

Metabolomics of disease resistance in crops

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Abstract

Plants are continuously exposed to the attack of invasive microorganisms, such as fungi or bacteria, and also viruses. To fight these attackers, plants develop different metabolic and genetic responses whose final outcome is the production of toxic compounds that kill the pathogen or deter its growth or semiotic molecules that alert other individuals of the same plant species. These molecules are derived from the secondary metabolism and their production is induced upon detection of a pathogen-associated molecular pattern (PAMP). These PAMPs are different molecules that are perceived by the host cell triggering defense responses. PAMP-elicited compounds are highly diverse and specific of every plant species and can be divided into preformed metabolites or phytoanticipins that are converted into toxic molecules upon pathogen perception, and toxic metabolites or phytoalexins that are produced only upon pathogen attack. Moreover, plant volatile emissions are also modified in response to pathogen attack to alert neighboring individuals or to make plants less attractive to pathogen vector arthropods. Plant metabolite profiling techniques have allowed the identification of novel antimicrobial molecules that are induced upon elicitation. However, more studies are required to assess the specific function of metabolites or metabolite blends on plant-microbe interactions.

Introduction

Opposite to animals, vascular plants do not possess an immune system which involves the presence of cells specialized in detection, prosecution and elimination of invasive microorganisms. However, plants are still able to respond to the attack of microorganisms and predatory arthropods relying on different mechanisms, first an *innate immunity* comprising a sensitive system to detect Pathogen-Associated Molecular Patterns (PAMPs) based on membrane-bound specific pattern recognition receptors (PRR) (PAMP-Triggered Immunity or PTI), and a second layer named

Effector-Triggered Immunity or ETI, involving intracellular proteins that respond to pathogen effectors or virulence factors.

PAMP-Triggered Immunity (PTI)

PAMPs are characteristic molecular structures shared by large groups of pathogens that act as elicitors of defense responses in plants. Among PAMPs perceived by plants, fungal structures such as cell wall β -glucan, conserved epitopes from cell wall transglutaminases and elicitors (secreted lipotransfer proteins); or bacteria-derived molecules such as lipopolysaccharides, flagellin, bacterial cold-shock proteins, and EF-Tu are found. Perception of PAMPs is not necessarily a specific event as some of them can elicit defense responses in a wide range of plant species (e.g. flagellin or the isolated epitope flg22). Nevertheless, most specific PRRs responsible of this pattern recognition remain elusive and only a few have been so far characterized. Most plant PRRs are membrane-bound receptor-like kinases comprising a ligand-binding domain on the cell surface. These molecules can also contain a cytoplasmic kinase domain (Receptor-Like Kinases or RLKs) or not (Receptor-Like Protein types or RLPs) that initiates PRR-triggered immunity upon PAMP perception (Macho and Zipfel, 2014). For a proper immune response induction, these receptors dimerize upon perception of the PAMP. To this respect, several models showing homo-, hetero-dimerization and heteromultimerization exist. Chitin perception in Arabidopsis is mediated by LysM-RLK CERK1/RLK1/LYK1. This CERK1 receptor contains three extracellular domains that bind long fungal chitin oligomers (comprising seven to eight N-acetyl-glucosamine residues) leading to its homodimerization and formation of an active receptor complex (shorter chitin oligomers do not induce receptor homodimerization and do not trigger immune response). Upon homodimerization, CERK1 cytoplasmic kinase domains are brought together enabling intermolecular transphosphorylation, following a similar mechanism to that of animal tyrosine kinases (Macho and Zipfel, 2014). Interestingly, chitin perception in rice is mediated by a mechanism known as heteromultimerization in which a chitin binding protein CEBiP (a predicted GPI-anchored protein) with no kinase cytoplasmic domain (as an RLP). Therefore, transduction signal after chitin perception requires the cooperation with additional proteins. CEBiP homodimerizes in the presence of active chitin and also forms a hetero-oligomeric complex with OsCERK1 (an ortholog of Arabidopsis CERK1 but its only extracellular domain does not bind chitin) (Shimizu et al., 2010). Flagellin detection and signal transduction

carried out by PRRs is well studied in the model plant *Arabidopsis thaliana*. In this plant species, LRR-RLK-FLS2 is the PRR responsible for binding flagellin from flagellated phytopathogenic procariotes (such as *Pseudomonas syringae* or *Xanthomonas spp.*) or the 22-aminoacid epitope flg22 (Dardick et al., 2012). This is a Leucine Repeat-Rich RLK that forms heterodimers with LRR RLK-BAK1/SERK3 regulatory complex upon epitope binding. Although, dimerization of FLS2 and BAK1 is not required for a proper flg22 binding both molecules act as co-receptors for epitope binding and signal activation. A similar activation mechanism has been described for the brassinosteroid receptor BRI1 (another LRR-RLK) that binds to co-receptors BAK1 or SERK1. This interaction between LRR-RLKs (and LRR-RLP) and BAK1 or related SERK proteins seems to be a common trend as supported by increasing evidence (Macho and Zipfel, 2014). As for rice CERK1, some mechanistic differences are also found in this case as well: OsSERK2, an *Oryza sativa* ortholog of BAK1 binds constitutively to the LRR-RLK XA21 conferring resistance to *Xanthomonas oryzae* pv. *oryzae*, but no ligand has been confirmed yet therefore it cannot be precluded how PAMP interaction would modify OsSERK2-XA21 interaction (Bahar et al., 2014; Sun et al., 2013). A more complex interaction has been observed in tomato, where the BAK1 ortholog interacts with an LRR-RLP named Eix1 and negatively regulates signaling mediated by Eix2 (a related LRR-RLP). Upon ligand perception, receptor complexes interact with different cytoplasmic partners to trigger intracellular signaling leading to a rapid ROS burst and expression of genes involved in defense (Bar et al., 2010). One of these partners is the kinase BIK1 that has several protein targets in *Arabidopsis* such as AtRBOHD NADPH oxidase involved in apoplastic ROS production in response to PAMP perception. Another protein target in rice that responds to chitin-triggered signaling through OsCERK1 is the Rac GDP/GTP exchange factor (OsRacGEF1) that activates OsRac1 leading to resistance to fungal pathogens (Akamatsu et al., 2013). Moreover, the activation of defense responses on PAMP perception needs to be properly shut down after the pathogenic threat is over. To this respect, as signaling activation is driven by phosphorylation negative regulation modulated by protein phosphatases is a plausible explanation. Hence, several protein phosphatases 2C have been shown to interact with members of different PRR complexes: KAPP interacts with FLS2 (flg22-triggered responses) as well as with other plant RLKs (Gomez-Gomez, 2001), in rice XB15 phosphatase and XB24 ATPase associate with XA21 thus regulating the phosphorylation status of PRR (Park et al.,

2008). Additionally, PAMP-bound activated RLKs are also degraded to enable the restoration of free receptors at the plasma membrane. Degradation of receptors is carried out through polyubiquitination catalyzed by the E3-ubiquitin ligases PUB12 and PUB13 that constitutively interact with BAK1 and are recruited into the FLS2 receptor complex after perception of flg22 (Lu et al., 2011).

Effector-Triggered Immunity (ETI)

However, these defense responses can be inhibited by virulence factors that successful pathogens have evolved. In turn, adapted plants have also developed resistance proteins that detect these virulence factors and inhibit their action on cell targets. This response is mediated by resistance proteins (R proteins, most of them encoding highly polymorphic NB-LRR proteins) that recognize these virulence factors leading to the induction of a hypersensitive response (HR) including programmed death of infected cells inhibiting pathogen spread. This is considered an enhanced PTI response that leads to disease resistance. It is worth noting that this ETI branch is effective on obligate biotrophs (microorganisms that can only feed on living cells) or hemibiotrophs but not on pathogens that kill host cells during colonization or necrotrophs (Jones and Dangl, 2006). It is hypothesized that these NB-LRR proteins act as guards of intracellular targets of pathogen effectors (this recognition can be carried out directly or indirectly by detecting products of the action on host target molecules). As mentioned above, this layer of defense leads to HR and induction of programmed cell death contributing to arrest pathogen expansion. This programmed cell death has been associated to the intracellular production of ROS by mitochondria and chloroplasts and the activation of NB-LRR and metacaspases (Coll et al., 2011).

Metabolite profiling techniques in plant-pathogen interactions

In recent years, phytopathologists have gained access to modern metabolite profiling platforms that allow monitoring hundreds of metabolites in a single analysis. Moreover, these techniques are exhaustive enough to allow researchers to identify novel metabolites with potential antimicrobial activity (Allwood et al., 2008). Hence, comparison of resistant versus susceptible varieties, genotypes or mutants is a fundamental tool to decipher resistance mechanisms and identify defense compounds. In this context, instead of monitoring a few (known toxic compounds) the array of low molecular weight metabolites (metabolome) are analyzed and quantitated constituting

the whole metabolic phenotype of a plant. To this respect, it is then possible to identify pre- and post-invasion resistant and susceptible metabolic phenotypes and infer relationships among different pathways.

Most non-volatile secondary metabolites are semipolar chemical compounds that can be easily separated on a C18 column (or specifically modified versions of this packing) using reversed phase liquid chromatography coupled to a high resolution mass spectrometer (usually a hybrid quadrupole/time-of-flight or QTOF-MS) through an atmospheric pressure ionization source (either electrospray ESI or chemical ionization, APCI). This instrumental coupling is known as LC/MS. High resolution QTOF-MS instruments provide accurate mass measurements (less than 0.02 arbitrary mass units of difference) and real isotopic pattern (related to the particular elemental composition of each compound). In addition, these instruments also allow fragmentation of selected compounds, thanks to the presence of a quadrupole segment and a collision cell before the flight tube, to gain structural information. This configuration is usually sufficient to analyze different types of secondary metabolites: flavonoids and triterpenoids (Arbona et al., 2015; Böttcher et al., 2008), phenolics (Böttcher et al., 2009a), glucosinolates and related compounds (Böttcher et al., 2009b; Zandalinas et al., 2012), lipids (Zoeller et al., 2012), etc...Due to the high specificity of secondary metabolites and the little information available on them (mass spectra, fragmentation pattern, elution time, etc...), together with the poor cross-platform exchangeability of LC/MS data, make very often necessary their *de novo* identification using in-house built databases or matching the annotation (MS spectra and retention time) with pure standards. For these reasons, ensuring high quality spectral information is of key importance to facilitate mass spectra curation and interpretation (Figure 1).

Complementarily, volatiles and polar compounds are analyzed by means of gas chromatography coupled to mass spectrometry (GC/MS). Polar compounds such as carbohydrates, free aminoacids and carboxylic acids of the Krebs cycle can be analyzed by GC/MS as methoxime/trimethylsilyl derivatives (Roessner et al., 2001) whereas volatiles do not need any further processing (Beck et al., 2014). Identification of polar metabolites and volatiles in this case is carried out in a targeted fashion using publicly-available databases such as the Gölm Metabolite Database (<http://gmd.mpimp-golm.mpg.de/>), NIST or the Fiehn metabolite database (<http://fiehnlab.ucdavis.edu/db>). This is possible for several reasons: 1) In GC analyses, retention index markers, such as

fatty acid methyl esters or alkanes, are added to standardize retention times of all eluted compounds to retention indices; this makes GC a more robust chromatographic technique than LC, where retention index markers are not employed; 2) the ionization in GC/MS is achieved through electron impact (EI), essentially an electron beam collides with each eluted metabolite and generates a population of ions. Retention indices (RI) as well as fragment ions are then used to match metabolite identities in metabolite databases.

Analysis of metabolomics data (both LC/MS and GC/MS) requires the aid of bioinformatic tools that allow extraction of chromatographic and spectral data in a manageable file format, such as MS Excel spreadsheets. This step can be achieved using different software tools developed so far: xcms (Smith et al., 2006), metAlign (Lommen, 2009), MzMine (Katajamaa et al., 2006; Pluskal et al., 2010) or, more recently, GridMass (Treviño et al., 2015), etc...perform peak picking and alignment of mass chromatographic peaks (Arbona et al., 2009). There are also tools for integrated GC/MS analysis that allow performing peak picking and alignment, RI calculation and matching of MS spectra in databases: TargetSearch (Cuadros-Inostroza et al., 2009), TagFinder (Luedemann et al., 2008) or FiehnLib (Strehmel et al., 2013). In addition, it is possible to annotate unknown derivatized metabolites by using GC/APCI(+)-QTOF-MS. The APCI ion source generates predominantly protonated ions and very few fragments or none. Annotation of unknowns requires the evaluation of adduct ion formation and calculation of elemental composition. In addition, an estimation of the number of methoxime and trimethylsilyl units per derivatized molecule is needed followed by a deep examination of the corresponding collision-induced dissociation mass spectrum (Strehmel et al., 2013).

Analysis of volatiles does not require previous derivatization and due to the nature of the matrix detection limits can be very low. The main problem is that these compounds need to be collected *in vivo* from plant samples because they are released to the atmosphere as they are synthesized. There are two main collection strategies: 1) sampling the headspace of a hermetically closed vial or flask in which the plant material is contained and 2) force an inert gas in this hermetically closed flask to carry the metabolites that are collected in a special adsorbent material (a solid phase extraction technique, SPE, applied to gas matrices). The first option implies that the surrounding headspace gas of the vial is not renewed. Then, the equilibrium between the plant and

the atmosphere is quickly reached and the migration of volatiles from plant tissues to the headspace gas is therefore reduced. In addition, although the matrix (air) is quite inert to MS detectors, emission of volatiles is often very slow and, therefore, long incubation periods are required (Figure 1). The second option is more advantageous in terms of extraction efficiency and the possibility of including a pre-concentration step. Following this strategy, gas surrounding the plant is continuously forced through a SPE cartridge and emitted volatiles retained. Cartridges are subsequently eluted with *n*-hexane and injected in the GC/MS. An improved version is the SPME, the M standing for micro, in which cartridge elution volumes are significantly reduced (Beck et al., 2014; Tikunov et al., 2005).

Metabolic responses to pathogen infection

Plants produce a vast array of low molecular weight compounds (an estimated of 100,000 different metabolites in the plant kingdom). These molecules, collectively known as secondary metabolites, are not essential for cell survival but do have a specific role in the adaptation to a changing environment. Most of them can be primarily grouped in four branches: isoprenoids, phenylpropanoids, alkaloids and fatty acid/polyketides (Dixon, 2001). Importantly, most secondary metabolites are specific of a plant family/genus or even species, making their functional characterization in plant defense by conventional molecular and genetic techniques a daunting task. Although most antimicrobial metabolites synthesized by plants have a broad spectrum activity, their relative activity relies on the presence of detoxification enzymes in a pathogen strain. The synthesis and accumulation of these compounds is induced after perception of pathogen attack (perception of PAMPs or pathogen effector molecules). Defense compounds can be divided into *phytoalexins*, that are produced upon pathogen perception, and *phytoanticipins* that are synthesized in an inactive form and pathogen recognition triggers its biochemical modification into a toxic derivative (Piasecka et al., 2015). It is important to recall that plants challenged with pathogenic fungal or bacterial strains modify their metabolome in the attacked cells (local response) and this response can be extended to the rest of the plant (systemic response). However, not all secondary molecules (synthesized *de novo* or modified during pathogen attack) have a toxic role, and not only one molecule is responsible for the toxic effect. Therefore, to ascertain the role of each elicited metabolite, it should be isolated and tested for toxic activity under control conditions, which is not always possible.

In this review, we described some compounds which *in vivo* function in plant immunity against bacterial and fungal pathogens has been studied in detail (see Figure 2 for the most important groups of plant metabolites involved in defense).

Phytoanticipins

Saponins are glycosides from lipophilic polycyclic structures derived from the isoprenoid pathway, occurring as triterpenoids or steroids (sapogenin). These molecules are widely distributed in the plant kingdom (mainly in dicots but also in some monocots) (Huhman et al., 2005; Piasecka et al., 2015). This group includes triterpenoids from *Avena spp.* named avenacins (A1, A2, B1 and B2) only accumulated in roots whereas avenacosides, with steroidal sapogenins, are accumulated in leaves (Piasecka et al., 2015). In addition, steroidal glycoalkaloids found in solanaceous plant species constitute important representatives of this group (e.g. α -tomatine of *Solanum lycopersicum* L. Mill). This compound is highly abundant in green tomato fruits showing values of 500 mg kg⁻¹ fresh fruit weight and it is degraded as the fruit ripens (Friedman, 2002). This compound has been shown to have an important activity against fungal pathogens. Hence, expression of β_2 -tomatinase gene from *Septoria lycopersici* in *Nectria haematococca*, a fungus that can colonize red tomato fruits but not green ripe ones, enabled it to detoxify α -tomatine and its ability to parasitize green tomato fruits (Sandrock and VanEtten, 2001). More recently, it has been shown that mutant *Cladosporium fulvum* lines with suppressed CfTom1 glycosyl hydrolase activity (GH10 tomatinase) exhibited significantly lower virulence in tomato (Kmen et al., 2013). This is due to the accumulation of tomatidine upon cleavage of α -tomatine by CfTom1 hydrolase that suppresses induced defense responses in tomato (Ito et al., 2004). Moreover, the role of avenacins in immunity in oat has been studied by means of a genetic screening of a mutant population of *Avena strigosa* leading to the identification of *saponin deficient (sad)* mutants. These mutants are deficient in enzymes that are specifically involved in avenacin biosynthesis and not avenacosides. Therefore, roots of homozygous *sad* lines exhibited hyper-susceptibility to adapted and non-adapted fungal strains. (Mugford et al., 2013).

Glucosinolates are the most studied secondary metabolites. Globally, these molecules are β -D-thioglucosides-*N*-hydroxysulfates with different aliphatic, aromatic or indolic substitutions and are primarily present in Brassicales and some species of the

Euphorbiaceae family. Glucosinolates are derived from different aminoacids such as alanine, valine/leucine, isoleucine, methionine, phenylalanine/tyrosine and tryptophan. Nevertheless, not all possible structures are present in all brassicaceae species but a particular subset. For instance, in the model species *Arabidopsis thaliana*, aliphatic glucosinolates derived from methionine and indolic glucosinolates derived from tryptophan are abundant (Piasecka et al., 2015); in leaves of cultivated rapeseed, levels of some aliphatic glucosinolates such as progoitrin, gluconapin and glucobrassicinapin and indolics such as neoglucobrassicin are predominant whereas in kale, cabbage (Velasco et al., 2011) and wild radish, indolic glucosinolates were less abundant compared to aliphatics (Malik et al., 2010). There is a wealth of information on the interaction of glucosinolates with the environment. In their glycosylated form, glucosinolates are quite stable and biologically inactive but the activation of β -thioglucoside hydrolases or myrosinases upon pathogen attack initiates glucosinolate hydrolysis and release of toxic breakdown products (Grubb and Abel, 2006). Breakdown products are unstable and decompose in different types of molecules including isothiocyanates (ITCs), nitriles and epithioalkanes that have the toxic effect on microbes. Particularly, ITCs derived from aliphatic glucosinolates have a deterrent activity against predating arthropods such as spider mites (Piasecka et al., 2015; Zhurov et al., 2014); those derived from indolic glucosinolates have antifeedant effects on aphids (Kim et al., 2008). In *Arabidopsis thaliana*, tryptophan-derived metabolites conferred non-host resistance to *Plectosphaerella cucumerina*. For instance, *cyp79b2/cyp79b3* double mutant, that cannot convert tryptophan to the indol-3-acetaldoxime the first committed step in indol glucosinolate biosynthesis, exhibited an increased *P. cucumerina* entry rate compared to wt or phytoalexin deficient 3 (*pad3*) mutants (Sanchez-Vallet et al., 2010). In addition, challenging *Arabidopsis* or other brassicaceae with pathogens induces expression of PEN2 that encodes a lysosome-associated atypical myrosinase that metabolizes indolic glucosinolates generating particular breakdown products such as raphanusamic acid and indol-3-amine and also induces the expression of CYP81F2, a P450 cytochrome monooxygenase that catalyzes the hydroxylation of indol-3-ylmethyl glucosinolate into hydroxyindol-3-ylmethyl glucosinolate. Both loss-of-function *pen2* and *cyp81f2* *Arabidopsis* mutants exhibit similar penetration phenotypes in non-adapted fungal pathogens such as *Blumeria graminis* f. sp. *hordei* (Bednarek et al., 2009).

Cyanogenic glucosides are hypothesized to be the precursors of glucosinolates and are widespread in the plant kingdom including ferns and gymnosperms. Chemically, these molecules are β -D-glucosides of α -hydroxynitriles derived from tyrosine, phenylalanine, valine, leucine and isoleucine. As glucosinolates, these metabolites are quite stable and biologically inactive as synthesized, but after hydrolysis the α -hydroxynitriles released decompose spontaneously to generate hydrogen cyanide. These metabolites are known to have an influence on plant-arthropod interaction but their role in defense against microbial pathogens is not yet clear. To this respect, highly cyanogenic *Phaseolus lunatus* accessions showed an elevated susceptibility to the hemibiotroph *Colletotrichum gloeosporioides* compared to genotypes with a lower cyanogenic potential. These accessions also showed a significantly lower polyphenol oxidase activity; indeed, exogenous HCN application also depressed the activity of this enzyme. Likewise, highly cyanogenic varieties of *Hevea brasiliensis* (rubber tree) exhibited low scopoletin (a coumarin phytoalexin) accumulation and, hence, a strong susceptibility to the blight fungus *Microcyclus ulei* (Lieberei et al., 1989). This generalized depression of plant immunity upon hydrolysis of cyanogenic glucosides has been associated to the toxicity of HCN gas on neighboring plant cells that inhibits enzyme activity. On the contrary, in barley, cyanogenesis has proved to be beneficial against pathogenic microbes. However, accumulation of leucine-derived epiheterodendrin does not render cyanogenic barley plants since no specific β -glucosidase is expressed. Transient expression of the sorghum cyanogenic β -glucosidase *dhurrinase2* in barley reduced notably its colonization by *Blumeria graminis* f. sp. *hordei*, indicating that cyanogenesis is effective against this biotrophic fungus (Lipka et al., 2005; Nielsen et al., 2006).

Benzoxazinone glucosides are another group of phytoanticipins found primarily in crops of the Poaceae family including corn (*Zea mays*), wheat (*Triticum spp.*), rye (*Secale cereale*) and some barley wild relatives. In corn and wheat, the main accumulating benzoxazinone glucoside is DIMBOA (2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one) glucoside. Their role in plant immunity has been assigned based on a positive correlation between their accumulation in tissues and pathogen resistance. As in cyanogenic glucosides or glucosinolates, their function is attributable to aglycones whereas glycosylated forms are stable and harmless. As aglycones, these molecules have shown a remarkable antifungal activity in vitro against a wide range of plant

pathogens. In vivo, reverse genetics experiments also support the role of these metabolites in plant immunity. That is the case of the *bx1* corn, mutant unable to synthesize DIMBOA glucoside, which showed an extreme susceptibility to *Septosphaeria turcica*, responsible of corn leaf blight. Moreover, challenging corn plants with different pathogens not only induces accumulation and hydrolysis of DIMBOA glucosides but also its metabolism to other forms, such as the monohydroxylated and dimethoxylated HDMBOA, that can also be synthesized *de novo*, as demonstrated by its accumulation in *bx1* plants (Ahmad et al., 2011; Huffaker et al., 2011).

Phytoalexins

As mentioned above, phytoalexins are antimicrobial compounds that are toxic *per se* and produced upon pathogen attack.

Camalexin is the typical phytoalexin from *Arabidopsis thaliana* which is synthesized from tryptophan after its metabolism to indol-3-yl acetaldoxime catalyzed by CYP79B2 and CYP79B3, being the only step shared with indole glucosinolate biosynthesis (Böttcher et al., 2009b; Glawischnig, 2007). Afterwards, indol-3-yl acetaldoxime is converted to indol-3-yl acetonitrile (IAN) by CYP71A13, constituting another limiting step. This metabolite is then conjugated to cysteine and then converted spontaneously or in a reaction catalyzed by CYP71B15 to dihydrocamalexic acid, the direct precursor of camalexin and substrate of PAD3. Camalexin accumulates upon elicitation with pathogens (Mert-Türk et al., 2003; Schlaeppli et al., 2010) but also after subjecting *Arabidopsis* to Cd²⁺ or Ag⁺ stress (Mert-Türk et al., 2003). Although not all cruciferous plants accumulate camalexin on pathogen attack, most phytoalexins of this species are derived from tryptophan and, therefore, harbor an indole moiety. To this respect, cruciferous phytoalexins can be divided into six groups depending on the initial core structure: group I phytoalexins comprise indole-substituted compounds with thiocarbamate or dithiocarbamate and dithioimidate moieties, group II are based on cyclobrassinin sulfoxide, core structure of group III phytoalexins is indol-3-ylacetonitrile whereas group IV contain indol-3-ylacetaldehyde; phytoalexins from group V are known as ‘spiro’ and contain a methyl thiodihydrothiazole group attached to the indole moiety. Finally, group VI phytoalexins, in which camalexin is included, is constituted of relatively stable compounds with different structures and MS/MS

fragmentation patterns from those in the rest of groups (Pedras et al., 2006). As an example, rapeseed seedlings infected with *Plasmodiophora brassicae* produced at least six different indole phytoalexins: spirobrassinin (V), brassilexin (VI), brassicanate (IV), rutalexin (II), cyclobrassinin (II) and 4-methoxybrassinin (I) (Pedras et al., 2008). The model plant *Thellungiella halophila* accumulated wasalexins (I) and methoxybrassinin (has a dimethyl acetylcarbonimidodithioate substituent on the methoxyindole ring and not a methyl ethylcarbomodithioate as methoxybrassinin) upon elicitation with CuCl_2 whereas camalexin could not be detected (Pedras and Adio, 2008).

In other plant species, compounds derived from distinct pathways act as phytoalexins and are produced upon pathogen elicitation: phenols, acetophenones, biphenyls and dibenzofurans, stilbenes, coumarins, flavonoids, terpenes, etc. (Gottstein and Gross, 1992). In *Vitis vinifera*, the stilbenoid phytoalexin resveratrol can be present alone or forming different oligomers. Moreover, this metabolite has gained scientific relevance due to its attributed health properties (Vogt, 2010). Resveratrol synthesis is catalyzed by stilbene synthase (STS) from coenzyme A esters of cinnamic acid derivatives (particularly, resveratrol derives from *p*-coumaroyl-CoA derivatives). This STS enzyme is encoded by a multigene family in grapevine and is phylogenetically related to chalcone synthase that competes with STS by the phenolic acid-CoA derivatives. The expression of this gene is induced by biotic or abiotic elicitors, increasing phytoalexin levels (Jeandet, 2015; Jeandet et al., 2002). Mulberry (*Morus alba*) also produce stilbene phytoalexins (oxyresveratrol) upon elicitation with fungal pathogens such as *Fusarium solani* f. sp. *mori*. (Gottstein and Gross, 1992).

Another group of phytoalexins widely distributed in angiosperms are coumarins and the most important representative is scopoletin. This phytoalexin was first identified in rubber tree after infection with pathogenic fungi (Gottstein and Gross, 1992). Its synthesis arises from feruloyl-CoA in a single hydroxylation step catalyzed by a dioxygenase, subsequent isomerization, rotation and lactonization. Moreover, scopoletin can be further modified by UDP-glucosyl transferases rendering scopolin (Vogt, 2010). In Citrus, irradiation and infection with pathogens also induces accumulation of several coumarin-type phytoalexins. Scoparone was isolated from the bark tissue and fruit rind of sweet orange after infection with *Phytophthora citrophthora* (Afek and Szejnberg, 1988). Its accumulation was associated to resistance to *P. citrophthora* and antifungal treatments such as fosetyl-Al also increased scoparone levels in *Citrus* tissues (Gottstein

and Gross, 1992). Nevertheless, some coumarins might have an opposite effect on pathogen growth. Inoculation of wounded *C. limon* fruits with *Penicillium digitatum* rendered a 2% of fruits exhibiting green mold symptoms. However, when epicarp oil from lemons was isolated and applied to washed fruit wounds, green mold development increased up to 92%. Fractionation and spectrometric assays allowed identification of the coumarin prangolarin that could act as a facilitator of green mold penetration and invasion (Arimoto et al. 1995).

Flavonoids also constitute a widely distributed group of plant secondary metabolites. They are highly structurally diverse and are involved in many essential processes including defense against pathogens and protection against stress-induced damage. To this respect, there are 'preformed' flavonoids that are synthesized during the normal development of a plant, and could also be involved in pathogen defense, and also flavonoids that are synthesized on pathogen attack (Treutter, 2006). This group of compounds comprises anthocyanins (active against bacterial blight agent *Xanthomonas spp.* in cotton leaves), flavones, flavonols (that occur in the lesion margins after infection in *Eucalyptus globulus*), flavanones and dihydroflavonols (aglycones and glycosylated forms), chalcones, dihydrochalcones and aurones (Williams and Grayer, 2004). These compounds, as stilbenes and coumarins, are synthesized from *p*-coumaroyl-CoA. This compound is metabolized by chalcone synthase (CHS) into chalcone, the first precursor molecule in flavonoid biosynthesis (Vogt, 2010). The occurrence of these metabolites in several plant species as defense compounds has been extensively studied: the isoflavonoids glyceollins in soybean (Ebel et al., 1976), pisatin in pea (Perrin and Bottomley, 1961), medicarpin in alfalfa (He, 2000), etc. They are effective not only against fungal pathogens but also against bacteria (Piasecka et al., 2015). In grass species several examples of flavonoids after pathogen inoculation are found: flavanone sakuranetin in rice (Hasegawa et al., 2014) or 3-deoxyanthocyanidins in sorghum (e.g. apigenindin and luteolinidin) (Snyder and Nicholson, 1990). Indeed, impairment in the synthesis of any of these metabolites was associated to enhanced susceptibility (Piasecka et al., 2015). In legumes, the most important phytoalexins are of the isoflavonoid type including medicarpin, glyceollin II, kievitone and vestitol (Harborne, 1999). In mulberry, a group of flavanoids named kuwanons including approximately 12 members were identified as antifungal metabolites. Moreover, infection of mulberry with *Fusarium solani* or *Stigmina mori* allowed the identification

of morusin, a diphenylpropane derivative and its accumulation inhibits fungal and bacterial growth (Gottstein and Gross, 1992). Infection of sweet orange citrus fruits with *P. citrophthora* induced the accumulation polymethoxylated flavones (nobiletin, sinensetin and tangeretin) and the hydrolysis of flavanone glycosides (hesperidin and isonaringin) thus increasing aglycone levels. These compounds exhibited a significant antimicrobial activity *in vitro* and its induction appeared as a plausible defense mechanism in citrus (del Río et al., 2004). In addition, modulation of levels of polymethoxylated flavones in citrus fruits by hormonal treatments increased resistance to *P. citrophthora* (Ortuño et al., 2002). More recently, a sequential proteomics and metabolomics postharvest study on *C. unshiu* (satsuma mandarin) fruits revealed the induction of several flavonoid glycosides and aglycones after heat treatment, resulting in reduced germination of *Penicillium italicum* in rind tissue. Among the induced flavonoids, quercetin glycosides, hesperetin, naringenin, diosmin and rutin were found (Yun et al., 2013) which further supports their role in disease resistance. As mentioned above, flavonoids are broad spectrum antimicrobial metabolites being active not only against fungal pathogens but also bacteria. The disease known as Huanglongbing (HLB) (or citrus greening) is caused by different strains of the *Candidatus Liberibacter* (Hijaz et al., 2013). A preliminary study carried out on symptomatic and control leaves of sweet orange plants indicated important changes in secondary metabolites (Manthey, 2008). Subsequent metabolomics studies revealed a primary accumulation of several hydroxycinnamates and certain selected flavonoids such as hesperidin, naringenin and quercetin (Cevallos-Cevallos et al., 2009; Hijaz et al., 2013). Although the specific role of these metabolites on bacterial growth and expansion has not been assessed, hesperidin accumulation in sweet orange has also been associated to defense mechanism against the bacterial pathogen *Xylella fastidiosa* (Soares et al., 2015).

Terpenoid phytoalexins often coexist with other of different origin; such is the case of the phenylpropanoid sakuranetin that is accumulated upon pathogen attack along with momilactones, oryzalexins and phytocassanes in rice and zealexin and kauralexin in corn, all of them diterpenes derived from geranyl geranyl pyrophosphate (Horie et al., 2015; Piasecka et al., 2015). About 20 genes involved in the biosynthesis of these compounds have been identified and investigated in rice, including several diterpene synthases (copalyl diphosphate synthases or CPS, kaurene synthases-like or KSL and P450 monooxygenases). Among all, CPS4 is involved in the biosynthesis of

momilactones and oryzalexin S, as extracted from studies with a rice T-DNA insertion mutant *cps4-tos* devoid in both groups of terpenoid phytoalexins and also displayed significant sensitivity to rice blast fungus *Magnaporthe oryzae* and *Fusarium fujikuroi* responsible of the ‘bakanae’ or ‘foolish seedling’ disease (Piasecka et al., 2015). Nevertheless, although these plants showed significantly reduced levels of momilactones and oryzalexin S, they are not defective in the production of other terpenoid phytoalexins such as phytocassanes A to E and oryzalexins A to F; therefore, the putative role of these metabolites in rice immunity needs a further validation. The synthesis of zealexins and kauralexins is less understood, although it is thought that mediate interactions with microbial pathogens and insects in aerial tissues. However, recent studies have shown that *anther ear 2 (an2)* corn mutants deficient in kauralexin production are more prone to drought-induced damage (Vaughan et al., 2014). Under water deprivation, phytoalexin production is root-specific and does not have any effect in phytoalexin levels in shoots but could have an influence on the ability to induce their production in aboveground tissues. Moreover, treatment with the plant hormone ABA caused the accumulation of terpenoid phytoalexins in maize roots (Vaughan et al., 2015). Interestingly, in *Nicotiana plumbaginifolia*, the production of the sesquiterpenoid capsidiol was inhibited by ABA treatment (Mialoundama et al., 2009). Other phytoalexins are induced by jasmonates (Naoumkina et al., 2007) or SA (Durango et al., 2013). Citrus produce a large number of triterpenoids that have been associated to fruit and juice quality as well as responses of vegetative tissues to the environment. These compounds are synthesized from squalene by formation of a polycyclic molecule containing a furanolactone core structure; the main representative is limonin and its respective glucosylated form (Arbona et al., 2015). These compounds have been found to accumulate in vegetative *Citrus* tissues in response to HLB and could be part of the metabolic defense response (Manthey, 2008; Slisz et al., 2012).

ROS production and lipid peroxidation are major hallmarks of the HR in plants. The formation of lipid peroxides is catalyzed by lipoxygenases (LOX) and these molecules are involved in the execution of programmed cell death and also in signal transduction processes. In the cytoplasm, LOXs oxidize fatty acids rendering oxylipins that can diverge into the jasmonate or the hydroperoxide lyase biosynthesis pathways (Zoeller et al., 2012). Elicitation by pathogen attack or wounding induces cell membrane lipases that release linoleic acid from phospholipids into the cytoplasm (Aliferis and Jabaji,

2012; Dat et al., 2005; Zoeller et al., 2012). Subsequently, LOX enzymes catalyze the formation lipid hydroperoxides. A central molecule is 13-hydroperoxy octadecatrienoic acid (13-HPOT) that can be further metabolized into 13-keto octadecatrienoic acid, cleaved into *n*-hexenal, or diverted into jasmonate biosynthetic pathway (Schaller, 2001). Besides jasmonates that do have a role in stress responses (De Ollas et al., 2015), fatty acid hydroperoxides have been shown to have a role in the induction of HR after pathogen attack (Dat et al., 2005; Zoeller et al., 2012) although cell death is not always associated with lipid peroxidation (Dat et al., 2005). In animal systems, lipid signaling plays an important role in the development of inflammatory response. As examples, the induction of leukotrienes and thromboxanes from arachidonic acid occurs in acute and chronic inflammatory processes (Hammond and O'Donnell, 2012). These responses are also downregulated after application of non-steroidal anti-inflammatory drugs such as acetyl salicylic acid or ibuprofen (Savchenko et al., 2010). Unlike animal systems, higher plants do not accumulate arachidonic acid; however, certain moss species such as the model bryophyte *Physcomitrella patens* are reported to contain significant amounts of this fatty acid; in addition, they also contain linoleic and linolenic acids (Beike et al., 2014) and accumulate cyclopentenone oxylipins (such as 12-oxo-phytodienoic acid, OPDA) but not jasmonic acid (Beike et al., 2014; Stumpe et al., 2010). Nevertheless, this fact does not seem to affect their ability to cope with biotic threats (Ponce de León et al., 2007), what means that other oxylipins might have taken over the role that JA and JA-Ile have in higher plants, reinforcing the hypothesis of lipid signaling as a trans-kingdom key feature in the response to infections.

In plant-pathogen interactions, much attention is paid to soluble semipolar metabolites as most phytoalexins and phytoanticipins fall within this category. However, successful pathogens subvert plant metabolism in order to enable the efficient uptake, sequestration and utilization of nutrients derived from the host photosynthetic activity. Under these circumstances, a reduction in net CO₂ assimilation as well as a reprogramming of host carbon partitioning is often observed within the first stages of infection (Parker et al., 2009). It is expected that levels of polar metabolites from the primary metabolism: carbohydrates, aminoacids, tricarboxylic acids, etc. are affected by pathogen attack. In *Arabidopsis thaliana*, inoculation with *Pseudomonas syringae* caused metabolic changes that affected glucosinolates, phenolic compounds but also aminoacids (phenylalanine and tyrosine) and disaccharides (sucrose) (Ward et al.,

2010). Similarly, in symptomatic leaves of HLB affected citrus plants, phenylalanine accumulated whereas sucrose levels decreased (Chin et al., 2014), which indicates a different mode of action to that of *P. syringae*. Indeed, citrus varieties with enhanced susceptibility to HLB showed higher basal levels of L-proline, L-serine, and L-aspartic acid, galactose as well as butanedioic and tetradecanoic acids whereas resistant varieties showed higher levels of L-glycine and mannose. In addition, a stronger metabolic response was observed in sensitive varieties respect to resistant ones (Cevallos-Cevallos et al., 2012). Changes in primary metabolism indeed might influence the occurrence of secondary metabolites as their direct precursors. Therefore, microbes reprogram plants primary metabolism in order to provide themselves with nutrients; additionally, adapted plants might use this reprogramming to feed the secondary metabolism with precursors to synthesize lethal phytoalexins (Sanchez-Vallet et al., 2010). Moreover, it is also likely that plants respond to pathogen infection by reprogramming their own primary metabolism to deplete infected cells of the nutrients necessary to support microbial growth.

In response to pathogen elicitation, plants emit a number of volatile organic compounds (VOCs) that have several roles such as semiochemicals in plant-plant, plant-insect or plant-microbe interactions, responses to herbivore, biological control of entomological pests or invasive plants and as sensory and flavor attributes (Beck et al., 2014). Herbivory and fungal infections in different plant species induce the emission of several terpenes and fatty acid-derived volatiles that could be involved, directly or indirectly, in plant defense. For instance, when corn plants are attacked by herbivores synthesize β -caryophyllene from farnesyl diphosphate that is released to the atmosphere and attracts natural enemies of the herbivores (Beck et al., 2014). The induced VOC blend seems to exhibit attacker specificity and different strains of the same pathogen could induce different combinations of volatiles. Although their specific role is not yet clear, certain pathogen inducible volatile compounds such as 3-hexenol, 2-hexenal, methyl salicylate or linalool have been shown to inhibit pathogen growth (Ponzio et al., 2013). In corn, infection with different species of the phytopathogen *Fusarium* induced the production of diterpene VOCs (β - and α -selinenes, β -macrocarpene, β -bisabolene and trichodiene) and their production correlated with the induction of zealexins (Becker et al., 2014). The specific role of these volatiles in infection response is not known although C6 green leaf volatiles have been associated to priming neighboring plants against pest attack (Beck et

al., 2014). To this respect, it has been recently reported that the green leaf volatile 3-hexenyl acetate enhances defense against *Fusarium graminearum* in wheat (Ameye et al., 2015). In citrus, downregulation of D-limonene synthase altered monoterpene levels and also induced resistance to *Penicillium digitatum* infection (Rodriguez et al., 2013). Conversely, overproduction of plant volatiles by transgenic *Arabidopsis* plants overexpressing a terpene synthase involved in monoterpene production (35S:TPS23/27) did not have a resistant behaviour against *Verticillium longisporum*. Released monoterpenes had a positive effect on conidial germination and hyphal growth subsequently allowing a faster colonization (Roos et al., 2015). As mentioned above, the role of VOCs as semiochemicals transferring information between individuals of the same plant species, different plant species and also different kingdoms is of ecological relevance (Morrell and Kessler, 2014). For instance, *Candidatus Liberibacter Solanacearum* alters the volatile emission spectra in the host tomato plant. A number of VOCs were more abundant in the headspace of infected plants than in control plants: α -farnesene, α -pinene, terpinen-4-ol, β -terpineol, α -terpineol, and γ -terpineol along with aldehydes nonanal and octanal. This altered volatile emission influenced settlement behaviour of the *Candidatus Liberibacter Solanacearum* vector psyllid, as non-infected psyllids preferentially settle on an infected plant and non-infected plants attract infected psyllids (Mas et al., 2014). Other tri-trophic interactions have been described involving VOCs as mediators of this interaction, corn inoculation with the endophytic bacterium *Enterobacter aerogenes* increased resistance to blight fungus *Setosphaeria turcica* due to the increased production of 2,3-butanediol. However, these plants were more susceptible to the caterpillar *Spodoptera littoralis* (D'Alessandro et al., 2014), reinforcing the specificity of the VOCs blend production. Further research will focus in deciphering the specific roles of different VOCs blends and their implications in ecological interactions.

Metabolic response of plants to viruses

Viruses are invasive nucleic acids that use the host cell enzyme machinery to propagate throughout the entire organism. Infection of plants by viruses has different effects depending on virus-plant host compatibility, overall health status of plants, etc. Some viruses improve host plant quality and attractiveness to vectors (e.g. aphids), but other (e.g. Cucumber Mosaic Virus or CMV) significantly reduce plant 'appealing' through dramatic changes in phloem carbohydrate and aminoacid redistribution, resulting in

massive dispersal of vectors. Interestingly, in CMV-infected squash there is an increase in volatile emission (but not in the particular VOCs blend) that exerts an attractive effect on vector aphids (Mauck et al., 2014). Therefore, it seems clear that also viruses subvert plant metabolism in order to facilitate their own dispersal. Another example found in the literature is the interaction of tobacco with *Bemisia tabaci*, the tobacco whitefly. Tobacco is a relatively poor host for *B. tabaci* but infection with *tomato yellow leaf curl virus* (TYLCV) increased host suitability associated to changes in volatile terpenoid synthesis (Luan et al., 2013). A similar response was observed in tomato, whiteflies preferred settling on TYLCV-infected plants that on healthy ones that have higher emissions of the volatile terpenes β -myrcene, thymene, β -phellandrene, caryophyllene, (+)-4-carene, and α -humulene (Fang et al., 2013). In addition, there are also differences between resistant and susceptible cultivars. Tomato cultivars resistant to TYLCV exhibited a more coordinated response in the primary metabolism than susceptible ones, leading to the production of defensive secondary metabolites aimed to cope with the infection (Sade et al., 2014). There are also differences in locally-infected tissues or in organs that have developed a systemic response to the infection. For instance, in response to *tomato mosaic virus* (ToMV), tomato leaves responded to local infection by decreasing aminoacid, sucrose and phenolic acid levels and increasing tricarboxylic acid levels, systemic leaves showed similar profiles but also exhibited increased levels of tryptophan, sucrose and caffeoyl esters of glucaric acid. This was associated to a requirement of carbohydrates in locally-infected leaves to support the biosynthesis of defensive compounds such as rutin (López-Gresa et al., 2012). However, the specific role of changes in secondary metabolites on virus infection is not clear, as viruses are not real 'living' organisms and therefore their live cycle should not be affected by induced toxic compounds. A putative role of these metabolites could be directed to reduce virus spread by acting against the entomological vector. To ascertain the specific role, more *in vitro* and *in vivo* assays of potential defense compounds need to be performed both on virus replication and vector fitness.

Conclusions and future prospects

The now established high-throughput metabolite profiling techniques (LC/MS and GC/MS) have allowed the identification of a great number of novel molecules with putative antimicrobial, pathogen-growth deterring or semiotic functions. However, the great challenge is to undoubtedly assess their function *in vivo*. Modulation of toxic plant

products to respond to pathogens could constitute a unique tool to enhance resistance, improving crop productivity and reducing pesticide application. Hence, metabolomics in combination with other –omics techniques have significantly contributed to the identification of genes and pathways responsible for the biosynthesis and accumulation of a plethora of secondary metabolites. As a result, a number of candidate genes for transgenic approaches are now available. However, it is important to properly assess any potential side effects of increasing phytoalexin production as these metabolites could be significantly toxic for human consumption. In addition, the host-pathogen interaction plasticity has also to be taken into account, as phytoalexin-overproducing transgenic crops could in turn drive adaptation of pathogens (Großkinsky et al., 2012). For these reasons, although substantial progress has been made towards the identification of genes and metabolites with potential antimicrobial activities and their implementation in crop improvement strategies (Table 1, see also Dubey et al., 2014), there is still a long way until researchers could fully understand the complex plant-pathogen interactions.

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Table 1. Transgenic strategies to enhance secondary metabolite production and plant defense against pathogens (adapted from (Dubey et al., 2014; Großkinsky et al., 2012)).

Metabolite class	Plant species	Target secondary metabolite	Transgenic strategy/genetic modification	Observed result
Alkaloids				
	<i>Arabidopsis thaliana</i>	Camalexin	Knock out of phytoalexin deficient 3 (<i>PAD3</i>), responsible of the last step in camalexin biosynthesis (Böttcher et al., 2009b)	Decreased camalexin, biosynthesis, no effect on <i>Pseudomonas syringae</i> infection (Glazebrook and Ausubel, 1994).
			EtOH-inducible oomycete elicitor (PaNie)	Time-dependent induction of tryptophan and camalexin biosynthesis genes by PaNie (Rauhut et al., 2009).
			Overexpression of <i>miR393</i>	Re-direction of secondary metabolism from camalexin to glucosinolates, increased resistance against <i>Pseudomonas syringae</i> and <i>Hyaloperonospora parasitica</i> , increased susceptibility against <i>Alternaria brassicicola</i> (Robert-Seilaniantz et al., 2011).
			Dexamethasone inducible overexpression of <i>MEK2</i> ; <i>mpk3/mpk6</i> knock out	Camalexin accumulation after <i>MEK2</i> induction, reduced/delayed camalexin biosynthesis in <i>mpk3</i> and <i>mpk6</i> mutant lines, increased susceptibility against <i>Botrytis cinerea</i> of <i>mpk3</i> mutant line (Ren et al., 2008).
			Knock out of <i>WRKY33</i>	Reduced camalexin levels in <i>wrky33</i> mutant lines (Ahuja et al., 2012).
	<i>Catharanthus roseus</i>	Strictosidine	Overexpression of tryptophan decarboxylase and strictosidine synthase	Increased alkaloid production (Canel et al., 1998).
Terpenoids				
	<i>Oryza sativa</i>	Momilactones	Overexpression of transcription factor (e.g. <i>OsTGAP1</i>) and biosynthesis genes Knock down of biosynthesis genes	Increased momilactone levels in overexpression lines, constitutive momilactone accumulation in <i>OsTGAP1</i> overexpression lines, reduced momilactone levels in knock down lines (Okada, 2014)
			<i>CIPK14/15</i> RNAi and overexpression of <i>CIPK15</i>	Decreased momilactone accumulation in <i>CIPK14/15</i> RNAi-line, increased momilactone accumulation in <i>CIPK15</i> overexpression line (Kurusu et al., 2010)
		Momilactone A	Overexpression of Accelerated Cell Death and	Increased momilactone A accumulation and increased

		Resistance 1 (<i>ACDR1</i>) a putative Raf-like MAPKKK. Spotted leaf 18 <i>Spl18</i> mutant	resistance against <i>Magnaporthe grisea</i> in <i>ACDR1</i> overexpression lines (Kim et al., 2009). Increased momilactone A accumulation and increased resistance against <i>Magnaporthe grisea</i> in <i>spl18</i> mutant (Mori et al., 2007).
		Overexpression of Rac GTPase (<i>RAC1</i>)	Increased momilactone A accumulation and increased resistance against <i>Xanthomonas oryzae</i> in <i>RAC1</i> overexpression lines (Ono et al., 2001).
		Overexpression of <i>SBP</i> , a Se binding protein involved in plant defense.	Increased momilactone A accumulation and increased resistance against <i>Magnaporthe grisea</i> and <i>Xanthomonas oryzae</i> in <i>SBP</i> overexpression lines (Sawada et al., 2004).
	β -caryophyllene	Overexpression of <i>OsTPS3</i> encoding a beta-caryophyllene synthase	Plants with increased emission rates of (E)- β -caryophyllene (Ohara et al., 2003).
	Phytocassanes	<i>CIPK14/15</i> RNAi and overexpression of <i>CIPK15</i>	Decreased phytocassane accumulation in <i>CIPK14/15</i> RNAi-line, increased phytocassane accumulation in <i>CIPK15</i> overexpression line (Kurusu et al., 2010).
<i>Nicotiana tabacum</i>	Capsidiol	Overexpression of isopentenyl transferase (<i>ipt</i>)	Induction of capsidiol biosynthesis and increased resistance against <i>Pseudomonas syringae</i> , increased susceptibility to <i>Botrytis cinerea</i> (Großkinsky et al., 2011).
	Trichodiene	Over expression of trichodiene synthase gene, that converts farnesyl pyrophosphate to trichodiene - Inserted under constitutive action of CaMV35S	Expression of active enzyme, increases the production of the sesquiterpenoid product in leaves (Tijerino et al., 2011).
	Monoterpenes	Expression of <i>Perilla frutescens</i> limonene synthase	Increased synthesis of limonene (Doares et al., 1995).
	Patchoulol and sesquiterpenes	Overexpression of patchoulol synthase.	Catalyses the conversion of 2E,6E-Farnesyl diphosphate to patchoulol and diphosphate. Synthesis of patchoulol (volatile) and 13 additional sesquiterpene products (Rasmann et al., 2005).
	Cembratrienol	Antisense co-suppression of Cytochrome P450 hydroxylase gene specific to the trichome gland Involved in metabolism of sesquiterpenoids	Exudates of transgenic plants contain high concentrations of cembratrieneol and showed lower aphid predation (Wang et al., 2001).
<i>Nicotiana glauca</i>		ABA deficient mutants <i>Npaba1</i> and 2	Increased capsidiol accumulation in <i>Npaba1</i> and <i>Npaba2</i> mutant lines (Mialoundama et al., 2009).
<i>Mentha x piperita</i> L.	Several	Co-suppression of 1-deoxy-d-xylulose-5-	Transgenic plants showed a lower yield loss due to pests

		terpenoids	phosphate reducto-isomerase (DXR). Enzyme that catalyzes the first committed step of methyl erythritol phosphate (MEP) pathway	and pathogens (Mahmoud and Croteau, 2001).
	<i>Petunia hybrida</i> , <i>Solanum lycopersicum</i> , <i>Dianthus caryophyllus</i>	Linalool	Constitutive over expression in flowers of S-linalool synthase that catalyzes the conversion of geranyl diphosphate to 3S-linalool	Linalool and its derivatives produced by the transgenic plants act as repellents of <i>Myzus persicae</i> aphids and associated pathogens (Lücker et al., 2001).
	<i>Nicotiana spp.</i>	Volatile terpenoids, sclareol	<i>NpPDR1</i> overexpression, an ABC transporter.	Increased volatile diterpene emission and sclareol secretion upon elicitation with pathogens (Aharoni et al., 2003; Stukkens et al., 2005).
	<i>Artemisia annua</i>	sesquiterpenoids	Overexpression of farnesyl diphosphate synthase	Increased metabolic flux in the sesquiterpenoid biosynthetic pathway (Doares et al., 1995).
	<i>Lotus japonicus</i> <i>Nicotiana tabacum</i>	(E,E)-geranyl linalool and 4,8,12-trimethyltrideca-1,3,7,11-tetraene (TMTT)	Terpene synthase gene – PITPS2 from <i>Phaseolus lunatus</i>	Transgenic plants produce (E,E)-geranyl linalool and 4,8,12-trimethyltrideca-1,3,7,11-tetraene (TMTT). Attracts predatory mites to infested plants (Wu et al., 2006).
	<i>Gossypium hirsutum</i>	Gossypol	Overexpression of <i>NPRI</i> , that controls systemic acquired resistance (SAR).	Increased gossypol accumulation and increased resistance against <i>Rhizoctonia solani</i> and <i>Alternaria alternata</i> in <i>NPRI</i> overexpression lines (Parkhi et al., 2010).
Phenylpropanoid pathway				
<i>Cinnamates/Coumarins</i>	<i>Nicotiana tabacum</i>	Scopoletin	Overexpression of isopentenyl transferase (<i>ipt</i>) gene.	Induction of capsidiol biosynthesis and increased resistance against <i>Pseudomonas syringae</i> , increased susceptibility to <i>Botrytis cinerea</i> (Großkinsky et al., 2011).
	<i>Solanum lycopersicum</i>	Increased oxidation of phenolic substrates	Antisense expression of Tobacco O-glucosyl Transferase (<i>TOGT</i>) gene. cDNA transfer of polyphenol oxidase gene from <i>Solanum tuberosum</i> under constitutive action of CMV35S	Reduced scopoletin accumulation and increased TMV susceptibility in <i>TOGT</i> antisense lines (Chong, 2002). Transgenic plants showed increased resistance to <i>Pseudomonas syringae</i> and strong inhibition of bacterial growth (Brillada et al., 2013).
<i>Flavones</i>	<i>Arabidopsis thaliana</i> ,	Medicarpin	Overexpression of chalcone isomerase (<i>CHI</i>)	Increased flavonol levels in <i>CHI</i> overexpression lines

<i>Isoflavones</i>	<i>Solanum lycopersicum</i> , <i>Medicago sativa</i>		<i>gene</i> .	(Dixon et al., 2002).
			Overexpression of isoflavone <i>O</i> -methyl transferase (<i>IOMT</i>).	Increased medicarpin accumulation and increased resistance against <i>Phoma medicaginis</i> in <i>IOMT</i> overexpression lines (Dixon et al., 2002)
	Several plant species	e.g. Coumestrol, Glyceollins, Medicarpin	Various modulations of biosynthesis genes and regulatory elements	Diverse (Du et al., 2010).
	<i>Lotus corniculatus</i>	Vestitol	Antisense expression of chalcone synthase (<i>CHS</i>)	Reduced vestitol accumulation in <i>CHS</i> antisense lines (Colliver et al., 1997).
	<i>Medicago truncatula</i>	Formononetin, Medicarpin	Overexpression of isoflavone synthase (<i>IFS</i>)	Increased formononetin and medicarpin accumulation in <i>IFS</i> overexpression lines (Deavours and Dixon, 2005).
	<i>Pisum sativum</i>	Pisatin	RNAi lines for (+)6a-hydroxymaackiain 3- <i>O</i> -methyltransferase (<i>HMM</i>), isoflavone reductase (<i>IFR</i>) and sophorol reductase (<i>SOR</i>).	Reduced pisatin accumulation in <i>HMM</i> , <i>IFR</i> and <i>SOR</i> RNAi lines (Kaimoyo and VanEtten, 2008).
			<i>HMM</i> antisense, overexpression of pisetin demethylating activity (<i>PDA</i>).	Reduced pisatin accumulation in <i>HMM</i> antisense and <i>PDA</i> overexpression lines (Wu and VanEtten, 2004).
	<i>Oryza sativa</i>	Sakuranetin	Overexpression of <i>ACDR1</i> gene.	Increased sakuranetin accumulation and increased resistance against <i>Magnaporthe grisea</i> in <i>ACDR1</i> overexpression lines (Kim et al., 2009).
			<i>Spotted leaf 18 (Spl18)</i> mutant	Increased sakuranetin accumulation and increased resistance against <i>Magnaporthe grisea</i> in <i>spl18</i> mutant (Mori et al., 2007).
<i>Stilbenes</i>	Several plant species	e.g. Resveratrol	Overexpression of <i>STS gene</i>	Increased stilbene accumulation and increased resistance to various pathogens in <i>STS</i> overexpression lines (Delaunoy et al., 2009; Jeandet et al., 2002).
	<i>Lactuca sativa</i>	Resveratrol	Overexpression of <i>STS</i>	Transfer of resveratrol accumulation (Liu et al., 2006).
	<i>Oryza sativa</i> , <i>Solanum lycopersicum</i>		Overexpression of <i>STS</i>	Increased resistance against <i>Magnaporthe grisea</i> and <i>Phytophthora infestans</i> (Hammerschmidt, 1999).

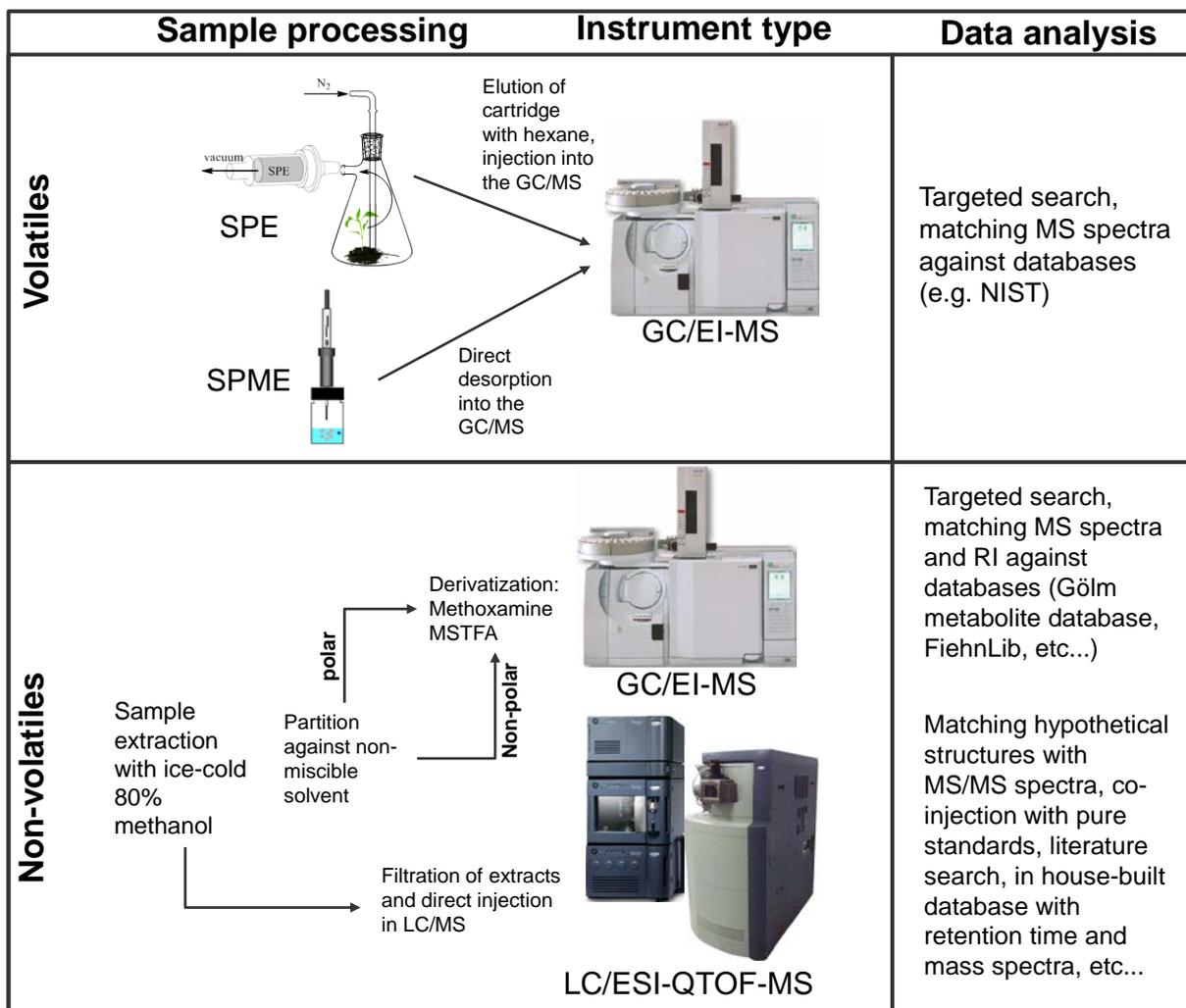


Figure 1. Different mass spectrometry-based metabolite profiling strategies according to sample type and metabolite class. Abbreviations: GC/EI-MS, gas chromatography/electron impact-mass spectrometry; LC/ESI-QTOF-MS, liquid chromatography/electrospray-quadrupole time-of-flight-mass spectrometry; MSTFA, methyl silyl trifluoroacetamide; SPE, solid phase extraction; SPME, solid phase microextraction.

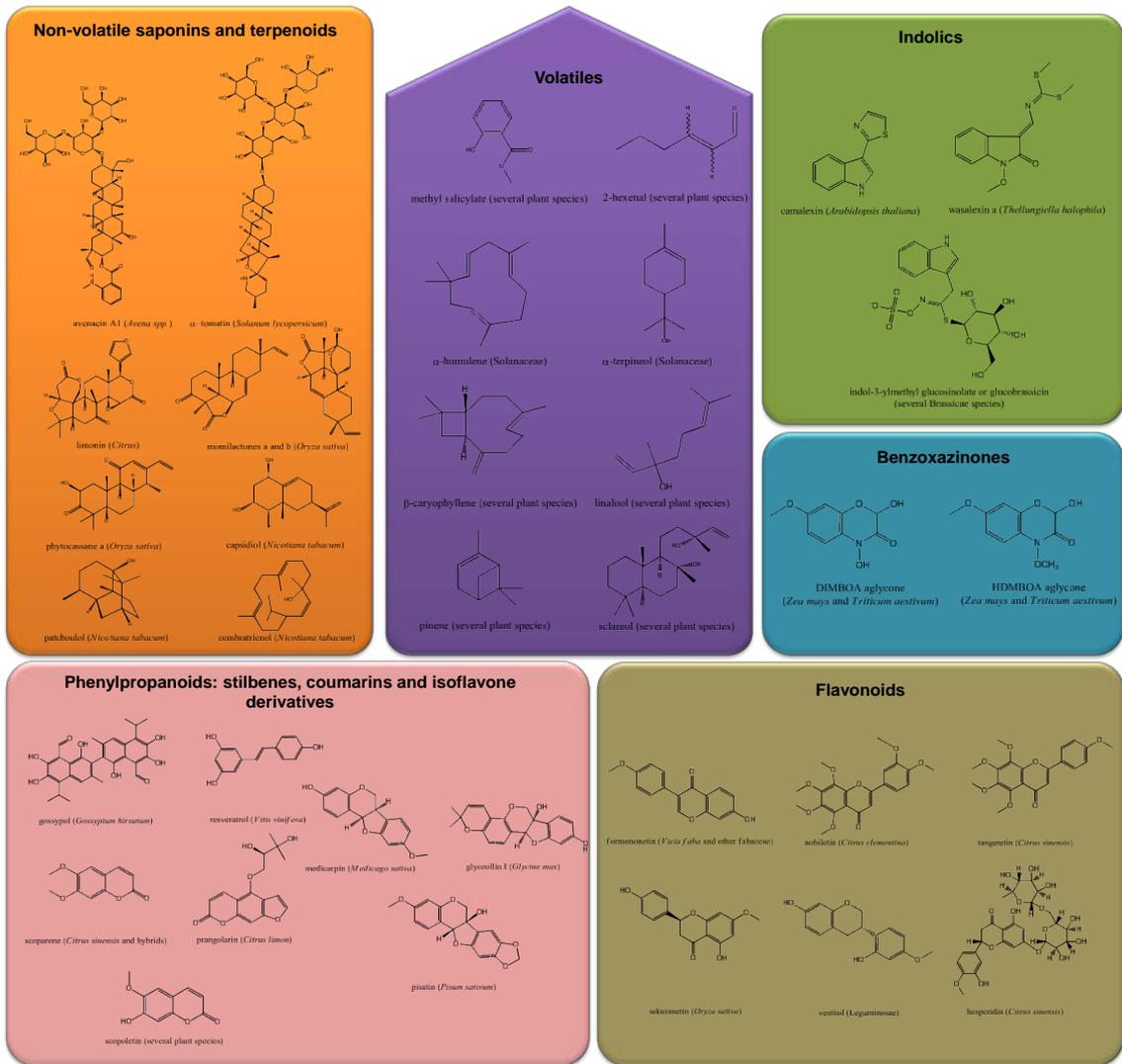


Figure 2. Compound classes and structures of metabolites involved in plant defense.