested to involve mitochondrial dysfunctions, photorespiration and ATP consumption, activation of caspases, activation of protein kinases and of MAPK cascade, ER stress and the Unfold Protein Response, increased expression of HR marker genes and zinc finger genes, with as final result defence responses or HR-like cell death (Takahashi et al., 2003, 2004; Uehara et al., 2005; Kusano et al., 2008; Mitsuya et al., 2009). Many other defence mechanisms triggered by abiotic stress are also activated. For example, increased expression of PAO induces peroxidase, superoxide dismutase, catalase and activities of enzymes, contributing to redox homeostasis and limiting oxidative damage. Furthermore H_2O_2 released by PAO is required for the peroxidase-mediated deposition of lignin and suberin polyphenolic domain in wounded tissues (Angelini et al., 2008).

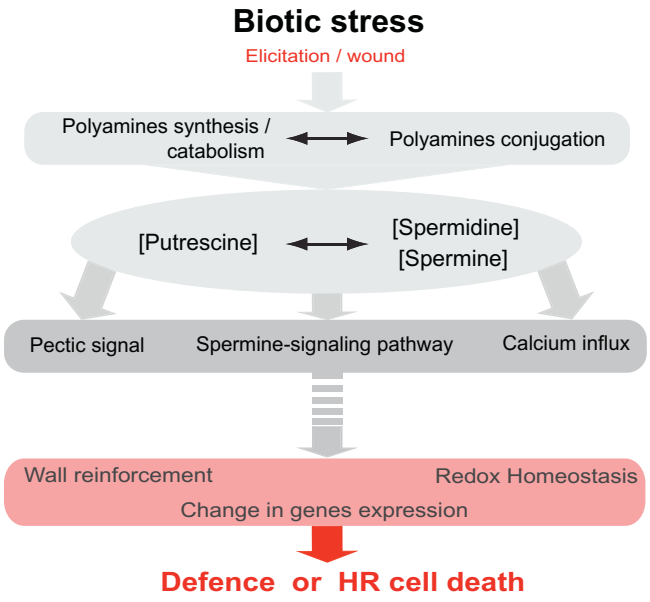

Fig. 2. Polyamine-mediated response in plant defence against biotic stress.

Polyamines thus have a dual function in defence response. A protective effect results from oxygen radical scavenging, increased expression of antioxidant enzymes, and generation of H_2O_2 as a signal. Toxic pro-oxidative effects occur when unrestrained increase in their content leads to intensive degradation, while H_2O_2 levels beyond a given threshold lead to PCD (Kuznetsov et al., 2006). Putrescine can prevent PCD induced by PAO-generated H_2O_2 (Yoda et al., 2009). The ratio of (spermidine + spermine)/putrescine and the ratio between polyamines, pectins and Ca^{2+} also seem to be important. A tight control of the homeostasis of putrescine and spermidine/spermine appears to predetermine cell fate between defence and PCD (Kusano et al., 2008).

3. Phenolamides (also termed phenylamides or hydroxycinnamic acids amides)

A large proportion of the polyamines found in plants are mono-, di- or tri-substitued with phenolic acids such as coumaric, caffeic and ferulic acids. These phenolic acids can also be conjugated with arylmonoamines like tyramine, tryptamine, octopamine or anthranilate. Phenolamides form a large class of plant secondary metabolites that are abundant in plants. A few examples are depicted in Fig. 3. Depending on the presence of a residual free amino group, the resulting conjugate can be basic or neutral which conditions its physicochemical properties (Edreva et al., 2007). Conjugation of polyamines with phenolics significantly reduces their polarity and hydrophilicity. This may favour their translocation, stability and compartmentation. While conjugation can be a mean to regulate the pools of both parent compounds and to store phenolics and bioactive polyamines, conjugates were often regarded as final and accumulated products. Turnover and translocation of conjugates were however quite early described (Martin-Tanguy, 1985, 1997; Havelange et al., 1996) and, more recently, interconversion between free and conjugated precursors has found some support (Luo et al., 2009). In addition, new data suggest that they might also be metabolic intermediates contributing to the complexity of the phenolic metabolism and possibly to its cross-talk with nitrogen metabolism (Morant et al., 2007; Matsuno et al., 2009).

Abundance, diversity and distribution of phenolamides are well documented (Martin-Tanguy et al., 1978; Martin-Tanguy, 1985; Bienz et al., 2005; Rogoza et al., 2005). However, surprisingly little is known about their biological functions. Potential roles of phenolamides in plant development and defence have been summarized in two reviews (Facchini et al., 2002; Edreva et al., 2007).

3.1. Phenolamides associated with plant growth and floral initiation

High concentrations of phenolamides are most often associated with organ growth, in particular floral induction and development (Martin-Tanguy, 1985; Kakkar and Rai, 1993).

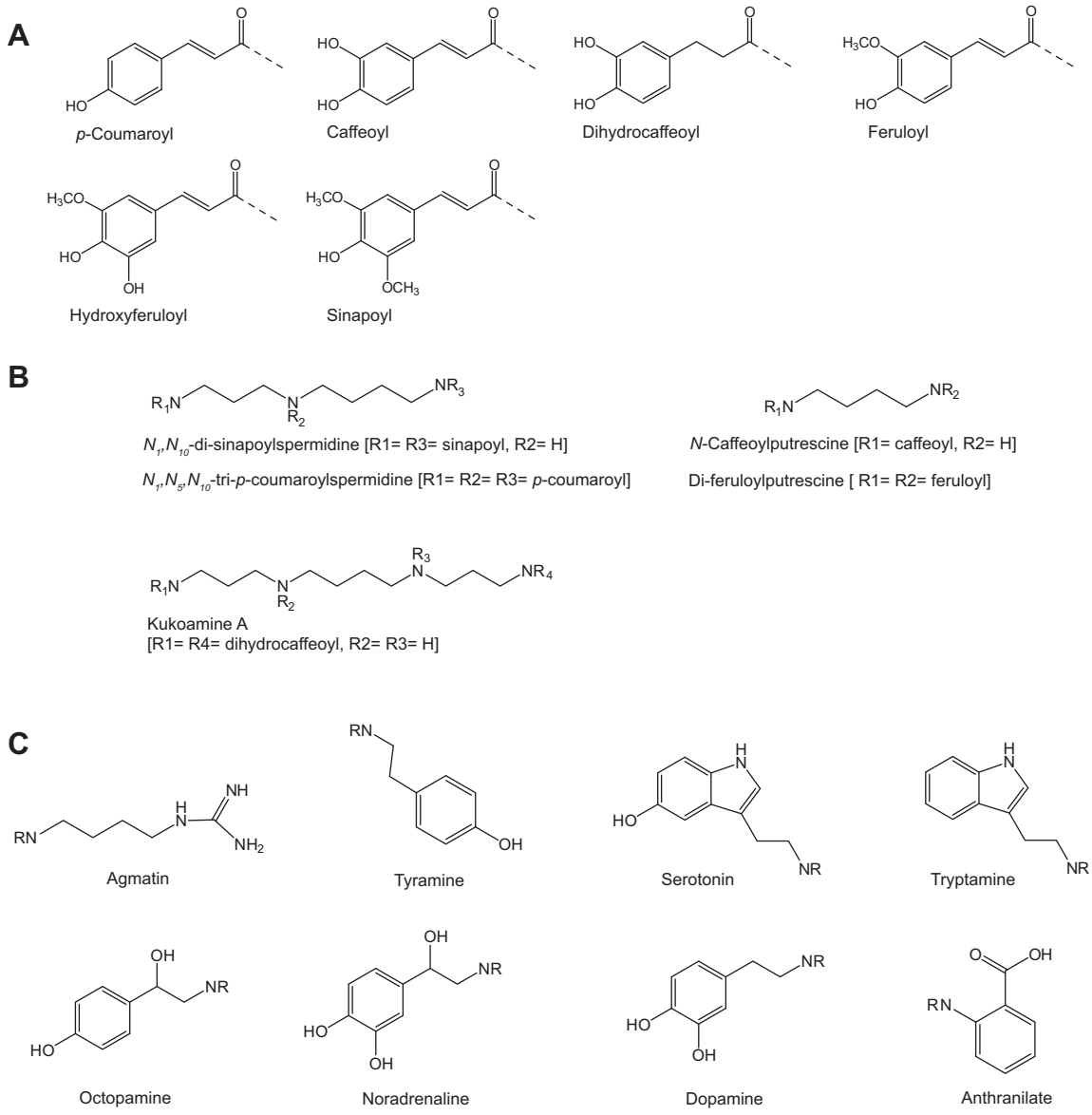

Fig. 3. Chemical structures of common phenolamides and their parent hydroxycinnamic acids. (A) Common acyl substituents found in phenolamides. (B) Examples of phenolamides derived from polyamines. (C) Most common arylamine moieties found in phenolamides.

Polyamides were proposed to be a necessary component of the mobile signal to flower development (Tarenghi and Martin-Tanguy, 1995; Havelange et al., 1996). Even if they are not considered as the florigen itself, their accumulation clearly correlates with flower initiation and development. Flowering plants usually contain little or no phenolamides in leaves. During floral transition however, phenolamides first accumulate in the upper leaves and apices (Martin-Tanguy, 1985, 1997). Subsequent accumulation in floral organs correlates with disappearance from the leaves. In *Sinapis alba*, free and conjugated putrescine were found in phloem leaf exudates after floral induction (Havelange et al., 1996). Their production and flowering response were inhibited with difluoromethylornithine, (an inhibitor of ornitine decarboxylase) and this inhibition could be reversed by application of putrescine to the roots. A role of phenolamides in floral development was also supported by their absence in mutants that do not flower (Martin-Tanguy, 1985) or by altered floral morphologies upon accumulation of spermidine conjugates (Malmberg and McIndoo, 1983).

Different classes of phenolamides are clearly associated with different floral organs (Martin-Tanguy, 1985; Aribaud and Martin-Tanguy, 1994a; Tarenghi and Martin-Tanguy, 1995). In tobacco, basic water soluble caffeoylputrescine and caffeoylspermidine accumulated in female organs are considered as markers of female fertility, while neutral di-*p*-coumaroylputrescines, di-*p*-coumaroylspermidines and *p*-coumaroyltyramine found in the anthers are considered as markers of male fertility (Cabanne et al., 1981). Diferuloyl conjugates are transiently detected after fertilization. In a similar way, high levels of neutral phenolamides were found in male flowers from Araceae while basic phenolamides were detected in female flowers (Ponchet et al., 1980). Sterile flowers of the same plants were devoid of both types of compounds. Male sterility in maize was also linked to the absence of phenolamides in the anthers (Martin-Tanguy et al., 1982). Various studies have been carried out in different plant species to further correlate male sterility with polyamine biosynthesis and accumulation. In most cases, male sterility could be associated with reduced content in polyamines and conjugates, in particular

insoluble conjugates (Aribaud and Martin-Tanguy, 1994b; Li and Li, 1997; Tian et al., 1998; Guo et al., 2003). Higher levels of polyamines were however reported in the male sterile stamenless-2 mutant of tomato and related to abnormal stamen development (Rastogi and Sawhney, 1990).

If accumulation of specific phenolamides seems clearly correlated with specific stages of reproductive development, the accumulated molecules usually differ among plant species and a causal role could only be assessed indirectly. One approach used inhibitors of polyamine biosynthesis, such as DFMO (α-DL-difluoromethylornithine), a specific inhibitor of ornithine decarboxylase, which results in delayed flowering, leaf wrinkling and branching and reduced height (Burtin et al., 1991; Havelange et al., 1996). These effects could be reversed by putrescine feeding. Inhibitors of spermidine biosynthesis did not delay flowering but interfered with anther development and caused flower abortion, lack of pollen, heterostyly, replacement of anthers by petals, and infertility (Burtin et al., 1991). These defects were reversed by supplying spermidine. Both free polyamines and their conjugates were reduced after inhibitor treatments and restored upon putrescine feeding.

Alterations of plant development similar to those observed by treatment with DFMO were obtained by tobacco transformation with the root-inducing (Ri) TL-DNA from *Agrobacterium rhizogenes* and with 35S-r*olA* or 35S-*rolC* constructs (Martin-Tanguy et al., 1996; Martin-Tanguy, 1997). Genes carried by the Ri TL-DNA have specific effects on plant signalling and development. Ri TL-DNA transformation resulted in wrinkled leaves, shortened internodes, increased branching, flower retardation and strongly reduced fertility, all correlated with a decrease in polyamine conjugates. This effect was reversed by putrescine and tyramine feeding that also restored accumulation of polyamine conjugates in the Ri LT-DNA plants. In plants expressing the 35S-*rolA* construct, with abnormal flower and stamen development (anthers contained little or no viable pollen), application of free putrescine and tyramine was not sufficient to restore fertility. However, grafting on a wild-type rootstock that was induced to flower restored flower development, but flowers aborted without additional supply of putrescine and tyramine (Martin-Tanguy et al., 1996). In 35S-*rolC* plants, male sterility seemed more specifically correlated with an inability to form feruloyl amine derivatives due to a lack of putrescine:feruloyl-CoA and tyramine:feruloyl-CoA transferase activities (Martin-Tanguy, 1997). Accordingly, free amines failed to reverse the sterility phenotype.

3.2. Major pollen constituents

From the plethora of analytical data collected over several decades, the presence of neutral phenolamides in the male gametophyte appears as a clear and constant feature. In particular, di- and tri-substituted hydroxycinnamoyl conjugates emerge as the major metabolites detected in the anthers and more specifically in pollen grains. Putrescine and/or spermidine conjugates have been detected in the anthers or pollen of all Angiosperms, including Dicots such Rosideae (Strack et al., 1990; Tarenghi and Martin-Tanguy, 1995), Brassicaceae (Havelange et al., 1996), Solanaceae (Leubner-Metzger and Amrhein, 1993; Kang and Back, 2006), Asteraceae (Aribaud and Martin-Tanguy, 1994b; Werner et al., 1995), Betulaceae, Fagaceae and Juglandaceae (Meurer et al., 1986, 1988; Bokern et al., 1995; Meurer-Grimes, 1995), Acanthaceae, (Werner et al., 1995), and of Monocots like maize (Martin-Tanguy et al., 1982), Liliidae (Youhnovski et al., 1998, 2001) or Araceae (Ponchet et al., 1980). High polyamine conjugate contents were also reported in Gymnosperm sexual buds (Daoudi and Bonnet-Masimbert, 1998; Fraga et al., 2004). Interestingly, while phenolamides were present in all taxa, they differed in the chain length of the polyamine (putrescine or most often spermidine), the degree and pattern of substitution of the polyamine chain (di- or tri-substituted) and the degree of hydroxylation of the phenolic rings (from *p*-coumaroyl to sinapoyl). Phenolamides thus seem essential for pollen development, viability or germination. The reason for their structural variability among taxa is however not understood.

A. thaliana mutants affected in the biosynthesis (detailed below) of the hydrocinnamoylspermidines recently provided tools to investigate their role in pollen development (Fellenberg et al., 2008, 2009; Grienenberger et al., 2009; Matsuno et al., 2009). Promoter-GUS fusions constructs for several genes in the biosynthetic pathway revealed a very high expression in the tapetal cells. N^1,N^5-di(hydroxyferuloyl)-N^{10}-sinapoyl spermidine represented a major constituent in the pollen coat, as shown by autofluorescence loss in mutant and easy methanol wash out (Grienenberger et al., 2009). In addition, total depletion led to occasional pollen grain distortions, which might be indicative of participation to the structure of the pollen wall (Grienenberger et al., 2009). Although more frequent silique abortion and reduced seed set was reported for some mutants (Fellenberg et al., 2008; Matsuno et al., 2009), no strong impact on pollen viability, germination and fertility was reported.

3.3. Other plant tissues and developmental stages

Basic and especially neutral polyamines such as di-feruloylputrescine, di-feruloylspermidine, feruloyl tyramine have been detected in large amounts upon seed development and maturation in Monocots like maize and rice (Martin-Tanguy, 1985; Bonneau et al., 1994a). In maize, quite large fluctuations in specific phenolamide compounds were observed in the embryo and endosperm at different develomental stages (Martin-Tanguy, 1985). Highest phenolamide concentrations correlated with maximal rates of DNA, RNA and protein synthesis. Fertility was reported to be correlated with the level of phenolamides in developing seed. Microanalysis and fluorescence localized the main sites of accumulation of basic phenolamides in the embryo and of the most abundant neutral di-*p*-coumaroylputrescine and di-feruloylputrescine in the pericarp and/or aleurone cells (Sen et al., 1994). In maize, fairly large amounts of phenolamine conjugates were recovered in corn bran or corn fibers extracts (Moreau et al., 2001). More recently, di-sinapoyl spermidine and its glucose conjugate were found as major phenolic derivatives in the seeds of *A. thaliana* (Luo et al., 2009), their synthesis relying on a sinapoyl-CoA-specific acyl transferase (SDT).

Both in rice and *Arabidopsis*, a sharp decline in phenolamides was observed upon seed germination, with a concomitant increase in polyamines in the case of rice (Bonneau et al., 1994a; Luo et al., 2009). Interestingly, the *Arabidopsis* SDT transferase was found to be able to catalyze the reverse reaction, regenerating spermidine and sinapoyl CoA, and was expressed at high level during early stages of seed germination (Luo et al., 2009). It can thus be proposed that phenolamides constitute a polyamine and hydroxycinnamoyl storage unit that can be remobilized upon seed germination. In agreement with this hypothesis, high amine conjugate content in rice seeds was positively correlated with seed viability (Bonneau et al., 1994a). As recently pointed by Luo et al. (2009), accumulation of phenolamides in tissues that express high levels of reversible SDT requires compartmentation of the products. Such compartmentation can be supported by glycosylation of the spermidine conjugates, as observed in *Arabidopsis* seeds. Alternatively, product accumulation may result from transport into sink (e.g. inflorescence) tissues.

In tobacco roots, abundant free and wall-bound feruloyltyramine were reported (Hagel and Facchini, 2005). Conjugated amines were also detected in rice seedling roots (Bonneau et al.,

1994b), and various hydroxycinnamoyl derivatives of tyramine or spermidine in the roots of tropical plants (Lee et al., 2004; Zamble et al., 2006). Interestingly, recent work of Luo et al. (2009) indicates that an *Arabidopsis* polyamine acyltransferase specific for *p*-coumaroyl-CoA and spermidine is specifically expressed in root tips while phenolamides could not be detected in the roots. They may thus be further converted in such plant tissues.

Finally, accumulation of basic phenolamides in the stolons before potato tuber initiation suggested a role in tuberization (Paynot et al., 1983). This was later questioned by work of Leubner-Metzger and Amrhein (1992, 1993) who found no correlation between *in vitro* tuberization and accumulation of phenolamides and distribution of phenolamides in different species of Solanaceae and their tuber development.

3.4. Phenolamides and cell-wall cross-linking

It progressively appeared that cell-wall cross-linking has essential roles in plant development and defence. Those include control cell of elongation and plant growth, nucleation of lignin, cell-wall stiffening and thickening upon ageing, response to wounding and pathogen attack, and biodegradability by microbial and endogenous enzymes (Passardi et al., 2004; Buanafina, 2009). Cross-linking largely results from the formation of diferuloyl bridges between polysaccharides (via arabinoxylans) and lignin (Buanafina et al., 2009). However, polyamines and conjugates, feruloyltyramine in particular, seem to constitute other important bridging agents, especially in grasses and upon wounding or pathogen challenge. Large amounts of polyamines were found strongly bound to cellular structures, especially in roots (Vallée et al., 1983; Hagel and Facchini, 2005). Feruloyltyramine and feruloyloctopamine are ether-linked to the cell-wall of natural and wound potato periderm (Negrel et al., 1996).

In tobacco thin layer cultures, polyamine synthesis inhibitors induced various developmental changes in the cell-wall and middle lamella as well as a loss of cell adhesion (Berta et al., 1997). Those were reversed when the culture was supplemented with polyamines. This suggested a role of polyamines in cell-wall cross-linking but did not imply involvement of phenolamides. Wound-healing and infection are usually associated with deposition of suberin in cell-walls. Feruloylamides such as feruloyltyramine and feruloyloctopamines seem to constitute major components of the suberin polymer (Borg-Olivier and Monties, 1993; Graça, 2009). In addition, phenolamides have been suggested as the preferential substrates for some amine oxidases and peroxidases, respectively involved in H_2O_2 generation in the apoplast and in H_2O_2-dependent polymerization in the cell-wall (Aribaud and Martin-Tanguy, 1994b; Bernards et al., 1999).

Clarke (1982) first described rapid accumulation of highly fluorescent hydroxycinnamoyl tyramine and octopamine conjugates in methanol-soluble granules and their subsequent binding to the cell-wall following exposure of potato tubers to *Phytophthora infestans*. Irreversible incorporation of radiolabelled hydroxycinnamoyltyramine into cell-wall residue of TMV-infected *Nicotiana tabaccum* was then reported by Negrel and Jeandet (1987). Feeding *Nicotiana* cell cultures with radiolabelled tyramine also demonstrated incorporation into polymeric material at higher rate after elicitation with chitosan (Villegas et al., 1990). A similar response was reported for fungal infection of potato cell cultures and leaves that triggered incorporation *p*-coumaroyltyramine and feruloyltyramine in the cell-wall (Keller et al., 1996; Schmidt et al., 1998).

In the case of *Botrytis*-infected onion epidermal cells, several autofluorescent hydroxycinnamoyltyramine derivatives were found accumulated as granular deposits near the penetration site with associated polarization of the actin microfilaments. The major autofluorescent compounds (feruloyltyramine and its 3′-methoxy derivative) were subsequently bound via ether links onto the cell-wall (McLusky et al., 1999). More recently, extraction of suberized potato scab (*Streptomyces*-induced) lesions confirmed the presence of large amounts of feruloyltyramines and feruloyloctopamines, and also revealed the presence of minor components identified as cross-linked dimers of feryloyltyramines and feruloyloctopamines (King and Calhoun, 2005). Elevated expression and activity of the enzymes involved in feruloyltyramine synthesis provided a track to investigate the significance and impact of its insertion in the cell-walls. Engineered constitutive expression of tyrosine decarboxylase and of hydroxycinnamoyl-CoA:tyramine hydroxycinnamoyltransferase were thus shown to lead to an increased incorporation of hydroxycinamoyltyramines in the cell-wall at the wound sites, and to reduced digestibility (Facchini et al., 1999; Hagel and Facchini, 2005; Guillet and De Luca, 2005).

3.5. Bioactive compounds

Phenolamides were often described as bioactive compounds with antiviral, antibacterial antifungal, insecticidal, deterrent or therapeutic activities. For example, *N*-feruloyltyramine was isolated as the most active garlic component, suppressing P-selectin expression (Park, 2009). It is thus expected to play a major role in garlic positive effect on cardiovascular system by the inhibition of platelet activation. *N-trans*-feruloyltyramine was also described as antitumoral (Park and Schoene, 2002), antimycobacterial (Mata et al., 2004), as melanogenesis inhibitor in mouse melanoma cell (Efdi et al., 2007) and inhibitor of cyclooxygenase (Park, 2007). This led to attempts to engineer its production in *Escherichia coli* (Kang et al., 2009) and rice (Park et al., 2009a).

Kukoamines, initially isolated from medicinal plants such as *Lycium chinense*, have attracted attention for their hypotensive effects and anti-trypanosomal activity (Funayama et al., 1980, 1995; Ponasik et al., 1995). Interestingly, kukoamine A (N^1,N^{12}-bis(dihydrocaffeoyl)spermine) and related dihydrocaffeoylated spermidines and spermines have recently also been identified in potato tuber and other Solanaceae such as tomato fruit or *Nicotiana sylvestris* leaves (Parr et al., 2005). Other phenolic amides isolated from the root bark of *L. chinense*, dihydro-*N*-caffeoyltyramine, *trans*-*N*-caffeoyloctopamine, *cis*- and *trans*-*N*-caffeoyltyramine displayed antifungal activity against *Candida albicans* (Lee et al., 2004) or anti-inflammatory properties via suppression of cycloxygenase expression (Han et al., 2010). Similarly, the tri-substituted spermidines representing the major *Quercus alba* pollen constituents were shown to decrease mycelial growth of *Pyronophora avenae* and to reduce powdery mildew infection of barley seedlings (Walters et al., 2001).

In other cases antioxydant and radical scavenging activities were reported (e.g. Bors et al., 1989; Calvin et al., 1998; Son and Lewis, 2002; Han et al., 2002; Zamble et al., 2006). Potentiated radical scavenging and quenching of singlet oxygen by phenolamides relative to parent compounds has been evidenced (Bors et al., 1989; Velikova et al., 2007). All (*E*)-spermidine conjugates such as those present in the pollen coat undergo very easy photoisomerization of the phenolic acid side chains at 365 nm. Their strong absorbance in the 270–330 nm range was thus postulated to have a protective function for germinal cells (Hu et al., 1998; Bienz et al., 2005).

3.5.1. For plant defence against microorganisms

While a defensive role of phenolamides against plant pathogens is well documented, in many cases their defensive function is only deduced from a correlation between metabolite accumulation and stronger resistance to the pathogen. For example, jasmonic acid was shown to promote a strong increase in the local and systemic concentrations of phenolic putrescine and spermidine conjugates

in barley leaves. This increase in phenolamide concentrations correlated with a reduction in powdery mildew infection (Walters et al., 2002). More documented is the impact of feruloylamines accumulated in tobacco upon hypersensitive response to TMV (Martin-Tanguy, 1985). Accumulation of these compounds correlated with the hypersensitive reaction. Their application on leaf discs caused a significant reduction of the number of virus-induced lesions (Martin-Tanguy et al., 1976). In addition, TMV inoculation was shown to promote incorporation of radiolabelled tyramine and feruloyltyramine into the acid-insoluble fraction of the cell-wall (Negrel and Jeandet, 1987). Interestingly, it has been proposed that the rare presence of viruses in flowers and seeds is related to their high contents in phenolamides (Martin-Tanguy, 1985; Edreva et al., 2007). Antimicrobial role of feruloyltyramine is often attributed to cell-wall strengthening and inhibition of pathogen penetration. As mentioned above, this is supported by the accumulation of fluorescent methanol-soluble granules observed in various plant-pathogen interactions near the penetration site and prior to papilla formation (Clarke, 1982; McLusky et al., 1999). Newman et al. (2001) also observed *in vitro* inhibition of bacterial growth by coumaroyltyramine and feruloyltyramine that appeared to determine incompatible interaction of the pepper plant with *Xanthomonas campestris* while antifungal activity was reported for the feruloyltyramine isolated from *Allium* roots (Fattorusso et al., 1999).

Putrescine, spermidine or tyramine derivatives appear ubiquitous in higher plants. A diversity of more specific phenolic amides accumulated upon pathogen attack were shown to behave as phytoanticipins or phytoalexins. The most extensively described are probably avenanthramides produced in oat leaves upon crown rust infection (Mayama et al., 1981, 1982; Miyagawa et al., 1995). These *N*-hydroxycinnamoyl anthranilate derivatives form a family of compounds also found in large amounts in seed groats and hulls (Collins, 1989) which concentrations appear tightly correlated with genetic resistance to crown rust (Wise et al., 2008). They are incorporated in the cell-walls of elicited oat leaf segments floated on stable isotope precursors (Okazaki et al., 2004).

In barley, *p*-coumaroyl-hydroxyagmatin accumulates in response to fungal attack by *Erysiphe graminis*. It exhibits antifungal activity both *in vivo* and *in vitro* and seems involved in cross-linking process of papillae (von Röpenack et al., 1998). Oxydative dimerization of *p*-coumaroylagmatin and feruloylagmatin leads to preformed and pathogen-inducible antifungal metabolites called hordatins that exhibit spore germination inhibition activity (Stoessl and Unwin, 1970). Kristensen et al. (2004) extensively discussed their significance and role in plant defence that may also include a contribution to polymerization products in papillae and cell-walls and to bacterial encapsulation. Unexpectedly and although detected in low amounts, *p*-coumaroylagmatine was recently described as a metabolic marker of rosette leaves in *A. thaliana* (Matsuda et al., 2009).

In the case of onion, feruloyl-3′-methoxytyramine was the major product accumulated in response to *Botrytis alii* (McLusky et al., 1999). A direct antifungal activity could not be demonstrated for this compound, but local accumulation as autofluorescent paramural granules at the penetration site was interpreted as participation to the local peroxidative cross-linking of the cell-wall. Other 7′- and 5′,7′-hydroxylated forms of *p*-coumaryoltyramine, *p*-coumaroyloctopamine and *p*-coumaroylnoradrenaline are the major metabolites accumulated by tomato carrying specific pathogen resistance genes (von Roepenack-Lahaye et al., 2003). In this case, *p*-coumaroylnoradrenaline but not *p*-coumaroyloctopamine was shown to have antibacterial activity against *Pseudomonas syringae*. Other examples of the diversification of defence compounds include clovamide (caffeoylDOPA) and the associated tyrosine phenolamide in red clover (Tebayashi et al., 2000) or *N*-*p*-coumaroylserotonin and *N*-feruloylserotonin accumulated in bamboo upon infection with *Phyllostachys bambusoides*, the causative agent of witch broom disease (Tanaka et al., 2003). *N*-*p*-coumaroylserotonin antifungal activity was confirmed.

Transient phenolamide accumulation was also reported upon beneficial symbiotic interactions with arbuscular mycorrhizal fungi in onion and barley and interpreted as the initiation of a defence response (Grandmaison et al., 1993; Peipp et al., 1997). Reduction of hyphal branching and of growth of mycorrhizal fungi has been reported by Grandmaison et al. (1993). In pine seedling, ectomycorrhizal fungus inoculation rather resulted in an early phenolamide accumulation in the needles (Niemi et al., 2006).

3.5.2. For defence against insects

Basic plant phenolamides are very closely related to the polyamine conjugates found in the venoms of predaceous spiders and wasps. It was thus proposed that they might be involved in protection against arthropods or used as natural insecticides. Investigation with synthetic basic phenolamides indicated no antifeedant nor toxic activity toward a variety of lepidopteran larvae using semi-synthetic diets laced with N^1- and N^8-coumaroylspermidine. *In vitro* inhibition of glutamatergic crustacean and mammalian synaptic receptors by such spermidine and spermine derivatives was however observed (Klose et al., 2002; Fixon-Owoo et al., 2003). Oxidative decomposition of the phenolamides in the diet was proposed to explain these contradictory results.

Well supported insect deterrence activity was however recently reported for caffeoylputrescine. Tebayashi et al. (2007) demonstrated that ovipositional deterrence of the leafminer *Liriomyza trifolii* acquired by sweet pepper tissues upon ageing or jasmonic acid treatment was related to their ability to accumulate caffeoylputrescine. Cotyledon treatment with synthetic *p*-coumaroylputrescine also decreased oviposition. These results were recently comforted by the work of Kaur et al. (2010) who demonstrated control of caffeoylputrescine and dicaffeoylspermidine synthesis by the jasmonic acid activated *Na*MYB8 transcription factor in *Nicotiana attenuata*. *Na*MYB8-silenced plant lacked these compounds and allowed better performance of both specialist (*Manduca sexta*) and generalist (*Spodoptera littoralis*) caterpillars than wild-type plants. Consistently, exogenous application of synthetic caffeoylputrescine at physiological doses impaired growth of *M. sexta* caterpillars.

3.5.3. For adaptation to abiotic stress

The role of phenolamides in abiotic stress is difficult to dissociate from that of its phenolic and polyamine constituents (Bouchereau et al., 1999; Groppa and Benavides, 2008). Their more specific functions seem to principally rely on their antioxydant and radical scavenging properties. Those have been extensively discussed by Edreva et al. (2007). Phenolamides that are good substrates for peroxidases may in addition support elimination of H_2O_2 and, as mentioned above, cell-wall strenghtening in the apoplast. Conjugation and turnover may in addition impact polyamine cross-talk with ethylene.

The best documented example is the accumulation of conjugated putrescine observed in O_3-sensitive and O_3-tolerant tobacco lines exposed to ozone (Bors et al., 1989; Langebartels et al., 1991). Leaf injury of the O_3-sensitive line by ozone treatments was prevented to a large extent by root application of polyamines. The titers of soluble free and conjugated polyamines were concomitantly increased and the amounts of polyamines associated with cell-wall or membrane pellet fractions were elevated four to six times above control. Reactivity assays of polyamines and conjugates towards hydroxyl, *tert*-butoxyl, sulphite radicals and superoxide anions indicated high rate constants for putrescine conjugates only (Bors et al., 1989). Accordingly, exposition to ozone doses that did not cause any visible injury resulted in rapid conjugated putrescine accumulation in the O_3-tolerant tobacco lines but only a slow

one in the O_3-sensitive plants. Monocaffeoyl-putrescine accumulated in the apoplastic fluid of the tolerant plants, in agreement with a potential role as an extracellular oxyradical scavenger (Langebartels et al., 1991). Either ethylene or polyamines were found to be early induced, in agreement with their biosynthetic antagonistic routes and shared precursor. Slow phenolamide accumulation correlated with the development of necrotic lesions similar to the hypersensitive response to TMV infection.

Accumulation of phenolic conjugates was also recorded upon a diversity of abiotic stresses, such as K, Ca, Mg and P deficiencies (Delétang, 1974), sulphur starvation (Klapheck, 1983), water excess (Edreva et al., 2007), heat shock (Edreva et al., 1998). Conversely, salt-stress was reported to reduce formation of polyamine conjugates in the roots (Shevyakova et al., 2006). Involvement of phenolamides in plant adaptation to UV-B irradiation was recently suggested by the existence of common regulatory elements with herbivore elicitation (Kaur et al., 2010). This prediction has yet to be confirmed. Interestingly, a spinach-specific phenolamide (*N*-feruloyl-(3-hydroxy-4-methoxyphenyl)ethylamine) accumulates in response to sublethal doses of diphenylether herbicides that promote tetrapyrrole accumulation and oxidative stress (Suzuki et al., 1981). Higher doses also result in necrotic lesions.

3.6. Biosynthesis of phenolamides

Phenolamides are essentially derived from aminoacids. Their biosynthesis branches, on one side, on the core phenylpropanoid pathway at the level of hydroxycinnamoyl-CoA esters (Vogt, 2010). The hydroxycinnamoyl moiety thus derives from deaminated phenylalanine. The amine moiety results either from the ubiquitous polyamine pathway for the most widespread putrescine, spermidine and spermine conjugates found in all seed plants, either from just decarboxylated amino acids (and occasionally from an aminoacid, e.g. from tyrosine) (Fig. 4). The most common conjugate derived from a decarboxylated amino acid is feruloyltyramine that has been found in a broad range of species (Smith, 1977). Accordingly, the tyrosine decarboxylase gene characterized initially in tobacco (Farmer et al., 1999), potato (Schmidt et al., 1999) and opium poppy (Facchini et al., 1999) was since described in a variety of plants such as tomato (von Roepenack-Lahaye et al., 2003), *Camptotheca acuminata* (Lopez-Meyse and Nessler, 1997), *Ophiorrhiza pumila* (Yamazaki et al., 2003), rice (Kang et al., 2007) and *A. thaliana* (Lehmann and Pollmann, 2009). In some plants, more specific agmatine, tryptamine or phenylethylamine conjugates result from arginine, tryptophan or phenylalanine decarboxylation. The evolution, biochemistry and regulation of aminoacids decarboxylases were reviewed by Facchini et al. (2000). Decarboxylation products can be hydroxylated to form, for example, octopamine, dopamine, serotonin and noradrenaline that can also be conjugated to form phenolamide products (Matsuda et al., 2005). The 5-hydroxylation of tryptamine for the formation of serotonin can be catalyzed by a soluble tetrahydrobiopterin-dependent enzyme (Kang et al., 2007) or by a cytochrome P450 CYP71P1 (Fujiwara et al., 2010) in rice, expression of the latter being correlated with defence against rice blast. In pepper, two tryptophan decarboxylases have been described. One of them is only expressed upon fungal attack or ethylene for the synthesis of the hydroxycinnamoylserotonin phytoalexins (Park et al., 2009b). Anthranilate, another common amine moiety, derives from the aromatic amino acid precursor chorismate via an elicitor-inducible anthranilate synthase AS subunit. This enzyme is insensitive to tryptophan feedback and constitutes a paralogue of the enzyme involved in tryptophan biosynthesis (Bohlmann et al., 1996).

The conjugates can be further decorated via species-specific hydroxylation, methylation, cyclisation or coupling reactions. The latter modifications are poorly described so far, except for the intramolecular phenol-oxidative coupling of N^1,N^{10}-bis(*p*-coumaroyl)spermidine forming the alkaloid lunarine in the seeds of *Lunaria annua*, and the final oxidative coupling of (*S*)-dihydroverbacine leading to the synthesis of macrocyclic spermine alkaloids aphelandrine and orantine from *Aphelandra* sp. (Nezbedova et al., 2001). In both cases, the reaction was proposed to be catalyzed by a cytochrome P450 enzyme, based on its regio/stereoselectivity, on the microsomal localization of the enzyme activity and NADPH-dependence. In the case of alephandrine formation, this assumption was further supported by the O_2-dependence and CO-inhibition of the reaction.

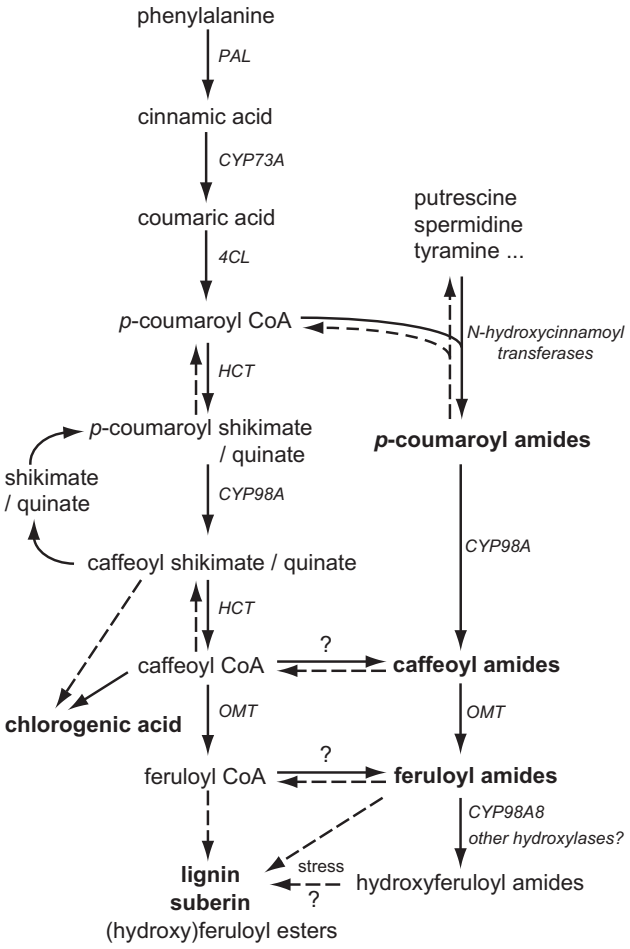

Fig. 4. Connection of phenolamides to the phenolic metabolism. PAL, phenylalanine ammonia lyase; 4CL, 4-coumaroyl-CoA ligase; HCT, hydroxycinnamoyl:shikimate/quinate hydroxycinnamoyl transferase; OMT, *O*-methyl transferase.

3.6.1. A panel of N-hydrocinnamoyl transferases

Coupling of the hydroxycinnamoyl and amine moieties is a critical step and can be considered as the real entry point to the phenolamide branch pathways. It is catalyzed by a diversity of soluble specific hydroxycinnamoyl transferases, several of them belonging to the superfamily of BAHD acyltransferases (D'Auria, 2006). In this family, five clades have been defined. *O*-hydroxycinnamoyl transferases producing phenolic esters belong to clade V (D'Auria, 2006; Petersen et al., 2009). *N*-hydroxycinnamoyl transferases using aliphatic amines as acyl acceptors (*N*-aliphatic AHCAT) belong to this same group, with the exception of barley agmatine:hydroxycinnamoyl transferase (ACT) found in clade IV (Burhenne et al., 2003) and *Arabidopsis* ACT surprisingly located in clade 1 (Muroi et al., 2009). A number of *N*-aliphatic AHCAT have

been purified from different plants and some related genes cloned and functionally characterized. Their properties are summarized in Table 1.

AHCAT are soluble enzymes and their specificity for the acyl acceptor and acyl donor varies, depending of the plant source. Di- and tri-acylated amines are commonly detected in plants. *Arabidopsis* spermidine transferases SCT (spermidine:dicoumaroyl transferase) and SDT (spermidine: di-sinapoyl transferase) can form di-acylated conjugates by sequential transfer of two hydroxycinnamoyl-CoA to spermidine (Luo et al., 2009), whereas the corresponding tobacco SHT transfers a single acyl group, the product formed *in vitro* identified as N^1-feruloylspermidine (Negrel et al., 1991). After incubation of *Arabidopsis* SHT (spermidine:hydroxycinnamoyl transferase) with spermidine and feruloyl-CoA, triferuloylspermidine was the major reaction product (Grienenberger et al., 2009). *N*-acylating enzymes using aliphatic tetra-amines (agmatine, spermine) or aromatic diamines (serotonin) have not yet been shown to transfer more than a single acyl group. Another group of *N*-hydroxycinnamoyl transferases uses aromatic amines as acyl acceptors. HCBT and HHT (Table 1) acylate anthranilate and 5-hydroxyanthranilate, respectively, for the biosynthesis of specific phytoalexins in carnation and *Arabidopsis*. Both belong to clade V of the BAHD family (Muroi et al., 2009).

All other enzymes described so far catalyze the synthesis of amides from tyramine. Tyramine:*N*-hydroxycinnamoyl transferases (THTs) were extensively studied in Solanaceae. THT enzyme and gene were first identified in tobacco (Negrel and Martin, 1984; Negrel and Javelle, 1997; Farmer et al., 1999). In addition to Solanaceae, THTs were biochemically and/or genetically characterized from Papaveraceae, Piperaceae and Gramineae (Table 1). Plant THTs are not members of the BAHD family. Analysis of their amino acid sequences (Farmer et al., 1999; Kang et al., 2006; Schmidt et al., 1999) revealed substantial homology with mammalian spermidine/spermine acetyltransferases (SSAT) and more generally GCN5-related *N*-acetyltransferases (GNAT). Eukaryotic SSAT (Lu et al., 1996; Pegg, 2008) and several microbial antibiotic *N*-acetyltransferases contain a highly conserved domain (RGFGIGS motif), where three amino acids residues (Arg, Gly, Gly) have been shown to be essential for activity and are conserved in plant THTs. Like human SSAT, plant THT enzymes also seem to be active as dimers (Lu et al., 1996; Schmidt et al., 1999). Analyses of purified proteins by SDS/PAGE and HPLC suggest that tobacco and opium poppy THT are homodimers with subunits of 24–28 kDa each (Farmer et al., 1999; Yu and Facchini, 1999).

Substrate specificities of THT enzymes have been investigated in detail. Their best acyl acceptor was in most cases tyramine. All share a marked preference for feruloyl-CoA compared to other hydroxycinnamoyl-CoAs. CASHT (serotonin:*N*-hydroxycinnamoyl transferase) and THT proteins isolated from pepper have clearly distinct substrate specificities. Their catalytic efficiencies were measured on purified, native or recombinant enzymes (Burhenne et al., 2003; Kang et al., 2006). Recombinant THT does not accept serotonin as substrate but efficiently catalyzes the synthesis of feruloyltyramine and *p*-coumaroyltyramine. When feruloyl-CoA is the acyl donor, CASHT has a sixfold lower catalytic efficiency with tyramine than with serotonin (Jang et al., 2004). A functional analysis using recombinant chimeric CASHT and THT proteins was performed to determine the specificity of the amine-binding domain (Kang et al., 2006) showing that tyrosine 149 is a critical amino acid residue controlling amine substrate specificity. Kinetic studies and product inhibition patterns on THT indicate that the mechanism of catalyzis is ordered (or iso-ordered) bi–bi, with hydroxycinnamoyl-CoA being the first substrate to bind the transferase (Hohlfeld et al., 1995; Negrel and Javelle, 1997).

The apparent K_m values reported for purified or recombinant *N*-hydroxycinnamoyl transferases usually vary between 1 and 10 μM for the best acyl donor (CoA ester) and from 22 to 76 μM for the preferred amine substrate (Table 1). This compares to values ranging from 50 to 600 μM for the acyl donors and 0.75–70 mM for quinate or shikimate in the case of the *O*-hydroxycinnamoyl transferases (Hoffmann et al., 2003; Niggeweg et al., 2004) that are assumed to drive the main flow of precursors to the cell-wall formation. Since phenolamides accumulate often at high concentrations in the plant tissues, an important question is "can they be remobilized to restore the amine and phenolic precursors?" The reversibility of the reaction was seldom evaluated. When feruloyl or *p*-coumaroyltyramine were incubated with potato THT and free coenzyme A, feruloyl or *p*-coumaroyl-CoA and free tyramine were formed (Hohlfeld et al., 1995). The high value calculated for the equilibrium constant $K = 1.3 \times 10^4$ and the relatively high negative value for $\Delta G'^{\circ}_{eq}$ (-23.5 kJ mol^{-1}) are in favour of a reaction mainly in the direction of the acylated products. The major spermidine conjugate found in *Arabidopsis* seeds is a 4′-*O*-glycosyl-di(sinapoyl)-spermidine (Luo et al., 2009). *In vitro*, di-sinapoyl spermidine was predominantly formed when recombinant SDT was incubated ($k_{cat} \approx 5$ s^{-1}) with spermidine ($K_{mapp} = 37$ μM) and sinapoyl-CoA (8 μM), but SDT also catalyzes di-sinapoyl spermidine hydrolysis in the presence of CoA with a $k_{cat} \approx 38$ s^{-1} ($K_{mapp} = 35$ μM for di-sinapoyl spermidine and 84 μM for CoA). During germination, the decreased level of spermidine conjugates correlates well with the higher SDT activity. The available data however were not sufficient to determine flux direction *in planta*, which may depend on metabolite concentrations and physiological context.

Expression of *N*-hydroxycinnamoyl transferases has been associated with different stages of the plant development (Table 1) and is most often activated by viral (Negrel and Martin, 1984), fungal, bacterial infections and chemical elicitation (Hohlfeld et al., 1995; Ishihara et al., 1998; Von Roepenack-Lahaye et al., 2003; Yang et al., 2004; Muroi et al., 2009), or by wounding (Ishihara et al., 2000) and abiotic stress such as UV treatment (Back et al., 2001).

3.6.2. A versatile P450 family for 3′-hydroxylation of phenolic conjugates

For a long time it was taken for granted that hydroxycinnamoyl transferases were assembling *p*-coumaroyl-, caffeoyl-, feruloyl- or sinapoyl- and amine-building blocks to form terminal conjugates. Recent data rather suggest that phenolamides could also be metabolic intermediates contributing to form multiple branches on the phenylpropanoid pathway (Fig. 4). This hypothesis was initially supported by flux analyses in potato tuber indicating that both phenolic esters and amides were turned over, and that turnover of amides of tyramine and octopamine was selectively and strongly increased upon elicitor treatment (Matsuda et al., 2005). These studies also suggested that feruloyltyramine and feruloyloctopamine could result from direct hydroxylation and methylation of *p*-coumaroyltyramine and *p*-coumaroyloctopamine. This assumption has recently found some support with the demonstration that cytochrome P450 enzymes belonging to the CYP98 family can catalyze the *meta*-hydroxylation of phenolamides. It was first shown that coumaroyltyramine was taken as a substrate for *meta*-hydroxylation by CYP98 enzymes from different plants including *Arabidopsis* CYP98A3, sweet basil CYP98A13 and wheat CYP98A11 and CYP98A12 (Morant et al., 2007). The reaction catalytic parameters of wheat CYP98A11 and CYP98A12 indicated that gene duplication might have led to the evolution of more competent isoforms for the hydroxylation of phenolamides.

A striking example of such specialization has recently emerged from a reverse genetics approach of CYP98A8 and CYP98A9 function in *A. thaliana* (Matsuno et al., 2009; Fellenberg et al., 2009). Both of them were shown to result from a retroposition and accel-

Table 1
Characteristics of purified or recombinant hydroxycinnamoyl-CoA:amine *N*-(hydroxycinnamoyl) transferases.

Enzyme	Plant source	Size of monomer/ highest or tissue specific expression	Major acyl acceptors (in decreasing order of catalytic efficiency)		Major acyl donors (in decreasing order of catalytic efficiency)	NCBI Genbank Nucleotide Accession No.	References
ACT (EC 2.3.1.64)	*Hordeum vulgare*	Monomer 48 kDa	Aliphatic amines	Absolute specificity for agmatine (K_m: 5–8 μM)	– Cinnamoyl-CoA – Coumaroyl-CoA($K_m \approx 2$ μM) – Feruloyl-CoA – Caffeoyl-CoA	AY228552 AY234333	Bird and Smith (1981), Bird and Smith (1983) and Burhenne et al., 2003
ACT	*Arabidopsis thaliana*			Agmatine	– *p*-Coumaroyl-CoA – Feruloyl-CoA	BT011800(At5g61160)	Muroi et al. (2009)
SHT	*Nicotiana tabacum*			High specificity for spermidine	– *p*-Coumaroyl-CoA – Feruloyl–caffeoyl CoA		Negrel et al. (1991)
SpmHT/SHT	*Aphelandra tetragona*			Spermidine spermine	– *p*-Coumaroyl-CoA – Feruloyl-CoA		Hedberg et al. (1996)
SCT	*Arabidopsis thaliana*	54 kDa		Absolute specificity for spermidine ($K_m \approx 52$ μM)	– Absolute specificity for *p*-coumaroyl-CoA ($K_m \approx 10$ μM)	NM_128072 (At2g25150)	Luo et al. (2009)
SDT		53 kDa siliques, emerging seedlings		High specificity for spermidine ($K_m \approx 37$ μM)	– High specificity for sinapoyl-CoA ($K_m \approx 8$ μM)	NM_127915 (At2g23510)	
SHT		51 kDa tapetum of anthers		Absolute specificity for spermidine	– Feruloyl-CoA – Caffeoyl/coumaroyl-CoA – Cinnamoyl-CoA	NM_127464 (At2g19070)	Grienenberger et al. (2009)
PHT (EC 2.3.1.138)	*Nicotiana tabacum*	≈50 kDa		Putrescine cadaverine	– Caffeoyl-CoA – Feruloyl/cinnamoyl-CoA		Meurer-Grimes et al. (1989), Negrel (1989), Negrel et al. (1991) and Negrel et al. (1992)
HCBT (EC 2.3.1.44)	*Dianthus caryophyllus*	Monomer 53 kDa	Arylamines	High specificity for anthranilate	– Cinnamoyl/*p*-coumaroyl-CoA – BenzoylCoA	Z84383	Yang et al. (1997)
HHT	*Avena sativa*	48 kDa		5-Hydroxyanthranilate	– Feruloyl-CoA – Avenalumoyl-CoA – Cinnamoyl-CoA	AB076980 AB076981 AB076982	Ishihara et al. (1998) and Yang et al. (2004)
THT (EC 2.3.1.110)	*Nicotiana tabacum*	Homodimer subunits of ≈24 kDa		Tyramine ($K_m \approx 24$ μM) octopamine	– Feruloyl-CoA ($K_m \approx 1$ μM)	AJ005062AJ131767 AJ131768	Negrel and Martin (1984), Negrel and Javelle (1997) and Farmer et al. (1999)
THT	*Solanum tuberosum*	Heterodimer (≈49 kDa, gel filtration) subunits of ≈25 kDa		Tyramine ($K_m \approx 22$ μM) octopamine	– Cinnamoyl/feruloyl-CoA($K_m \approx 36$ μM) – *p*-Coumaroyl-CoA		Hohlfeld et al. (1995) and Hohlfeld et al. (1996)
THT	*Solanum tuberosum*	Dimer (63 kDa, gel filtration) subunits of ≈30 kDa		Tyramine ($K_m \approx 40$ μM) octopamine	– Cinnamoyl-CoA ($K_m \approx 60$ μM) – Feruloyl-CoA	AB061243	Schmidt et al. (1999)
THT	*Papaver somniferum*	Homodimer subunits of 25 kDa mature roots		Tyramine ($K_m \approx 76$ μM)	– Feruloyl-CoA – Cinnamoyl-CoA ($K_m \approx 2$ μM) – Sinapoyl-CoA – *p*-Coumaroyl-CoA		Yu and Facchini (1999)
THT	*Solanum lycopersicum*	≈27 kDa		Tyramine ($K_m \approx 4$ μM) noradrenaline dopamine, octopamine	– *p*-Coumaroyl-CoA (the only acyl donor tested)	AY081907 AY081905 AY081908 AY081906	Von Roepenack-Lahaye et al. (2003)
THT	*Capsicum annuum*	28 kDa young stems and roots		Tyramine ($K_m \approx 40$ μM) but not serotonin	– Feruloyl/*p*-coumaroyl-CoA ($K_m \approx 20$ μM)	AY819700	Kang et al. (2006)
CASHT	*Capsicum annuum*	28 kDa flowers		Serotonin ($K_m \approx 73$ μM) tyramine	– Caffeoyl-CoA – Feruloyl/*p*-coumaroylCoA	AF329463	Back et al. (2001), Jang et al. (2004) and Kang et al. (2006)
THT	*Zea mays*	40 kDa (gel filtration)		Tyramine, tryptamine ($K_m \approx 59$ μM) dopamine phenethylamine	– Feruloyl-CoA ($K_m \approx 5$ μM) – Sinapoyl-CoA – *p*-CoumaroylCoA		Ishihara et al. (2000)

Table 1 (*continued*)

Enzyme	Plant source	Size of monomer/ highest or tissue specific expression	Major acyl acceptors (in decreasing order of catalytic efficiency)	Major acyl donors (in decreasing order of catalytic efficiency)	NCBI Genbank Nucleotide Accession No.	References
THT	*Triticum aestivum*	Seedling, roots	Tyramine	– Sinapoyl-CoA – Feruloyl-CoA		Louis and Negrel (1991)
THT	*Hordeum vulgare*	Germinating barley roots	Tyramine phenylethylamine	– Sinapoyl-CoA – Feruloyl-CoA – *p*-CoumaroylCoA		Louis and Negrel (1991)

ACT, agmatine:*N*-coumaroyl transferase; CASHT, serotonin:*N*-hydroxycinnamoyl transferase; HCBT, hydroxycinnamoyl/benzoyl-CoA:anthranilate:hydroxycinnamoyl/benzoyl transferase; HHT, hydroxyanthranilate:hydroxycinnamoyl transferase; PHT, putrescine:*N*-hydroxycinnamoyl transferase; SCT, spermidine:*N*-dicoumaroyl transferase; SDT, spermidine:*N*-di-sinapoyl transferase; SHT, spermidine:*N*-hydroxycinnamoyl transferase; SpmHT, spermine:*N*-hydroxycinnamoyl transferase; THT, tyramine:*N*-hydroxycinnamoyl transferase.

erated evolution leading to the apparition of enzymes dedicated to the *meta*-hydroxylation of tri-*p*-coumaroylspermidine and, for CYP98A8, also to the *meta*-hydroxylation of tri-feruloylspermidine for the formation of N^1,N^5-di(hydroxyferuloyl)-N^{10}-sinapoyl spermidine, a major pollen coat constituent (Matsuno et al., 2009). These enzymes thus seem to be acting downstream of the SHT transferases mentioned above (Fig. 4). However, the function of these phenolamide hydroxylases does not appear restricted to the tapetum during pollen development. CYP98A9 gene expression was also detected in vascular tissues and root stele and tip (Matsuno et al., 2009). Expression of CYP98A8 was detected in seeds (Fellenberg et al., 2008). It is thus possible that pathways involving phenolamide hydroxylation are involved in other aspects of plant development and are active downstream of other polyamine hydroxycinnamoyl transferases such as those described by Luo et al. (2009). Interestingly, Matsuno et al. (2009) pointed the fact that, while the canonical *Arabidopsis* CYP98A3 preferred substrate is the shikimate ester of *p*-coumaric acid, it is also able to hydroxylate the tri-*p*-coumaroyl amide of spermidine, though with a lower efficiency.

3.6.3. Further decoration of the resulting phenolamides

Final products accumulated by the plant usually include feruloyl derivatives and sometimes sinapoylated compounds. Fellenberg et al. (2008) have recently demonstrated that AtTSM1, an *O*-methyltransferase belonging to the *A. thaliana CCoAMT* gene family, was exclusively expressed in the tapetum, where it was catalyzing the terminal methylation of tri-(5-hydroxyferuloyl)spermidine into N^1,N^5-di(hydroxyferuloyl)-N^{10}-sinapoyl spermidine. Further investigations also suggested that CCoAMT1, the methyltransferase involved in caffeoyl-CoA methylation in lignin biosynthesis and also associated with flavonoid and sinapoylmalate biosynthesis (Do et al., 2007), catalyzes methylation of tri-caffeoylspermidine into tri-feruloylspermidine (Fellenberg et al., 2009). While hydroxycinnamoylspermidines are apparently not the preferred substrates for AtTSM1 and CCoAMT1 in vitro, it is interesting to note that their roles as phenolamide methylases seem to be validated *in vivo* (Fellenberg et al., 2008).

Enzymes involved in sugar conjugation of phenolamides have not been described yet. Glycosylated derivatives have however been identified during seed (Luo et al., 2009) and flower (Fellenberg et al., 2009) development. Their abundance was higher in *cyp98a8* mutants as compared to wild-type. Glycoside formation was also detected upon SCT overpression in *Arabidopsis* leaves (Luo et al., 2009). Glycosylation thus seems to occur upon intracellular accumulation of hydroxycinnamoylspermidines.

3.6.4. Regulatory genes

The different branches of the phenolamide pathways are obviously differentially regulated with, for example, basic and neutral phenolamides being under separate transcriptional control (see for example Matsuda et al., 2005; Kaur et al., 2010). However, information on their regulatory cascades is still scarce. It is well established that accumulation of caffeoylputrescine in Solanaceae is under the control of the jasmonate signalling pathway (Chen et al., 2006; Tebayashi et al., 2007; Paschold et al., 2007) and, recently, the first regulatory elements involved in this JA-response have been identified. Two homologous R2R3MYB transcription factors, *Nt*MYBJS1 from *N. tabaccum* and *Na*MYB8 from *N. attenuata*, were found to control *p*-coumaroyl- caffeoyl- and feruloylputrescine accumulation in BY-2 cell cultures and intact plants respectively (Gális et al., 2006; Kaur et al., 2010). Overexpression and antisense constructs of *Nt*MYBJS1 showed that it specifically controls the JA-response of a subset of genes in the phenylpropanoid and polyamine pathways. The MYB DNA-binding domain was able to specifically bind PAL-A and PAL-B promoters. Consistently,

*Nt*MYBJS1 overexpression led to the accumulation of hydrocinnamoylputrescines (Gális et al., 2006). The role of *Na*MYB8 was analyzed by Kaur et al. (2010) in the more ecologically relevant context of *N. attenuata*. They showed that its expression was activated by mechanical wounding, and amplified by the simultaneous application of *M. sexta* oral secretions. Caterpillar feeding-induced local and systemic accumulation of caffeoylputrescine and dicaffeoylspermidine was suppressed in *Na*MYB8 inverted-repeat silenced plants. *Na*MYB8 silencing also reduced transcriptional activation of a large set of genes related to the phenylpropanoid and polyamine pathways. As mentioned above, suppression of phenolamide production in the silenced plants was associated with an improved performance of generalist and specialist herbivores. The same study also demonstrated that *Na*MYB8 silencing suppresses the constitutive accumulation of caffeoylputrescine in young leaves and reproductive tissues. Thus *Na*MYB8 also controls caffeoylputrescine synthesis during plant development.

A similar study has been carried out by Shinya et al. (2007) and led to the identification of a β-glucan and laminarin responsive elements in tobacco BY-2 cells. This work allowed the identification of another R2R3MYB-type transcription factor termed *Nt*MYBGR1 that targets phenylpropanoid genes for glucan-induced accumulation of caffeoylputrescine and feruloylputrescine and might be representative of plant response to fungal attack.

3.7. Overlooked branching in the phenylpropanoid metabolism?

It gradually appears that phenolamides have to be regarded as metabolic intermediates rather than just final products. They undergo hydroxylation and methylation of the phenolic rings, can be stored as hexose conjugates and remobilized upon demand. High polyamine concentrations accumulate in the plant cells. The low K_m of *N*-hydroxycinnamoyl transferases for their amine acceptors results in the formation of significant pools of amide derivatives where *p*-coumaroyl shikimate/quinate concentrations are usually below detection threshold (Matsuda et al., 2005; our own observations). This might be corrected by a higher affinity of P450 enzymes for ester conjugates than for the corresponding amides, but the large gap in the size of the pools of precursors suggests that the role of phenolamides in relation to the phenylpropanoid pathway has to be reassessed. To evaluate the potential contribution of hydroxycinnamoyl amides in the formation and storage of guayacyl and syringyl units, a more extensive description of the enzymes catalyzing their hydroxycinnamoyl transfer, hydroxylation, methylation and glycosylation/deglycosylation steps will be required. Of particular interest will be the reverse reactions catalyzed by hydroxycinnamoyl transferases to convert the amides into CoA esters. More information is also required about fluxes through the amides and esters branches of conjugates in different plant tissues, and about their storage and transport.

Besides participation to branched pathways, phenolamides have clearly acquired specific functions in plant development and defence. Two of them seem to be shared by several compounds. Those are (1) a contribution to cell-wall cross-linking and reinforcement and (2) a direct toxicity for predators and pathogens, either as built-in or inducible defence. Differences in species-specific metabolites also account for evolution of specific defence or recognition systems. Some amine conjugates such as putrescine, spermidine or tyramine derivatives are present in all Angiosperms and probably beyond. They are expected to have more generic functions in plant structure, signalling, in particular in the reproductive cycle. Mutants isolated so far failed to identify clear effects on plant reproduction resulting from the suppression of the putrescine and spermidine conjugate pathways (Fellenberg et al., 2008, 2009; Matsuno et al., 2009; Grienenberger et al., 2009; Kaur et al., 2010). This might be due to partial functional redundancies or to the fact that the phenotypes were usually evaluated with RNAi or antisense lines or under laboratory conditions. Kaur et al. (2010) interpreted the absence of developmental phenotype as a demonstration that phenolamides are exclusively involved in defence and are accumulated in reproductive tissues to protect them against abiotic or biotic stresses, according to the Optimal Defence Theory. Further studies involving KO suppression mutants tested under field conditions are however required to confirm this hypothesis.

Phenolamides are quantitatively major plant metabolites that are tightly regulated during plant ontogeny and adaptative responses. Their role in plant biology is still surprisingly overlooked but raises an increasing interest. While biological functions of phenolamides are obviously linked to those of their polyamine and phenolic constituents, evolution of species-specific branched pathways is indicative of the acquisition of a diversity of metabolite-specific functions. It should also be considered that phenolamides, in particular via polyamines, offer an opportunity of cross-talk between nitrogen and phenolic metabolisms.

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