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Revealing insect herbivory-induced phenolamide metabolism: from single genes to metabolic network plasticity analysis

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Abstract

The phenylpropanoid metabolic space comprises a network of interconnected metabolic branches that contribute to the biosynthesis of a large array of compounds with functions in plant development and stress adaptation. During biotic challenges, such as insect attack, a major rewiring of gene networks associated with phenylpropanoid metabolism is observed. This rapid reconfiguration of gene expression allows for the prioritized production of metabolites that help the plant solve ecological problems. Phenolamides are a group of phenolic-derivatives that originate from the diversion of hydroxycinnamoyl acids from the main phenylpropanoid pathway after *N*-acyltransferase-dependent conjugation to polyamines or aryl-monoamines. These structurally diverse metabolites are abundant in reproductive organs of many plants and have recently been shown to play roles as induced defenses in vegetative tissues. In the wild tobacco, *Nicotiana attenuata* in which the herbivory-induced regulation of these metabolites has been studied, rapid elevations of phenolamide levels that function as induced defenses result from a multi-hormonal signaling network that reshapes connected metabolic pathways. In this review, we summarize recent findings in the regulation of phenolamides obtained by mass spectrometry-based metabolomics and outline a conceptual framework for gene discovery in this pathway. We finally introduce a multifactorial approach useful in deciphering metabolic pathway reorganizations among different tissues in response to stress.

Keywords

Phenolamides; Phenylpropanoid pathway; *N*-acyltransferase; Metabolomics; Systems biology; Self-organizing maps; *Nicotiana attenuata*

Introduction

Over the last three decades, it has become abundantly clear that the plethora of small molecules produced by plants which are not directly required for growth play important

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roles as signal and defense molecules rather than being 'waste' products (Berenbaum and Zangerl 2008, Hartmann 2007, Pichersky and Gang 2000, Pichersky and Lewinsohn 2011). In contrast to those derived from 'primary' metabolic pathways, the biosynthesis of these metabolites is restricted to selected plant taxa. This suggests that specific biosynthetic pathways have been positively selected throughout the course of evolution in particular plant lineages when a compound or group of compounds addressed specific ecological needs. Consistent with this view, ecological and/or evolutionary insights gained from field studies and lab-based functional genomic analyses has clearly established that a plant's interaction with insects has sculpted many aspects of plant metabolism. This includes the composition and size of metabolic classes as well as regulatory networks that determine their fluxes (Agrawal 2012, Berenbaum and Zangerl 2008, Prasad *et al.* 2012). The frequently invoked functional distinction between secondary and primary metabolites is therefore more a reflection of our ignorance of the genes controlling their biosynthesis and their biological function (Pichersky and Gang 2000). Metabolites derived from the shikimate/ phenylpropanoid core pathway illustrate how sophisticated rearrangements of central metabolic pathways allow plants to solve ecological challenges imposed by their interactions with insects.

Phenylpropanoid derivatives are found ubiquitously across the plant kingdom and share at least one aromatic hydrocarbon ring with one or more hydroxyl groups attached to it, a common feature derived from the skeleton of phenylalanine (Vogt 2010). Simple hydroxycinnamoyl acids and esters produced by the core part of the shikimate pathway serve, after oxidation, reduction, methylation, decoration with different kinds of small molecules and/or polymerization, as metabolic units for the production of an enormous array of compounds such as, among others, flavonoids, coumarins, lignin and phenolamides. These different metabolic classes play essential roles in development but also as plant defenses against biotic challenges. For example, phenolic-derived floral scents and pigments are essential determinants of a plant's fertility and outcrossing rates by attracting insect or bird pollinators (Dudareva and Pichersky 2000, Kessler *et al.* 2013). On the other hand, many structurally different phenolics rapidly accumulate to higher levels as components of an induced defense arsenal against hervivore attack (Karban and Baldwin, 1998), processes involving plant species specific transcriptionally-mediated rearrangements of metabolic pathways (Howe and Jander 2008). Important insights into the structural and regulatory genes of the core phenylpropanoid pathway have been summarized in several review articles (Costa *et al.* 2003, Tohge *et al.* 2013, Vogt 2010). In contrast, how stressed cells re-channel metabolic fluxes of the phenylpropanoid core pathway for the production of a specific spectrum of metabolites required to solve ecological problems remains largely unknown. The same holds for the well-studied downstream branches for which some branch-specific transcription factors have been identified.

In this review, we describe recent insights into the regulation of phenolamide production, a pathway that originates from the diversion of hydroxycinnamoyl acids from the main phenylpropanoid pathway after conjugation to polyamine or aryl-monoamine molecules by hydroxycinnamoyl-CoA:amine *N*-(hydroxycinnamoyl)transferases -- hereafter referred to as N-acyltransferases --(Bassard *et al.* 2010, Edreva *et al.* 1998, Facchini *et al.* 2002). These metabolites,sometimes referred to as phenylamides or more accurately as *N*-

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hydroxycinnamoyl-amine conjugates, are a diverse group of phenolic-derived secondary metabolites found in many dicotyledonous as well as monocot plant lineages (Bassard *et al.* 2010, Edreva *et al.* 2007, Facchini *et al.* 2002, Martin-Tanguy *et al.* 1978, Martin-Tanguy 1985). Organ-specific pools of phenolamides were originally thought to function only in developmental homeostasis, but more recently these products have been shown to accumulate during stress and to function as induced defenses (Demkura *et al.* 2010, Kaur *et al.* 2010, Martin-Tanguy 1985, Muroi *et al.* 2009, Newman *et al.* 2001). Different plant species have been shown to accumulate phenolamides during insect herbivory, but important advances in the defensive function of these metabolites have been mainly obtained in transgenic *Nicotiana attenuata* plants for which precise genetic manipulations of phenolamide transcriptional regulation and structural genes were conducted. The dramatic increases in the production of some of these metabolites during insect herbivory in *N. attenuata* (Gaquerel *et al.* 2010, Kaur *et al.* 2010, Onkonkesung *et al.* 2012) have been shown to *(i)* reshape many other connected metabolic pathways (Gaquerel *et al.* 2013), *(ii)* be indicators of herbivory-induced hormonal signals spreading throughout a plant and *(iii)* decrease insect performance (Kaur *et al.* 2010).

In a nutshell, probing this complex metabolic grid may provide an interesting framework for assessing transcriptional and metabolic controls that prioritize the activation of metabolic branches when a plant is attacked by insect herbivores. In the conceptual framework we outline here, understanding metabolite regulation through metabolomics approaches is the first step in the gene discovery process, which we will illustrate with recent work in *N. attenuata*, a plant with well-known herbivory-induced signaling (Wu and Baldwin, 2010). As the herbivory-specific co-expression patterns among genes shaping phenolamide metabolism deployed throughout the plant are likely organ-specific, we describe a newly developed multifactorial approach for deciphering whole-organism metabolic pathway reorganizations.

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Mass spectrometry-based profiling of the phenolamide metabolic grid

Phenolamide structural diversity has been summarized by Bassard *et al.* (2010). Briefly, phenolamides have been identified in many dicotyledonous plants as well as in monocots, including wheat (*Triticum aestivum*), barley (*Hordeum vulgare*), rice (*Oryza sativa*) and maize (*Zea mays*) (Bassard et al. 2010, Edreva *et al.* 2007, Facchini *et al.* 2002, Martin-Tanguy *et al.* 1978, Martin-Tanguy 1985). Abundance and organ-specific profiles of phenolamide-producing plant lineages have been carefully documented in several studies (e.g. Martin Tanguy *et al.* 1978, Martin-Tanguy 1985, 1997). Activated forms of coumaric, caffeic and ferulic acids in combination with either aryl-monoamines (tyramine, tryptamine, anthranilate, etc…) or polyamines (putrescine and spermidine) are the most commonly encountered building blocks. In the case of spermidine-containing metabolites, the phenolamide ‘codebook’ produces mono-, di- and tri-substituted metabolites, which results in a large repertoire of structures that can be additional decorated *a posteriori* (Fellenberg *et al.* 2008, 2009, 2012a, Matsuno *et al.* 2009). The degree of acylation, and in turn, the resulting number of free residual amino groups, determines the phenolamides’ physicochemical properties as well as the biological functions mediated by these metabolites. Representative acylated putrescine and spermidine phenolamide structures are presented in Figure 1.

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Profiling phenolamide levels across tissue types by medium to high-throughput mass spectrometry-based metabolomics has proved decisive in revealing the genomic basis of phenolamide biosynthesis. Ontogenically-tuned increases in phenolamide levels have for instance been reported during herbivory (Kaur *et al.* 2010, Onkokesung *et al.* 2012). High levels of polyamine-based phenolamides have long been known as a characteristic feature of developing flower buds of many species and floral organ-specific phenolamide profiles are known to correlate with floral developmental stages (Fellenberg *et al.* 2009, Fellenberg *et al.* 2008, Fellenberg *et al.* 2012a, 2012b, Grienenberger *et al.* 2009, Matsuno *et al.* 2009). Phenolamides appear to be absent from mutants that do not flower or produce abnormal flowers suggesting that these metabolites fulfill important roles during normal flower development, however the exact function of these metabolites in these tissues, including the pollen coat, where they can be particularly abundant, remains puzzling.

The modular structure of phenolamides renders them easily tractable for elucidation by mass spectrometry, as spectrometry fragmentation patterns measured with high-resolution and even low-resolution mass spectrometers are frequently highly diagnostic and sufficient for identification of building blocks (Gaquerel *et al.* 2013, Matsuno *et al.* 2009, Onkokesung *et al.* 2012). Many members of the phenolamide library, especially poly-acylated metabolites, undergo fragmentation during in-source ionization so that, among others, coumaroyl- (*m/z* 147.05), caffeoyl- (*m/z* 163.04), or feruloyl (*m/z* 177.05) moieties resulting from cleavage from different core molecules (e.g. polyamine, monoarylamines but also sugars or quinate molecules) can be queried to compute extracted ion currents from mass spectrometry-based chromatograms recorded in profiling mode without bias towards specific metabolic classes (Onkonkesung *et al.* 2012). More specific *m/z* signals corresponding to either the mono-acylated spermidine molecules or fragments from polyacylated spermidines (e.g. *m/z* 308.21 for a mono-caffeoylspermidine molecule or fragment) can be used to visualize acylated spermidine profiles within an extract (Figure 1). To comprehensively mine these data, compound-specific pseudo-spectra collected in the profiling mode can be deconvoluted in an automated fashion by Open Access programs such as the R package CAMERA (Kuhl *et al.* 2012) as a preliminary step prior to statistical analysis to evaluate the enrichment of phenolamide diagnostic ions (Onkonkesung *et al.* 2012). The location of the phenolic moieties within the polyamine skeletons cannot be rigorous assigned from mass spectrometry data alone to positions N_1, N_5 or N_{10} of spermidine due to rearrangements occurring during fragmentation (Fellenberg *et al.* 2009, Gaquerel *et al.* 2010). Characterization of the *m/z* signals of the phenolamide building blocks make these metabolites amenable for fragmentation rule-based dereplication approaches (the analytical process by which known metabolites are identified from novel matrices based on established analytical rules).

Gene discovery in the phenolamide metabolic grid

The biosynthetic pathways of the amine moieties and of the phenolic blocks from deaminated phenylalanine have been thoroughly reviewed (Bassard *et al.* 2010, Kusano *et al.* 2008, Vogt 2010). The conjugation of phenolic units to a polyamine or an aryl monoamine molecule represents the key metabolic entry point in phenolamide biosynthesis, but the *N*-acyltransferases enzymes catalyzing these important conjugation reactions have remained

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unknown for many years. Recent functional genomic work on floral organ-specific phenolamide biogenesis has identified a panoply of *N*-acyltransferases, most belonging to the BAHD gene family (D'Auria 2006), that catalyze phenolamide biosynthesis. Phylogenetic relationships among the characterized hydroxycinnamoyl-CoA:amine transferases and Arabidopsis BAHD genes are presented in Figure 2. Phylogenetic clustering of these genes according to the type of amine molecule used as the acyl acceptor has been reported (Luo *et al.* 2009), but species-specific divergences are also known among *N*-acyltransferases acting on the same amine skeleton. Three *N*-acyltransferases for phenolamide biogenesis that compete for an overlapping set of phenolic acyl donors during herbivory (Figure 3 and Figure 4) have recently been identified in *N. attenuata* (Onkokesung *et al.* 2012). AT1 controls the production of *N*-coumaroylputrescine and *N*-caffeoylputrescine. *N*-hydroxycinnamoyl-CoA:putrescine transferase enzymatic activities were first characterized by Negrel *et al.* (1989, 1991, 1992), from which the formation of diacylated spermidine phenolamides in tobacco was shown to likely be a two-step reaction. DH29 controls the first acylation step on spermidine and silencing its expression disrupts the accumulation of most wound- and herbivory-induced spermidine-based phenolamides. CV86 is involved in the production of certain diacylated spermidine isomers from DH29-dependent mono-acylated spermidines, suggesting that additional *N*-acyltransferases are required to produce the full spectrum of metabolites found in *N. attenuata*. These two genes are distantly related to Arabidopsis *N*-acyltransferases that use spermidine as an acyl acceptor to catalyze in a single gene manner the sequential synthesis of specific polyacylated spermidine-based phenolamides (Luo *et al.* 2009, Onkokesung *et al.* 2012).

Gene and metabolite expression data sets are increasingly being integrated to accelerate gene discovery in this pathway. Previous MS-based metabolomics investigations in *Arabidopsis thaliana* using a multiple sampling experimental design, publicly available at AtMetExpress webpage (http://prime.psc.riken.jp/lcms/AtMetExpress/) and compatible with the developmental and single-tissue-based experiments available in AtGenExpress that are commonly used by the Arabidopsis community, demonstrated that transcriptional programs largely regulate the tissue-specific production of diverse phytochemicals, with the phenolamides being a case in point (Matsuda *et al.* 2010).

In this pioneering study, the visualization of gene-to-metabolite co-linearity patterns was enabled by the use of an 'Electronic Fluorescent Pictogram' browser (Winter *et al.* 2007) and of co-expression analysis based on self-organizing maps (Hirai *et al.* 2004). These tools pave the way for bioinformatics association studies for the discovery of *N*-acyltransferases with regio-isomer-specific activity for the acylation of spermidine skeletons, as the isomeric profiles of polyacylated spermidines are frequently tissue-specific. Following a similar approach (Ehlting *et al.* 2008), Matsuno *et al.* (2009) discovered the role of two tandem duplicated cytochrome P450 genes, *CYP98A8* and *CYP98A9*, arising from successive retroposition and duplication events. These two genes with pollen-specific expression act downstream of spermidine phenolamide-forming *N*-acyltransferases and control the meta-hydroxylation of the hydroxycinnamoyl moieties of specific phenolamides (Matsuno *et al.* 2009). Co-expression analysis using these two genes as baits identified an alternative phenylpropanoid pathway specifically supplying hydroxycinnamoyl units for the production of pollen coat phenolamides (Matsuno *et al.* 2009).

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Phenolamide metabolism as part of the anti-herbivore defensive arsenal

Robust elevations in the levels of *N*-caffeoylputrescine and of certain *N', N''*-dicaffeoylspermidine isomers occurring during insect feeding have been used to screen alterations in herbivory-induced signaling networks in leaves of *N. attenuata* transformants. MS-based metabolomics of the herbivory-regulated metabolome of this plant has shown that almost every aspects of the phenolamide metabolic grid are reconfigured during insect herbivory but these changes occur differently in locally-treated and systemic leaves of the same plant (Figure 3, Gaquerel *et al.* 2010, Kaur *et al.* 2010, Onkokesung *et al.* 2012). Increased phenolamide production following insect herbivory has also been reported in maize (Marti *et al.* 2013) and pepper (Tebayashi *et al.* 2007). Wounding a source leaf with a fabric pattern wheel on both sides of the midrib and immediately applying *M. sexta* oral secretions to the fresh puncture wounds (hereafter referred to as W+OS or OS-elicitation) provides a convenient means of accurately standardizing herbivore elicitation (McCloud and Baldwin 1997) and activating the associated defense/tolerance/escape responses in *N. attenuata* plants. This procedure recapitulates the major reconfigurations in the phenolamide metabolic nework that are repeatedly induced during insect feeding and allows researchers to conduct replicated time series metabolomics analysis. The resulting metabolic traces can, after appropriate data processing, be used to reconstruct metabolic networks in which phenolamide responses are resolved as the main induced responses.

Most previous analyses on the complete phenolamide profile of *N. attenuata* leaves have shown that, in line with the amplitude of the responses of their underlying biosynthetic genes, putrescine-based phenolamides exhibit more dramatic responses to the W+OS treatment than do the spermidine conjugates and that the caffeic acid-containing metabolites accumulate to higher levels in *N. attenuata* leaves than other types of phenolamides (Figure 3). Putrescine-based phenolamides occur at low levels in non-stressed leaves whereas high amounts of developmentally-regulated spermidine conjugates are regularly detected in most vegetative and reproductive tissues (Kaur *et al.* 2010, Keinanen *et al.* 2001, Onkokesung *et al.* 2012). Dynamics of spermidine-based phenolamides are relatively complex, which may not only reflect the multiple metabolic interconnections existing among these different metabolites but also be a signature for their highly specific functions. Only small amounts of mono-acylated spermidine are typically detected during W+OS treatment while these intermediates accumulate during insect feeding, suggesting a rapid conversion into diacylated forms (Onkokesung *et al.* 2012). Perhaps, as proposed by Bassard *et al.* (2010), these complex patterns illustrate a plant's ability to separately control the accumulation of different diacylated spermidine isomers for specific functions in plant defense and/or development. The turnover and interconversions among phenolamides and their respective free precursors remain to be explored and these may also contribute to the changes in metabolite levels seen during herbivory.

Interestingly, levels of some of these spermidine-based phenolamides rapidly decrease following simulated herbivory in *N. attenuata* such as the unindentifed isomers of *N',N''*-dicaffeoylspermidine while others, such as *N',N''*-caffeoyl,feruloylspermidine, exhibit inversely correlated accumulation patterns. Several studies have shown that further decoration can be added to phenolic residues when conjugated to polyamines. Notably,

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Fellenberg *et al.* (2008) identified an *O*-methyltransferase from the Arabidopsis *CcOAMT* gene family which controls the terminal methylation of tri-(5-hydroxyferuloyl)spermidine into N_1, N_6-di(hydroxyferuloyl)-N_{10}-sinapoylspermidine in the tapetum. Such enzyme-dependent interconversions between pre-existing diacylated spermidine pools (Fellenberg *et al.* 2008, Matsuno *et al.* 2009) are most likely shaping the complex dynamics seen in the accumulation of different spermidine-based phenolamides during herbivory and could for instance involve the methylation of *N',N''*-dicaffeoylspermidine into *N',N''*-caffeoyl,feruloylspermidine.

Regulation via the jasmonate-MYB8 transcriptional module

The accumulation of *N*-caffeoylputrescine in different solanaceous plants (e.g. Tebayashi *et al.* 2007) and the more recently characterized profound reconfigurations of most branches of the phenolamide metabolic network of *N. attenuata* during insect feeding are transcriptionally regulated by the jasmonic acid (JA) signaling pathway (Keinanen *et al.* 2001, Onkokesung *et al.* 2012, Paschold *et al.* 2007, Stitz *et al.* 2011, Ullmann-Zeunert *et al.* 2013) (Figure 4). Previous work with transformants or mutants directly impaired in jasmonate accumulation or perception accumulate much lower levels of *N*-caffeoylputrescine and stress-/herbivory-inducible phenolamides but exhibit less pronounced changes in basal levels of several spermidine-based phenolamides (Onkokesung *et al.* 2012, Paschold *et al.* 2007, Ullmann-Zeunert *et al.* 2013). In general, alterations due to jasmonate signaling deficiency are more pronounced in systemic leaf positions where induced phenolamide accumulation is thought to translate from major transcriptional adjustments initiated by jasmonate-dependent mobile signals transmitted from OS-elicited leaves. Jasmonate-dependent phenolamide accumulation requires the F-box protein COI1. This receptor protein after interaction with jasmonoyl-isoleucine (JA-Ile) targets JAZ transcriptional repressors for degradation by the proteasome, a transcriptional machinery controlling many secondary metabolic pathways (De Geyter *et al.* 2012). The strict requirement of JA-Ile in this process is clearly discernible in lines ectopically expressing an Arabidopsis jasmonic acid-specific methyltransferase (*sJMT*) which specifically depletes JA-Ile accumulation (Stitz *et al.* 2011).

Approaches exploiting natural variations are commonly used in Arabidopsis to infer associations between genes or phytohormone signals, including jasmonates, and quantitative traits of a plant's phenotype. Recent work on naturally variable traits in *N. attenuata* populations have highlighted that there exist important variations in the amplitude of the JA and JA-Ile bursts produced after simulated herbivory in these populations (Machado *et al.* 2013). In panel (b) of Figure 5, we show that herbivory-induced levels of *N*-caffeoylputrescine, as well as of other phenolamides, vary greatly in native populations when grown under controlled conditions of the laboratory, indicating the existence of genetically-determined variations at the level of the regulation and biosynthesis of these metabolites. These patterns of natural variation are significantly positively correlated with the amplitude of JA-Ile bursts.

Jasmonate-dependent activations in phenolamide metabolism do not translate solely from increases in the expression of genes of the phenylpropanoid pathway, most of them being

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well-known expression markers for wound and jasmonate responses. The expression of *N*-acyltransferases required for phenolamide production is also controlled by the jasmonate pathway through the transcriptional activity of *N. attenuata* MYB8 and *N. tabacum* MYBJS1, members of the R2R3 MYB transcription factor family (Galis *et al.* 2006, Kaur *et al.* 2010). The DNA-binding domain of the homologous gene of MYB8 in tobacco, MYBJS1, has been shown to bind to the promoter regions of copies of the *PAL* gene to regulate the expression of core genes of the phenylpropanoid pathway as well as a few from the polyamine pathway (Galis *et al.* 2006). Additionally, silencing *MYB8* in *N. attenuata* abolishes herbivory-induced elevations as a result of strong reductions in *AT1*, *DH29* and *CV86* expression (Onkokesung *et al.* 2012). *MYB8*-silenced plants do not show developmental alterations, indicating that this transcription factor may control herbivory-induced elevations in the phenylpropanoid flux guided towards phenolamide production rather than steady-state parameters of this pathway. In this respect, the current view is that COI1-based perception of JA-Ile alleviates a negative transcriptional control exerted by one or several yet-to-be-characterized JAZ proteins leading to the expression of *MYB8* (Figure 4). Recent work suggests that *N. attenuata* MYC2, a basic helix-loop-helix Leu zipper transcription factor regulating several jasmonate-dependent responses, regulate the expression of MYB8, but only minor alterations of the phenolamide profiles were detected in *MYC2*-transiently silenced plants (Woldemariam *et al.* 2013). As already demonstrated for other pathway specific transcription factors (Dal Cin *et al.* 2011, Mehrtens *et al.* 2005), the high specificity of *MYB8* in regulating phenolamide metabolism opens up interesting perspectives for increasing the rate of gene discovery in this pathway using transcriptional screens.

Herbivory-induced phenolamide profiles reveal interaction between phytohormone signaling pathways and nitrogen metabolism trade-offs

Virtually any signaling nodes influencing jasmonate pools may alter induced phenolamide levels (Heinrich *et al.* 2013). In this respect, high-throughput metabolomics profiling of transgenic lines with sufficient knowledge regarding disturbed signaling pathways can rapidly contribute to our understanding of phenolamide regulation (Figure 5, panel (a)). This includes the possibility of revisiting how phytohormone crosstalks and downstream transcriptional regulators shape defense metabolite production. The role of ethylene in regulating phenolamide production is particularly noteworthy, because in addition to its signaling function, ethylene biosynthesis connects with the putrescine-to-spermidine conversion (Kumar *et al.* 1996).

Phenolamide biosynthesis interacts with nitrogen metabolism through their polyamine component (Fellenberg *et al.* 2012b, Matsuno *et al.* 2009, Ullmann-Zeunert *et al.* 2013). Activation of herbivory-induced responses in tobacco plants in which patterns of stress-induced nitrogen accumulation have been tracked in different tissue compartments represents an ideal system for testing the nature of this interaction. The phenolamide profile is also strongly influenced by the soil type used. We have shown that sand-grown plants have their induced spermidine and putrescine-based phenolamide pools replaced by tyramine-based ones (Kim *et al.* 2011). It is currently unclear whether this metabolic shift is related to the differential nitrogen supplies between these two soils and allocation in the plant (Lou

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and Baldwin 2004). A recent flux study has demonstrated that elevations in phenolamide levels involves significant trade-offs for nitrogen allocation during insect herbivory (Ullmann-Zeunert *et al.* 2013). This study is of central importance to understand how nitrogen allocation costs may tune phenolamide metabolism inducibility throughout a plant's development. Mechanisms behind this metabolic trade-off will be investigated in the near future using transcriptomic approaches such as the one presented in the next section.

New systems-based approaches for the discovery of gene regulation in phenolamide metabolism

As frequently detected in transcriptomic screens, herbivore attack activates specific reorganizations of metabolic pathways which are different between locally-attacked and distal tissues from the same plant (Gulati *et al.* 2013b, Schittko *et al.* 2001). Spatially-coordinated modulations in gene expression networks may be a key mechanism to regulate changes in metabolite pools throughout the plant but this remains under-studied. Influential work in the regulation of the glucosinolate biosynthesis and distal networks connected to it, has shown that upstream genes of the pathway with important flux control and which are subjected to intense purifying selection (Olson-Manning *et al.* 2013) are central in shaping the glucosinolate chemotype according to the 'genomic context' or network of genes with which they are co-expressed (Malitsky *et al.* 2008). Instrumental data analysis tools for mining these gene networks may be found in a series of inspiring recently published 'evo-devo' transcriptomic studies. Several recent studies have highlighted the unprecedented perspective into the developmental regulation of genes that appropriate statistical analysis of transcriptomic data-sets provides. For instance, the elegant statistical and data visualization approach developed by Chitwood *et al.* (2013) has been used to demonstrate that changes in gene networks, rather than sequence divergence patterns, are responsible for the significant anatomical differences between cultivated and wild tomato species. Here we discuss the insights into phenolamide metabolism that resulted from the implementation of such a bioinformatic approach.

'*Interactive effect*' genes are enriched in metabolism-encoded processes

Surprisingly, the fact that most herbivory-inducible secondary metabolites also increase in systemic tissues has hardly been exploited in the context of gene function analysis. The case of phenolamides is particularly germane as the dynamics of these metabolites differ between local and systemic leaf tissues and these differences are known to be essential determinants of systemic defense induction in *N. attenuata*. Onkokesung *et al.* (2012) successfully selected *N*-acyltransferase candidates for the production of phenolamides based on their greater amplification by insects' OS cues in systemic tissues compared to mechanical wounding. Indeed, most induced defense secondary metabolism genes investigated in *N. attenuata* in the context of W+OS treatments have their expression amplified by OS-activated mobile signals that are transported into systemic tissues from OS-elicited ones (Gulati *et al.* 2013b, Kim *et al.* 2011, Schittko and Baldwin 2003, Schittko *et al.* 2001). This further underscores that only OS perception, and not mechanical wounding alone, leads to the deployment of robust systemic signals (Gulati *et al.* 2013a, Gulati *et al.* 2013b).

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Experiments designed to capture the dynamic rewiring of the gene networks that control the spread of herbivory-induced systemic responses often have a complex factorial structure resulting from the different conditions/treatments and tissue types analyzed and necessarily involve time-series analysis. Informed by the targeted interpretation of the metabolic gene regulation presented above, we designed, in a recent study, a dimensionality reduction method based on multifactorial analysis to categorize genes according to their degree of tissue-specificity and responses to W+OS elicitation (Gulati *et al.* 2013a, Gulati *et al.* 2013b, Figure 6). The procedure is based on bootstrap-based non-parametric ANOVA models implemented in the R package TANOVA (Zhou and Wong 2011, Zhou *et al.* 2010). We applied this method to the analysis of a time-course microarray data-sets for tissues collected from control and W+OS treated plants (Kim *et al. 2011*). When applied to the statistical comparison of gene expression between locally-treated leaves and systemic tissues collected from the same plant, we identified four mutually exclusive groups of genes with different ANOVA structures. We detected ANOVA structures significant for an *interactive effect* (two leaf positions behaving differently across the time series in response to W+OS elicitation), an additive effect (W+OS-induced responses independent of tissue type), or corresponding to independent effects derived from the main experimental factors (major treatment effects in both treated and untreated tissues or differences in tissue type with no response to treatment) (Gulati *et al.* 2013b). The interactive gene-set represents 69 % of the non-constantly expressed genes analyzed and is highly enriched in genes involved in metabolic processes. Most processes connected with secondary metabolic pathways map to this group of genes (red sector in Figure 6 panel (c)). Remarkably, genes of the phenylpropanoid and phenolamide pathways are among those exhibiting the largest *interactive effects*. Additionally, many other metabolic pathways and their transcription factors share similar behavior and have yet to be explored. This necessitates classifying temporal dynamics within this large group of promising genes for metabolic pathway exploration that constitutes the *interactive effect* group.

Tissue x treatment self-organizing maps visualize the sequential arrangement of metabolic pathways

Metabolic genes belonging to a common biosynthetic pathway tend to be co-regulated as a result of the activation of a robust regulatory system (Saito *et al.* 2008). Basic statistical approaches used to identify such strong co-expression patterns are however often plagued by the problems of gene prioritization (Bittner *et al.* 1999, Getz *et al.* 2000) that arise from conducting clustering analysis of gene expression under all experimental conditions (Swindell 2006). Indeed, patterns revealed by simple co-expression analysis essentially represent the static rewiring of the network, which does not realistically capture the plants' phenotypic plasticity that results from the ability of cells to activate transient gene associations which represent intermediate biological states. The need for condition-dependent algorithms to resolve functional gene associations which are affected only by a subset of the experimental conditions -- in our study, by the transmission OS-induced signaling to systemic leaf positions -- has been reviewed by Krouk *et al.* (2013).

We used the time-specific ANOVA coefficients reflecting the degree of significance for the interactive effect between the treatment and leaf positions and scaled them with the

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difference in amplitudes of responses to OS-elicitation to obtain a metric that characterize the behavior of a given gene in more than one tissue (here two leaf positions). We then applied self-organizing maps (SOMs) to delineate gene network assemblies. SOMs result from an iterative process in which neighboring clusters influence each other. The resulting maps clearly visualize the main expression patterns in the analysis of molecular responses to perturbations (Chitwood *et al.* 2013, Gulati *et al.* 2013b, Hirai *et al.* 2005, Hirai *et al.* 2004). SOMs colored according to the cluster's average intensity at each time point are presented in Figure 6. Gene network assemblies along the time-course are visualized by changes in the size of the groups of clusters similarly colored according to the tissue-specificity of gene expression. Of the sequential arrangements of the group of genes termed '*interactive*' motifs, we isolated one motif (S5) detectable 5h post-elicitation for systemic leaves, which contained an overrepresentation of metabolic pathway-encoded processes (Figure 6 panel (e)). From this motif, we delineated, in a previous study, the acyclic diterpene glycoside pathway, another route leading to the production of anti-herbivore defense metabolites (Heiling *et al.* 2010). Here we confirm that *PAL* genes and downstream elements of the phenylpropanoid pathway map into a large interactive motif (L1) rapidly induced in locally treated leaves. Consistent with their induced regulation to supply phenolamide production in these tissues, *MYB8*, *AT1*, *DH29* and *CV86* map on different cells corresponding to *interactive effects* detected first in local leaves (L1) and then in systemic leaves (S5a and S5b).

In our previous study, a rigorous comparison of Pearson correlation patterns before and after extraction of the *interactive effect* metric by multifactorial analysis, revealed that the method greatly improves the detection of tight regulation between the phenylpropanoid and its downstream phenolamide branch (Gulati *et al.* 2013b). We therefore propose that, after delimitation and selection of relevant interactive motifs using bait genes for specific branches of the phenylpropanoid metabolism, SOMs can be quickly mined for phenolamide gene discovery. In Figure 6, the overall workflow is illustrated and phylogenetic relationships between predicted *N. attenuata* BAHD genes are used as queries for the SOMs. This process based on multidimensional clustering of gene expression is specifically designed to mine enzyme-coding gene families for which substrate specificity and enzymatic functions are not readily predictable from phylogenetic relationships. Finally, the involvement in phenolamide biosynthesis and metabolism of a set of genes analyzed by this method can be tested by transient virus-induced gene silencing (VIGs), a rapid technique with many advantages for screening the role of metabolic genes at the interface between developmental and defense processes (Gaquerel *et al.* 2013, Galis *et al.* 2013, Steppuhn *et al.* 2010). Genes inferred from this analysis in motifs L1, S5a and S5b and exhibiting high sequence similarity with *AT1* and *CV86* are currently characterized for their respective involvement in the production of *N*-feruloylspermidine and specific *N*', *N*''-dicaffeoylspermidine isomers.

Plastic gene networks shape developmental vs defensive allocations of phenolic residues to phenylpropanoid sub-branches

Loss-of-function approaches can in some cases highlight complex patterns of 'metabolic tension' and feedback regulation existing between interconnected metabolic branches

(Vanholme *et al.* 2012). Our previous study provided support for the existence of an exacerbated competition in the conjugation of phenolic residues to putrescine or spermidine molecules (Onkokesung *et al.* 2012). Silencing of one acyltransferase enzyme impairs the accumulation of several metabolites while increasing another set of metabolites. Following the same approach, we also uncovered complex interconnections between the lignin and phenolamide pathways by silencing *hydroxycinnamoyl-CoA:shikimate/quinate hydroxycinnamoyl transferase-like* (*HCT-like*), an *O*-acyltransferase catalyzing the production of upstream intermediates in the lignin pathway that branch at the level of the phenylpropanoid pathway (Hoffmann *et al.* 2004, Hoffmann *et al.* 2003). Interestingly, *HCT-like* expression is also controlled by the transcriptional activity of MYB8, but, consistent with its main function in developmentally-controlled lignin deposition, its expression is less pronounced than that of phenolamide biosynthetic genes during herbivory and is also much less affected than these latter genes during herbivory in *MYB8*-silenced plants (Gaquerel *et al.* 2013). Widely-targeted metabolomics analysis on plants transiently-silenced for *HCT-like* revealed large metabolic shifts due to a large diversion of activated coumaric acid units, not channeled into lignin production by the action of *HCT-like*, into the production of developmentally and herbivory-induced coumaroyl-containing phenolamides (*N*', *N*''-dicoumaroylspermidine, *N*', *N*''-coumaroylputrescine, etc…). The fact that metabolic shifts in the production of unusual coumaroyl-containing phenolamides are largest during herbivory in *HCT-like*-silenced plants identifies *HCT-like* as a large effect gene within the gene network underlying phenylpropanoid metabolism plasticity.

Exploring the type of effect revealed by the multifactorial analysis for a given group of genes can be used to track the dynamic behavior of gene expression involved in connected branches of a metabolic pathway. At first blush, this approach could be used to mine the above mentioned interactions between the phenolamide and lignin branches. Interestingly, unlike the phenylpropanoid core pathway and phenolamide genes, preliminary work revealed that most previously characterized lignin biosynthetic genes tested, with the exception of *HCT-like*, do not exhibit an *interactive effect* regulation following insect herbivory. Individual expression patterns are presented in Figure 6 panel (f). These patterns suggest that steady-state coordinated expression patterns between the phenylpropanoid and lignin pathways are relaxed after herbivore attack. As a result of the profound reconfigurations of gene expression, tighter co-expression patterns seem to be established between the core phenylpropanoid module and the structural genes of the phenolamide pathway during herbivory compared to control conditions. More research is needed to understand the central function of MYB8 in assembling these coexpression networks between high amplitude regulation genes of the phenylpropanoid and phenolamide pathways in order to prioritize phenolamide production during insect herbivore attack.

Conclusion

The importance of phenolamides as central players in a plant's defenses is rapidly being recognized. The advances outlined here in understanding the transcriptional regulation and biosynthesis of these metabolites offer new possibilities for manipulating these dynamic phenolamide pools and understanding the many subtle adjustments at the interface between development and stress metabolic responses that determine phenolamine levels. Tissue-

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specific genetic silencing approaches such as recently established by Schäfer *et al.* (2013) will likely reveal novel facets to the functions of phenolamide metabolism.

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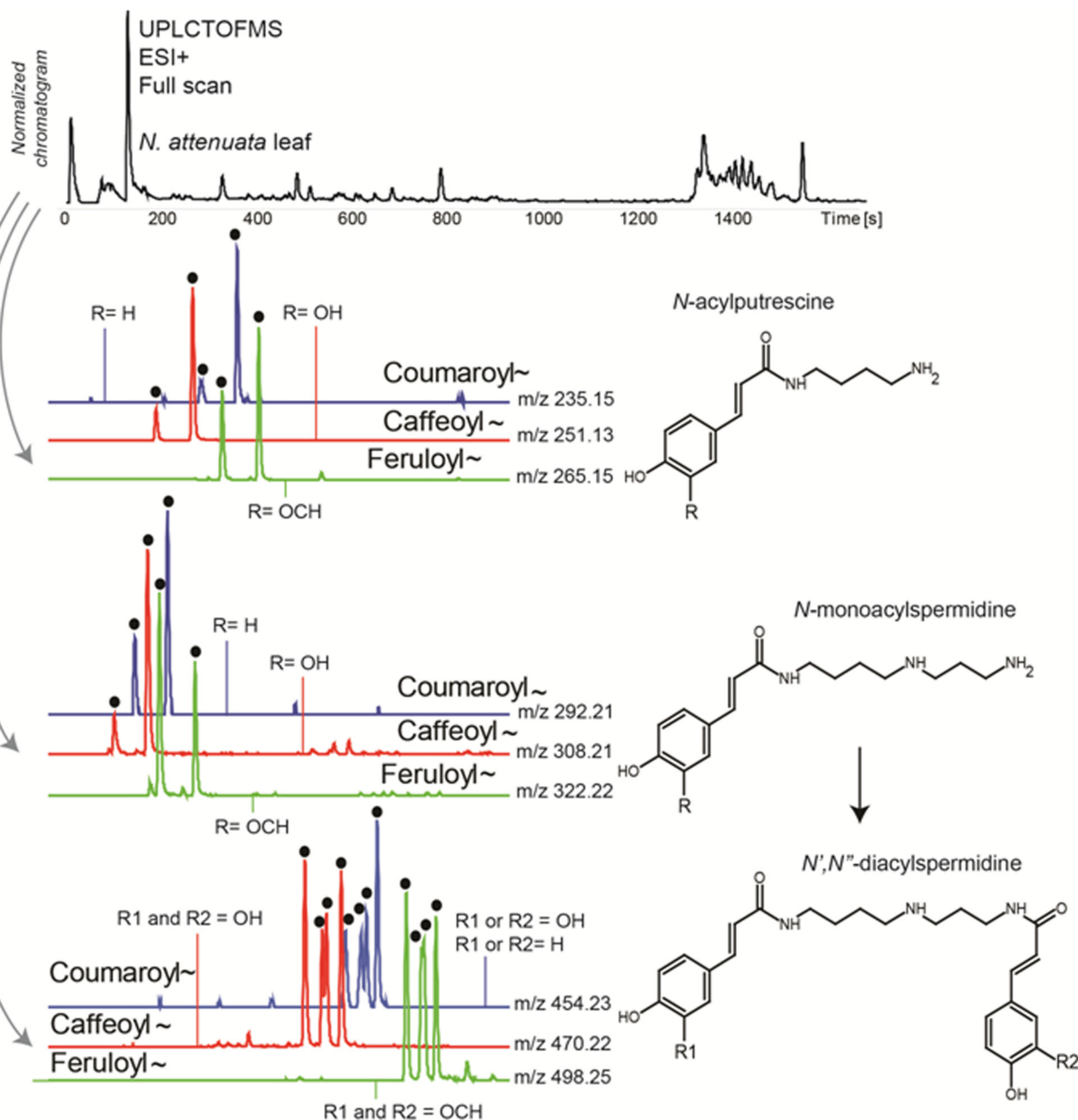

Figure 1. Mass spectrometry-based metabolomics of main phenolamides in *N. attenuata* leaves.
Phenolamides can readily be analyzed by ultra-high performance liquid chromatography coupled with a mass spectrometer. A representative UHPLC-time-of-flight-mass spectrometry (TOFMS) full scan chromatogram recorded in the positive ionization mode for an extract of an herbivory-induced leaf of *Nicotina attenuata* is shown. Coumaroyl- (*m/z* 147.05), caffeoyl- (*m/z* 163.04), or feruloyl (*m/z* 177.05) moieties resulting from the cleavage from different core molecules can, for example, be queried rapidly to compute extracted ion currents from the chromatogram. Specific *m/z* signals corresponding to either coumaroyl-, caffeoyl- and feruloyl-containing mono-acylated putrescine molecules or mono-

and diacylated spermidines (*N',N''*-coumaroyl,caffeoylspermidine, *N',N''*-dicaffeoylspermidine, *N',N''*-diferuloylspermidine) can be queried to reveal phenolamide peaks (highlighted with black dots). Representative structures are shown.

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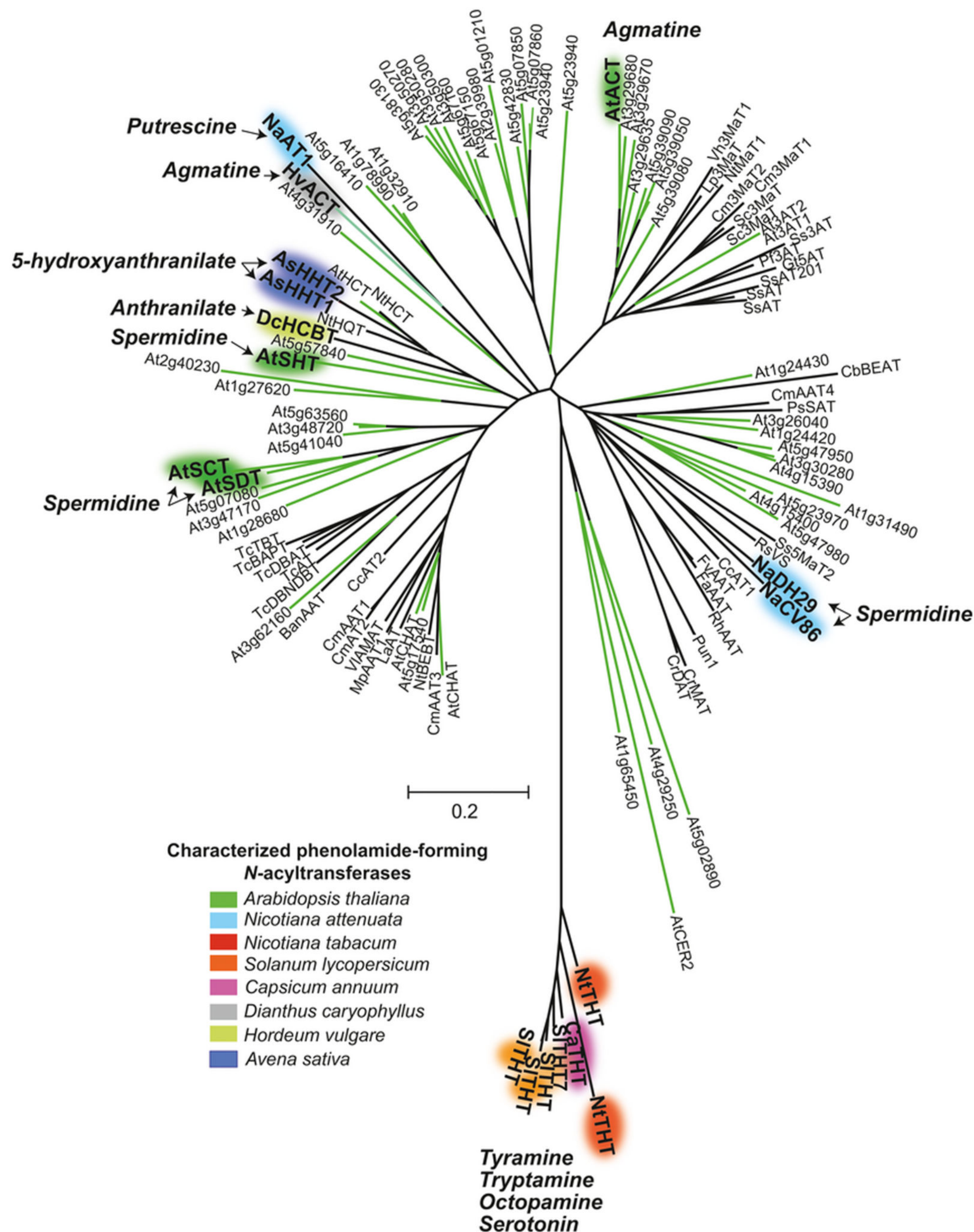

Figure 2. Phylogenetic relationships among phenolamide-forming *N*-acyltransferases.

A phylogenetic analysis was conducted for Arabidopsis *BAHD* (green branches of the tree) and functionally characterized *N*-acyltransferases including phenolamide-forming ones (acyl acceptor indicated) summarized in Bassard *et al.* (2010) and additional characterized *N*-acyltransferases reported in Luo *et al.* (2009). The phylogenetic tree reveals that *N. attenuata* DH29 and CV86 (highlighted in blue), which control the two-step synthesis of diacylated spermidines, cluster far from the Arabidopsis polyacylated spermidine-forming *N*-acyltransferases (AtSCT and AtSHT). Phenolamide *N*-acyltransferases with different acyl

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acceptor specificities (indicated next *N*-acyltransferases) are located on different parts of the tree. Sequences were aligned with Muscle and the alignment was trimmed with Gblocks to obtain 133 positions in 16 blocks that were used to calculate the phylogenetic tree using MEGA 4 and the Neighbor-Joining clustering method with 1000 iterations to calculate bootstrap values (Onkokesung *et al.* 2012). Colored ellipses of the tree connected to gene name in bold -- plant species names are reported in the color key -- denote for characterized phenolamide-forming *N*-acyltransferases. Plant species names are abbreviated as follows: *Arabidopsis thaliana*, At; *Avena sativa*, As; *Capsicum annuum*, Ca; *Catharanthus roseus*, Cr; *Clarkia breweri*, Cb; *Curcumis melo*, Cm; *Dianthus caryophyllus*, Dc; *Fragaria anassa*, Fa; *Hordeum vulgare*, Hv; *Lupinus albus*, La; *Malus pumila*, Mp; *Nicotiana attenuata*, Na; *Nicotiana tabacum*, Nt; *Papaver somniferum*, Ps; *Salvia splendens*, Ss; *Solanum lycopersicum*, Sl; *Taxum cupsidata*, Tc; *Vitis labrusca*, Vl.

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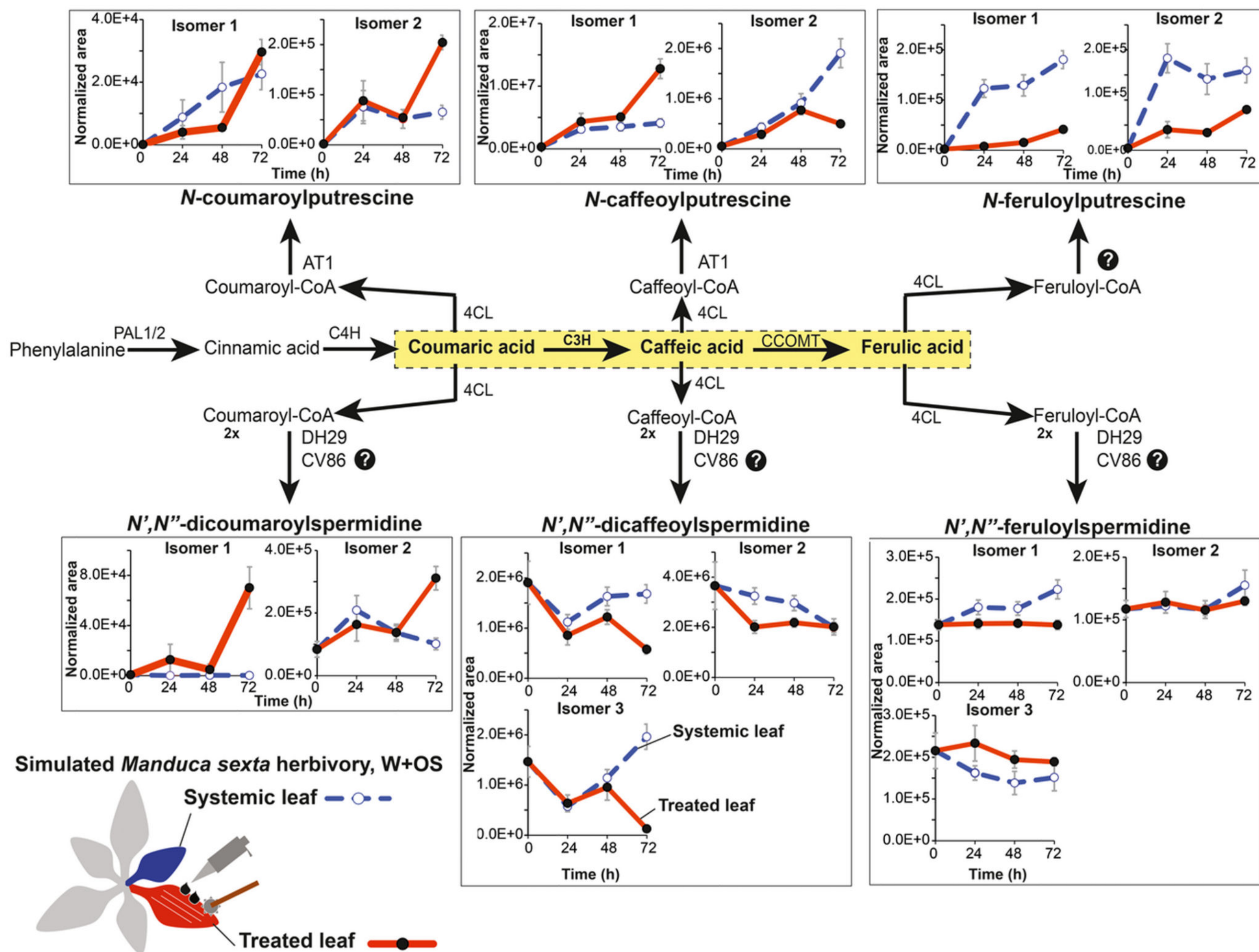

Figure 3. Herbivory-induced changes in *N. attenuata* leaf phenolamide metabolism.

Gene names and functions are given in the main text. Dark blue lines depict metabolite accumulation patterns in W+OS-treated leaves and red dashed lines depict responses in a systemic leaf from the same plant. Putrescine conjugates show greater induced changes than do spermidine conjugates. Dynamics differ among the predicted isomers.

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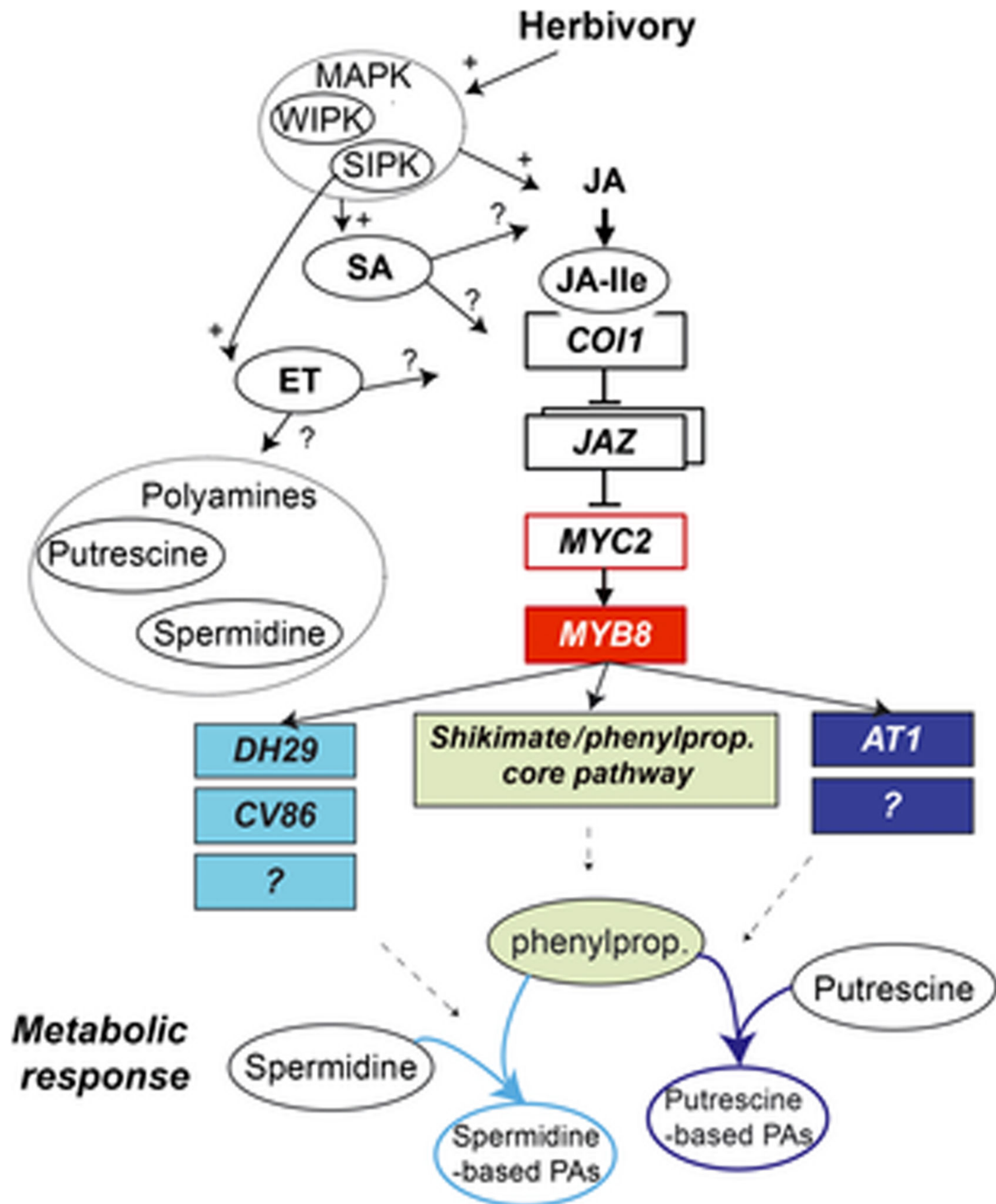

Figure 4. Current view of the regulation of herbivory-induced phenolamide biosynthesis in *N. attenuata* as mediated by the core jasmonic acid biosynthetic and transcriptional pathway.
MAPK signaling and interactions among other hormonal signaling networks shape the amplitude of the jasmonate bursts and downstream signaling. The role of ethylene in cross-regulating polyamine metabolism has yet to be rigorously investigated. Specific JAZ proteins inhibiting *MYB8* transcription are not yet known. MYB8 regulates induced changes in the core phenylpropanoid pathway and DH29, CV86, AT1 and yet unknown phenolamide-forming *N*-acyltransferases.

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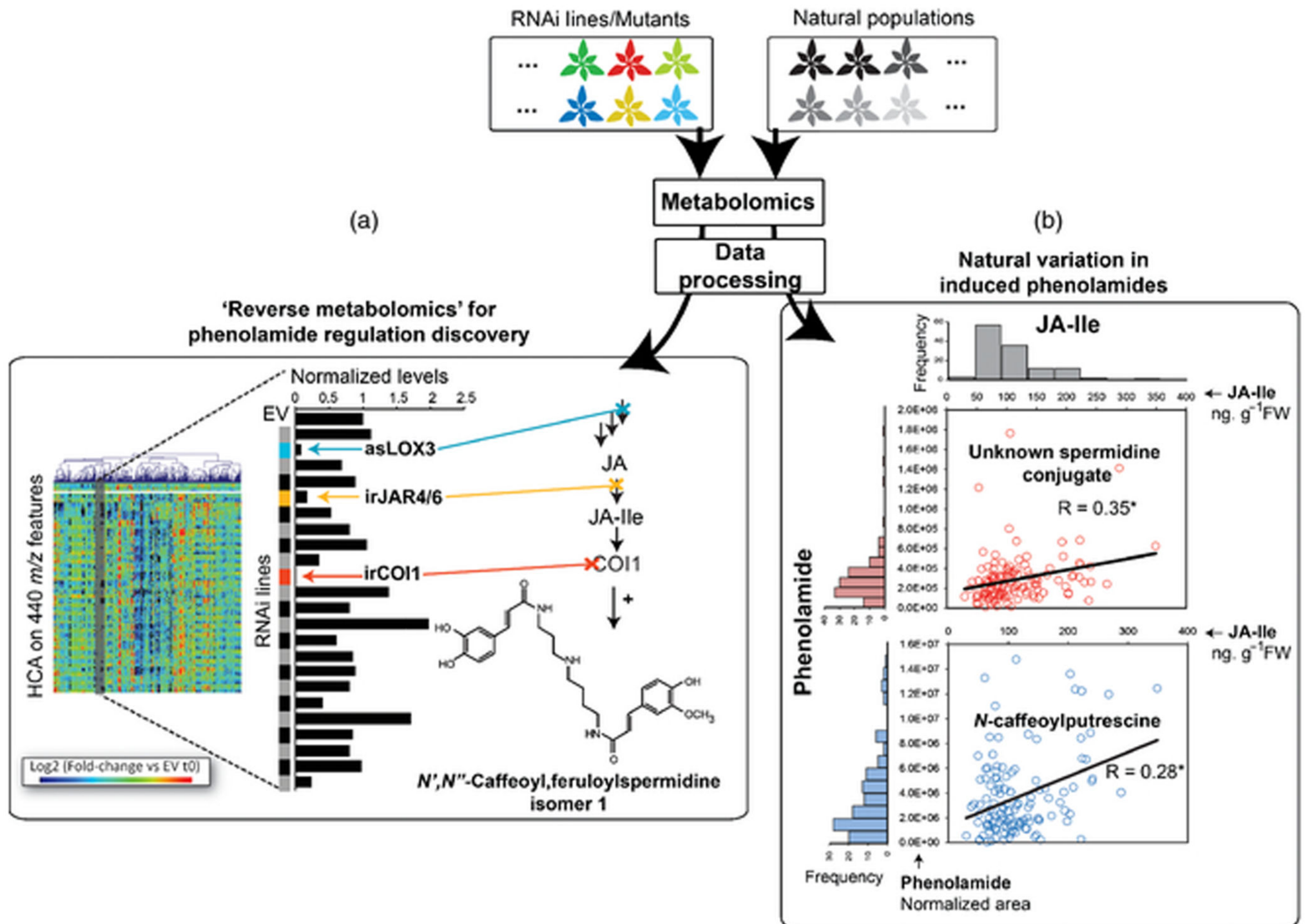

Figure 5. Novel approaches based on metabolomics to the discovery of regulatory mechanisms for herbivory-induced changes in phenolamide metabolism.

(a) High-throughput non-targeted metabolite profiling of herbivory-induced changes in a large collection of RNAi transgenic lines reveals regulators of metabolite accumulation. Processed data can be classified using hierarchical clustering and clusters of *m/z* signals of interest screened across the library of transgenic lines. The jasmonate regulation of *N',N''*-caffeoyl,feruloyspermidine is provided as an example. **(b)** Natural variation in W+OS-induced levels of *N*-caffeoylputrescine and of an unknown spermidine-based phenolamide in 176 natural accessions of *N. attenuata* positively correlate with natural variation in the OS-induced JA-Ile bursts.

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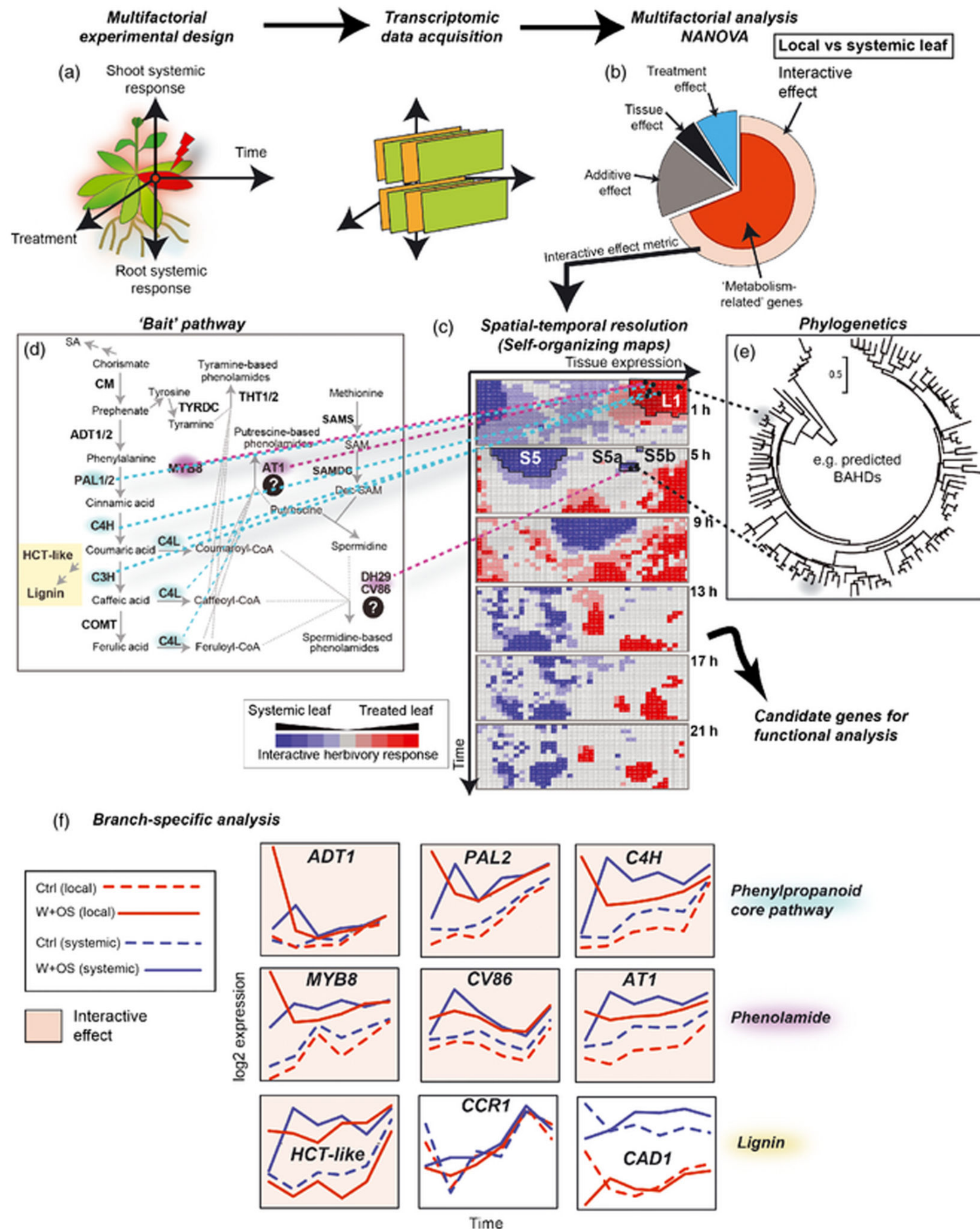

Figure 6. A multifactorial-based coexpression analysis work-flow for delineating systemically-induced secondary metabolic pathways.

A multifactorial analysis work-flow has been developed by Gulati *et al.* (2013) and its use in delineating genes in the acyclic diterpene glycoside pathway has previously been reported. This strategy is applied to the analysis of multidimensional transcriptomic data-sets acquired from multifactorial experimental designs (different tissue types, treatment, etc…) including time-series experiments **(a)**. Transcriptomic data collected at each time point are combined into a data matrix used for multifactorial analysis. The statistical group corresponding to the

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interactive effect genes (those genes that respond to the treatment differently according to the tissue type, here locally vs systemically treated leaves) is highly overrepresented with metabolism-related genes (red sector) **(b)**. Self-organizing maps are used to impose structure **(c)** and to cluster genes within this bin according to their temporal dynamics using a metric derived from the multifactorial analysis. Bait genes (here from the phenylpropanoid and phenolamide pathways) can be localized on the maps to identify clusters of genes of interest (phenylpropanoid genes: L1 for early *interactive effects* in local leaves; phenolamide genes: L1, S5a and S5b for local and then systemic *interactive effects*) **(d)**. These clusters of genes can be subsequently mined in accordance with the predictions of phylogenetic relationships **(e)**. **(f)** Genes from specific branches of the phenylpropanoid space can be classified according to the detection of an *interactive effect* regulation. Most lignin-related genes, except *HCT-Like*, do not show interactive effect regulation in response to herbivory, unlike the core phenylpropanoid and phenolamide genes.