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Endogenous plant metabolites against insects

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Abstract Herbivorous insects are responsible for large losses to agriculture through feeding damage itself but also by vectoring pathogens such as viruses. Plants have evolved various mechanisms to defend themselves against insects, including the production of specialised metabolites that act as natural insecticides. Throughout history, plants and plant extracts harbouring specialised metabolites have been exploited by man for their anti-insect properties. Also, commercial insecticides are often based on plant-specialised metabolites and adapted further e.g. to give them the physicochemical properties that render them systemic. The development and application of these synthetic pesticides has made it possible to scale up the production and to increase the yield of food crops worldwide. However, their adversary effects on the environment and on non-target organisms, first discussed in Rachel Carson's *Silent Spring*, continue to cause debate and recently resulted in a ban on the use of certain pesticides in the European Union. The trait of insect defence has been largely overlooked during crop domestication and resulted in basically unarmed crop plants. Endogenous production of specialised metabolites could be re-introduced in modern crop varieties to retrieve self-defences. However, despite the fact that a plethora of specialised metabolites from plants has been described, studies that validate their deterrent or insecticidal effect *in planta* (crop), let alone in field situations,

are sparse. To answer the question whether it is feasible to re-introduce endogenous insecticides to bring about insect resistance, the defence metabolites of plants, their synthesis and regulatory mechanisms underlying their production are examined.

Keywords Specialised metabolites · Herbivores · Insecticide · Resistance · Breeding

Plant defence and disease

In an attempt to battle the destructive behaviour of herbivores, many plants have evolved their own, broad or insect-specific, chemical defence. People have been using these compounds in agriculture, purposely, or without any knowledge about the underlying chemical and/or its mode of action on herbivores (Pavela 2016). Wild plants were harvested, and their extracts used to treat food crops, or the metabolite-producing plants were planted amidst crops in an intercropping manner. However, when food crops started to be produced on a large scale, the focus of growers and breeders was, next to maximised productivity and yield, predominantly on traits related to the ease of cultivation and harvesting. The effects of domestication, i.e. selection in favour- or against traits like seed size and leaf hairiness translated into increased herbivore susceptibility (Chen et al. 2015). Domesticated susceptible varieties were instead protected by cultivation in semi-controlled environments (e.g. greenhouses) and/or treatment with chemical insecticides. The application of chemicals might

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nowadays have a negative ring to it as the knowledge of adverse effects on ecosystems increased (Whitehorn et al. 2012; Hallmann et al. 2014). However, the use of insecticides has been a major factor behind improved agricultural productivity necessary for global food production. Plant-feeding insects are responsible for large losses in the production of edible plants (Oerke 2006; Pavela 2007).

In addition to feeding on plants, insects can host otherwise rather immobile plant pathogens that use them as mode of transport and as a mechanical tool to enter the host plant (intracellularly). Such insect-transmitted pathogens include fungi and bacteria but the most reported examples, especially in crop production, are viruses (Whitfield et al. 2015). Whereas fungi can be carried externally on the legs and body of various insects including chewing herbivores, viruses and some bacterial pathogens travel and even propagate inside their specific host insect, after which they are transmitted to plants via the insects' piercing mouthparts (i.e. stylet) as the insect salivates. In the case of phloem or xylem-feeding insects, the pathogen directly enters the sap stream, whereas cell-feeding insects like thrips, notorious transmitters of Tospoviridae, insert viral particles into epidermal cells (Rotenberg et al. 2015). In general, the most significant phloem-feeding vectors of viruses are whiteflies, aphids and planthoppers that, among various different viruses transmit the agronomically important Geminiviridae, Potyviridae and Rhabdoviridae respectively (Whitfield et al. 2015). However also phytoplasmas, bacterial pathogens, are transferred by phloem-feeding Hemiptera (Hogenhout et al. 2008). Psyllids transfer the phloem-limited *Liberibacter* bacteria responsible for Huanglongbing or Citrus greening disease (Haapalainen 2014). Xylem-sucking herbivores are usually bigger-sized hemipterans (e.g. leafhoppers) compared to phloem feeders, as sap extraction from xylem vessels involves dealing with a high-pressure gradient (Novotny and Wilson 1997). Xylophagous leafhoppers and sharpshooters are regarded as pest-insects transmitting for instance *Xylella* bacteria that cause disease in grape and in citrus (Redak et al. 2004). Insects with either chewing or piercing feeding modes elicit different (metabolite-based) defence responses (Li et al. 2017). Accordingly, certain metabolites that target sap-feeding insects for instance, indirectly affect the pathogen that is dependent on its vector for disease epidemiology.

Treatment of the insect vector using insecticides is common practice in the control of the pathogens they carry. Anti-insect chemicals, whether man-made or natural, can be effective in the battle against enemy insects through different modes of action, ranging from damaging the exoskeleton of the insect, to neurotoxic effects (e.g. natural nicotine and synthetic nicotinoids), to preventing the insect from feeding.

Plant-produced defence compounds, first thought to be mere by-products of the primary metabolism, are extremely diverse in structure, shaped by millennia of herbivore-selection pressure (Mithöfer and Boland 2012). Hence, nowadays these metabolites are no longer termed secondary, but rather specialised metabolites (Pichersky and Lewinsohn 2011). Plants often have tailor-made tissues where specialised metabolites are produced, stored and excreted while their production is regulated by enzyme kinetics, precursor flux, feedback inhibition and is under complex (post)-transcriptional control. This review focuses on the plants' chemical, direct defence against herbivorous insects and addresses the potential for its use in agricultural practises.

Historical use of specialised metabolites and synthetic pesticides in agriculture

From ancient times, plant extracts have been exploited as medicine, fragrance, food preservatives or pest deterrents (Pavela 2004). The first use of botanicals as anti-insect compound dates back to 2500–1500 BCE (Oerke 2006) but plant-based biopesticides are still relevant in e.g. organic farming or integrative pest-management approaches. One of the first specialised metabolites to be, unknowingly, used as an insecticide was the alkaloid nicotine extracted from tobacco (Pavela 2016). Other examples of extracts long used in agriculture include pyrethrum found in Asteraceae, capsaicin from hot pepper extract and azadirachtin isolated from Neem seeds (Pavela 2007; Benelli et al. 2017). The mechanisms of action by which they exert their effect on insects are various, e.g. blocking of receptors and channels that interfere in the insect nervous system, inhibition of cellular respiration and disruption of the insect hormone balance (Rattan 2010). Many historically used plant-derived compounds have later been developed into synthetically produced insecticides. The controlled synthesis of a single bioactive compound has the advantage that both quantity and quality are less variable compared

to a botanical extract. Furthermore, synthetic pesticides can be designed more stable (e.g. less reactive to air or light) making their effect durable after application in the field (Miresmailli and Isman 2014).

A multitude of synthetic pesticides have been utilized since the nineteenth century. The first generation of insecticides consisted of highly toxic substances such as arsenic-containing compounds. Paris Green, a copper acetoarsenite, was widely used in the United States as an insecticide from 1867 to 1900, effectively killing the Colorado potato beetle (Hughes et al. 2011). Arsenic, mostly in the trivalent form, has a high lipid solubility and is readily absorbed. It covalently binds oxygen and sulphur causing toxic injury to the nervous system, lipid peroxidation and protein oxidation (Zaman et al. 1995). Despite the adverse effects, and persistence of arsenic residues, modern arsenic-based pesticides are still in use (Bonmatin et al. 2015; Wood and Goulson 2017). Inorganic pesticides were largely replaced in the 1940s by a new generation of synthetic organic compounds, such as dichlorodiphenyltrichloroethane (DDT), lindanes and also organophosphorus compounds. These compounds were relatively inexpensive and therefore widely applied. DDT acts on voltage-gated sodium channels in the insect's sensory nerves (Davies et al. 2007). Contact with DDT leads to rapid firing of nerves, causing tremors and eventually resulting in excitatory paralysis and death of the insect. DDT has a broad-spectrum activity on insects but was (relatively) harmless to humans (Davies et al. 2007). However, chlorinated hydrocarbon compounds like DDT proved to be extremely persistent in the environment and had catastrophic side-effects accumulating in the fatty tissues of non-target organisms. These negative effects, described first in Rachel Carson's book "Silent Spring" (Carson 2002) eventually led to restrictions and even bans of the product in most countries (Schumann 1991). Nowadays, the most widely used synthetic pesticides consist of one of the following major classes: neonicotinoids, pyrethroids, organophosphates, carbamates, and phenyl-pyrazoles (Jeschke and Nauen 2008; Jeschke et al. 2010; Casida and Durkin 2013). An overview of the major groups, and their mode of action, is provided in Table 1.

All organic pesticide groups depicted in Table 1 act on the insect nervous system. As mentioned earlier these synthetic compounds are often chemocopies of plant-based biopesticides. Examples of these include the development of synthetic nicotine derivatives

Table 1 Most important groups of chemical insecticides, based on their characteristics, targets inside the insect and mode of action and the decade since introduced in agriculture

Group	Characteristics	Target	Introduced	Reference
organochlorides	non-systemic, persistent, e.g. DDT	nervous system, opening sodium ion channel in neurons causing spontaneous firing	40s	Davies et al. 2007
organophosphates	phosphorus esters e.g. parathion	nervous system, acetylcholinesterase inhibition, disruption of electrical impulse across synapses	40s	Fukuto 1990
carbamates	carbamate esters e.g. carbofuran	nervous system, acetylcholinesterase inhibitors (carbamylation)	50s	Fukuto 1990
pyrethroids	analogues of pyrethrin type I and II, neurotoxins e.g. deltamethrin	disrupt voltage-gated sodium channels of insect nerves.	80s	Soderlund 2012
phenyl-pyrazoles	systemic insecticide e.g. fipronil	blocking glutamate-activated chloride channels in insects, binds GABA receptors in the nervous system.	90s	Simon-Delso et al. 2015
neonicotinoids	systemic insecticide e.g. imidacloprid	binds to nicotinic acetylcholine receptors	90s	Simon-Delso et al. 2015

(collectively named ‘neonicotinoids’), currently one of the most widely used synthetic insecticides. Both natural and synthetic versions are acetylcholine receptor antagonists, or neurotransmitter mimics (Matsuda et al. 2001; Jeschke et al. 2013). Neonicotinoids have, however, been designed to possess the physicochemical characteristics that enable systemic distribution throughout the plant (Simon-Delso et al. 2015). Another example comes from the synthetic pyrethroid-derivatives which are related to pyrethrin compounds abundantly produced in the flowers of *Tanacetum cinerariifolium* or Dalmation Daisy (Casida and Quistad 1995). Natural pyrethrins and the early synthetic pyrethroids (e.g. allethrine), were easily broken down in contact with light and air. Therefore, later generations of synthetic pyrethroids (e.g. deltamethrin) were further modified, rendering enantiomers less instable exposed to air and less persistent in soil compared to the early generation (Qin et al. 2006; Davies et al. 2007). Both plant-produced metabolites and synthetic analogues affect the insect peripheral and central nervous system (Davies et al. 2007; Soderlund et al. 2002). Lastly, antagonism on GABA receptors found in commercial phenyl-pyrazole insecticides such as fipronil is also the mode of action of the antifeedant silphinene sesquiterpenes from *Senecio palmensis* (González-Coloma et al. 2002). These compounds have been shown to antagonise the inhibitory function on neuron firing by GABA in the central nervous system of *Drosophila* larvae by blocking the GABA-gated chloride-channels (Bloomquist et al. 2008; Bloomquist 1993).

Over the past decades the awareness of adverse effects of intensive pesticide application on the environment and on human health has grown (Henry et al. 2012; Whitehorn et al. 2012; Hallmann et al. 2014). With neonicotinoids (e.g. imidacloprid) crop growers were provided with tailor-made, systemic and broad working insecticides in the 1990s. However, the use of these very effective compounds in agricultural practise is currently a topic of debate in light of their side-effects on local ecosystems (Klatt et al. 2016). This has led to a severe restriction on their use in the European Union (EU Regulation No 485/2013) and a complete ban on the outdoor use of three neonicotinoid-based substances was endorsed in 2018. The search for natural alternatives to synthetically produced insecticides has therefore yet again gained interest.

Intercropping and push-pull

Monoculturing, growing a single crop type on the same plot, is the main agricultural approach in most places. It facilitates large scale-crop handling, planting and harvest and reduced manual labour. In this setting however, the risk of disease outbreak is substantial. Intercropping, growing two or more crops simultaneously in a field, resembles a simplified ecosystem, in which the ecological balance is better maintained. Its origin is not exactly known but its practise dates back to Paleolithic times (Anders et al. 1995). Crop plants can also be grown together with non-crop plants like oilseed radish (*Raphanus sativus ssp. oleifera*) and tansy (*Phacelia tanacetifolia*) that deter or kill herbivorous insects. For example, the roots of *R. sativus* produce toxic isothiocyanides that kill the eggs of the potato cyst nematode *Globodera pallida* (Ngala et al. 2015). Tansy, but also dill (*Anethum graveolens* L.) and coriander (*Coriandrum sativum* L.) can be used to attract natural enemies of pest insects (Landis et al. 2000). Various members from the *Allium* genus used in intercropping include, garlic, leek and chives to repel herbivore species like rabbits, aphids, beetles, spider mites, whiteflies and caterpillars (Hata et al. 2016; Debra and Misheck 2014; Sulvai et al. 2016; Sharaby et al. 2015).

Many of the beneficial effects found in intercropping systems can be attributed to volatile-mediated signalling (Pickett and Khan 2016). In sub-Saharan Africa a system termed “push-pull” was introduced to answer the threat of different pests of important staple crops (Khan et al. 2003). A successful example of push-pull strategy are cereals like maize and *Sorghum* intercropped with the “push” plant *Desmodium spp* and “pull” plant Napier grass (*Pennisetum prupureum*; (Khan et al. 2002). *Desmodium* emits volatiles that repel the stemborer moth (*Busseola fusca*) thereby providing protection to the intercropped cereals (Khan et al. 2000). As a bonus, the flavonoid-glycoside iso-schaftocide produced by *Desmodium* roots inhibits the germination and attachment of parasitic witch-weed *Striga hermontica* (Hooper et al. 2015). Napier grass is a remarkably effective trap-plant, emitting volatiles (i.e. β -caryophyllene) that attract both the pest insect as well as its natural enemies and secretes a viscous substance upon herbivory that traps and kills the larvae (Khan et al. 2007).

Specialised metabolites in plant defence

Specialised metabolites for which insecticidal or anti-insect activity has most often been proven are isoprene-derived terpenoids followed by alkaloids and phenolic compounds (Boulogne et al. 2012). Less reported on, but also significant, are acylsugars and cyanogenic glycosides. Although not regarded as a ‘specialised metabolite’ per se, direct defence against insects can also be based on the synthesis of defence proteins or polymers including cuticular waxes (Constabel and Barbehenn 2008; Gonzales-Vigil et al. 2011; Mahanil et al. 2008; Hartl et al. 2010). The discussion below however, is limited to metabolites in the stricter sense only. Estimates on the divergence of specialised metabolites reach over 200.000 different structures (Pichersky and Lewinsohn 2011). They branch off from primary metabolism (e.g. amino-acids, fatty acids, hormones) and have precursor pathways that are rather conserved among plant species. The diversification in the end-products is, however, largely the result of (stereo-)isomeric configuration, skeleton rearrangements and modifications of the final product which can be versatile (Degenhardt et al. 2009; Chen et al. 2011; Nelson and Werck-Reichhart 2011; Bleeker et al. 2011b). With >20.000 papers on metabolites exhibiting insecticidal properties published since the 1980s, the mass of information is overwhelming. However, after critical review, Isman and Grieneisen (2014) concluded that only one third of these publications were based on appropriate chemical characterizations of the tested (putative) insecticides and even less (27%) included positive controls. Moreover, the majority of these are based on assays with artificial diets, often supplied with a relatively high dose of a (purified) metabolite. Nawrot and Harmatha (2012) reviewed the literature describing plant-derived metabolites acting on insects in the scope of protecting post-harvest products, which might be less relevant for application in the field. In this section we focus on examples of endogenously produced anti-insect metabolites with potential for application in breeding, provided there was evidence for a chemotype-to-phenotype correlation *in planta*, where metabolites can either be toxic, repellent, inhibit development or feeding rates of the insect (Table 2).

Terpenoids

Terpenoids (or isoprenoids) are the most described family of compounds in the plant kingdom. They serve as a

basis for many structures with diverse functions in both specialised- and primary metabolism, ranging from rather small and volatile molecules (e.g. mono- and sesquiterpenes) down to hormones (e.g. cytokinins, abscisic acid, gibberellins) and structural cell components like pigments (e.g. carotenoids). All terpenoids are made from the same isoprene building blocks: isopentenyl diphosphate (IPP) and its isomer dimethylallyl diphosphate (DMAPP). Stacking and subsequent cyclisation or chimeric modification of the products by terpene synthases, results in a plethora of terpenoids. Classified by carbon-structure, volatile monoterpenes (C10) sesquiterpenes (C15) and non-volatile diterpenes (C20), sesterterpenes (C25), triterpenes (C30), tetraterpenes (C40) and polyterpenes (>C40) are distinguished (Hemmerlin et al. 2012). In total, the number of terpenoids structures produced within the plant kingdom is estimated to exceed 25.000 different structures (F. Chen et al. 2011). This great biosynthetic plasticity may enable plant species to adapt relatively quickly in response to contemporaneous herbivorous threats.

Terpenoids are also among the most reported bioactive compounds in relation to insects (Aharoni et al. 2005). Volatile allelochemicals could be used as repellents, preventing or decreasing plant-insect contact and with that, transmission of (viral) disease. The sesquiterpene 7-epizingiberene from *Solanum habrochaites* for instance, repels the silverleaf whitefly *Bemisia tabaci*, a vector for begomoviruses (Bleeker et al. 2011a; Rosen et al. 2015). Introgression of the biosynthetic pathway into a tomato cultivar resulted in the production of 7-epizingiberene making the plant less attractive to whitefly and apparently toxic for spider mites (*Tetranychus urticae*; Bleeker et al. 2012). Another well-described repellent sesquiterpene is (*E*)- β -caryophyllene. Overexpression of the β -caryophyllene synthase in *Arabidopsis* leads to an increased (*E*)- β -caryophyllene emission from the flowers and deterrence of the Asian citrus psyllid *Diaphorina citri*, a vector of the pathogen that causes Citrus Greening Disease (Alquezar et al. 2017; Hall et al. 2013). A recent study identified several citrus accessions emitting repellent (*E*)- β -caryophyllene from the peel of the fruit, which opens possibilities for an application in breeding programs (Zhang et al. 2017a).

Concentrated mixtures of volatile and non-volatile terpenes are found in tree resin. The Norway spruce (*Picea abies*) accumulates terpenoids in its resin upon attack by its natural enemy *Ips typographus*; the bark

Table 2 Endogenous specialised metabolites with *in planta* insecticidal activity

Phytochemical	Group	Plant source	Target pest	Evidence	Reference
leptin	alkaloid	<i>Solanum chacoense</i>	<i>Leptinotarsa decemlineata</i>	variation	Sinden et al. 1986
nicotine	alkaloid	<i>Nicotiana attenuata</i>	<i>Spodoptera exigua</i> ; <i>Manduca sexta</i>	transgenic	Steppuhn et al. 2004
α -chaconine	alkaloid	<i>Solanum tuberosum</i>	<i>Tecia solanivora</i>	variation	Karlsson et al. 2013
α -solanine	alkaloid	<i>Solanum tuberosum</i>	<i>Tecia solanivora</i>	variation	Karlsson et al. 2013
dhurrin	cyanogenic	<i>Sorghum bicolor</i>	<i>Phyllotreta nemorum</i>	transgenic	Tattersall et al. 2001
2/3-methylbutyronitrile	cyanogenic	<i>Populus trichocarpa</i>	<i>Lymantria dispar</i>	transgenic	Irmisch et al. 2014
kaempferol-3,7-dirhamnoside	phenolic	<i>Arabidopsis thaliana</i>	<i>Pieris brassicae</i>	transgenic	Onkokesung et al. 2014
rutin	phenolic	<i>Nicotiana tabacum</i>	<i>Spodoptera litura</i> ; <i>Helicoverpa armigera</i>	transgenic	Misra et al. 2010
<i>O</i> -acylsugars	sugar ester	<i>Solanum pennellii</i> , <i>S. pimpinellifolium</i>	<i>Manduca sexta</i> ; <i>Bemisia tabaci</i>	variation	Leckie et al. 2012; Silva et al. 2014
17-hydroxygeranyl-linalool	terpenoid	<i>Nicotiana attenuata</i>	<i>Manduca sexta</i>	transgenic	Heiling et al. 2010
(<i>E</i>)- β -farnesene	terpenoid	<i>Solanum berthaultii</i>	<i>Myzus persicae</i>	transgenic	Bhatia et al. 2014
7-epizingiberene	terpenoid	<i>Solanum habrochaites</i>	<i>Bemisia tabaci</i> ; <i>Tetranychus urticae</i>	transgenic	Bleeker et al. 2012
(<i>E</i>)- β -caryophyllene	terpenoid	<i>Arabidopsis thaliana</i>	<i>Diaphorina citri</i>	transgenic	Alquezar et al. 2017
(-)-limonene	terpenoid	<i>Picea abies</i>	<i>Ips typographus</i>	variation	Schiebe et al. 2012
hydroabietic acid	terpenoid	<i>Picea sitchensis</i>	<i>Pissodes strobe</i>	variation	Robert et al. 2010
cucurbitacin-C	terpenoid	<i>Cucumis sativus</i>	<i>Tetranychus urticae</i>	variation	Balkema-Boostrma et al. 2003
oleanolic-acid cellobioside	terpenoid	<i>Barbarea vulgaris</i>	<i>Phyllotreta nemorum</i>	variation	Kuzina et al. 2009

Indicated is how the evidence is provided for its activity: correlation of metabolite levels and phenotypes by the use of distinct genotypes (‘variation’) or transgenic plants (‘transgenic’)

beetle (Schiebe et al. 2012). Trees within a natural population that accumulate high concentrations of D-limonene and eucalyptol in the resin are less damaged by the beetle, suggesting these compounds have repellent and/or-toxic properties. Other such resin-based terpenoids appear to play a role in the interaction between the white-pine weevil (*Pissodes strobe*) and the Sitka spruce (*P. sitchensis*) that accumulates the diterpene dehydroabietic acid for protection (Robert et al. 2010).

Diterpene 17-hydroxygeranylinalool glucosides are terpene-derivatives synthesised in high amounts by wild tobacco *Nicotiana attenuata*. Silencing of a GGPP-synthase that generates the diterpene precursor molecule geranylgeranyl pyrophosphate, resulted in decreased levels of the diterpene glucosides and, while primary metabolites remained unaffected, there was a 10-fold increase in growth of *M. sexta* larvae on the silenced plants (Heiling et al. 2010). Furthermore, in field experiments these transgenic plants also suffered significantly more from attack by other herbivores, such as mirids and grasshopper species.

Triterpenoids are well known for their structural and regulatory role in plants as membrane sterols (e.g. campesterol) and steroids (e.g. brassinosteroids). Moreover, they are implicated to antagonise steroid receptors of insects (Clouse 2011; Dinan et al. 1999). Examples of insect-affecting triterpenoids include the cucurbitacins, common specialised metabolites in cucurbit species. The oxygenated triterpene cucurbitacin-C is effective against spider mites in cucurbits, however, it appeared to promote the growth of *Spodoptera exigua* larvae (Balkema-Boomstra et al. 2003; Barrett and Agrawal 2004). Other examples include limonoids like azadirachtin and xylogranatins, which are commonly described as anti-feedants or repellents in experiments with plant extracts. However so far, the ecological relevance in-plant remains to be investigated (Bezzar-Bendjazia et al. 2016; Dere et al. 2015; Gonzalez-Coloma et al. 2011; Wu et al. 2008). Glycosylated triterpenes, or saponins, are water-soluble metabolites believed to be involved in plant defence against herbivores but also pathogens (Osborn et al. 2011). Natural variation in saponin production in a population of winter cress (*Barbarea vulgaris*), led to the identification of saponin-aglycones hederagenin cellobioside and oleanolic-acid cellobioside as responsible for the observed resistance to the flea beetle *Phyllotreta nemorum* (Kuzina et al. 2009). Finally, saponins were correlated to resistance against the Diamondback moth (*Plutella*

xylostella) in several independent studies, where they were indicated as effective feeding deterrents against this Brassicacea specialist (Shinoda et al. 2002; Agerbirk et al. 2003; Badenes-Perez et al. 2014).

Alkaloids

Approximately 20% of all plant species produce alkaloids, and roughly 12,000 structures have been identified, including well known compounds such as caffeine, nicotine, cocaine and morphine (Ziegler and Facchini 2008). Alkaloids are non-volatile organic-molecules containing a nitrogen base in a cyclic-ring structure. They are typically derived from nitrogen containing molecules of the plant's primary metabolism e.g. phenylalanine, arginine, purins and putrescine (Ashihara et al. 2008; De Luca and St Pierre 2000).

Probably the best known alkaloid is nicotine, that has been shown to be essential for protecting (wild) tobacco species from a wide variety of native herbivorous insect e.g. *Diabrotica undecimpunctata*, *Spodoptera exigua* and *Trimerotropis spp.* Silencing of the nicotine biosynthetic pathway in *N. attenuata* led to a 3-fold increase in feeding damage by various insects (Steppuhn et al. 2004; Steppuhn and Baldwin 2007). There are however also insects that perform well on cultivated tobacco, e.g. *Helicoverpa zea* (Gog et al. 2014) and the Hawmoth caterpillar *Manduca sexta*, that not only tolerates nicotine but even uses the metabolite to deter its natural enemy (Kumar et al. 2014).

Also plants of the nightshade family such as potato (*Solanum tuberosum*) are known for the toxic alkaloids in their leaves (Müller 1998). In potato, the (glyco)alkaloids α -solanine and α -chaconine cause toxicity to the tuber-eating larvae of the Guatemalan potato moth (*Tecia solanivora*; Karlsson et al. 2013). In several wild potato accessions (*S. chacoense*), α -solanine and α -chaconine are acetylated into leptines, a metabolite that strongly affects the development of the Colorado potato beetle (*Leptinotarsa decemlineata*; Sinden et al. 1986). Later, leptin synthesis was successfully transferred to potato hybrids whereby resistance against the beetle was successfully introduced (Rangarajan et al. 2000; Lorenzen et al. 2001). Conversely, knocking down an essential step of the potato's alkaloid biosynthetic pathway resulted in accelerated development of the Colorado potato beetle (Paudel et al. 2017).

Tomatines, the main alkaloids of tomato, are spyrrosolane-type glycoalkaloids consisting of α -

tomatine and dehydrotomatine (Fontaine et al. 1948). Though many studies on their insecticidal properties have been published, conclusions were all based on experiments with artificial diets and there is no evidence yet that tomatines exert an insecticidal effect in nature (Barbour and Kennedy 1991).

Phenolics

Phenolics are defined as molecules with one-or more hydroxylated benzene-ring structures. Branching off from the primary metabolism, *L*-phenylalanine is converted to cinnamic acid by the key enzyme *L*-phenylalanine ammonia-lyase (PAL) and enters the phenylpropanoid pathway leading to the formation of the thousands of the phenolics described up to date, including flavonoids, anthocyanins, benzoic acids and polyphenols like lignins (Zhang and Liu 2015). Flavonoids occur as aglycones, sugar conjugates, or as polymers (e.g. tannins). Despite the fact that anthocyanin accumulation is enhanced by the herbivore-response hormone Jasmonic Acid (JA) (Shan et al. 2009), flavonols and anthocyanins themselves appear not to have direct insecticidal properties. Other than that, they are precursors in the formation of tannins, compounds with insect deterring and toxic effects that upon oxygenation inside the insect gut cause formation of reactive oxygen species (Barbehenn and Constabel 2011). The increased accumulation of anthocyanins at the site of aphid feeding on *Sorghum* could not be linked to the observed effect on aphid fecundity (Costa-Arbulú et al. 2001). Also, Malone et al. (2009) noticed accumulation of anthocyanins and other polyphenolics upon expression of a specific *MYB* transcription factor (*AtMYB75/PAP1*), which at first sight appeared to affect herbivore performance. However, upon closer investigation it was found that the precursor flux (quercetin and kaempferol) was rewired which indeed resulted in high levels of anthocyanins, but also impaired the production of kaempferol-3,7-dirhamnoside. It was the latter metabolite that was causal for plant defence against the generalist caterpillars (Onkokesung et al. 2014).

Phenolics exert toxicity upon being ingested by the herbivore insect. Feeding by the larvae of the oriental leafworm *Spodoptera litura*, induced the production of the phenolic compounds rutin, vanillic- and synaptic acid in pepper (*Capsicum annuum*), which affected larval development and also the response of the adult moth (Movva and Pathipati 2017). However, efficient

detoxification of various phenolics by prophenoloxidases in the insect gut appears to be common (Wu et al. 2015).

Acylsugars

In the early 1960's a toxic exudate was discovered in the wild tobacco species *Nicotiana glauca* containing compounds later named acyl-sugars (AS; Thurston and Webster 1962). AS are sugar moieties (typically glucose or sucrose) with one or more C2-C12 aliphatic acyl-chains esterified to a specific site of the ring structure (Fobes et al. 1985). AS are widely synthesised within the Solanaceae family and, secreted to the leaf surface, can accumulate up to 20% of the leaf dry weight thereby negatively affecting a broad range of insects (Fobes et al. 1985). AS exudates from wild potato *S. berthaultii* have been shown to affect the sap-feeding Green Peach aphid *Myzus persicae* (Neal et al. 1990) and protect *N. attenuata* from chewing-insect *Manduca sexta* (Weinhold et al. 2017). More examples come from wild tomato species, such as *S. galapagense*, *S. cheesmaniae*, *S. pimpinellifolium* and *S. pennellii*, where varying AS affect a whole range of insects including *B. tabaci*, *Tuta absoluta*, *T. urticae* and several thrips species (Leckie et al. 2012; Resende et al. 2006; Alba et al. 2009; Lucini et al. 2015; Rakha et al. 2017).

While the chemical structure and synthesis of AS is relatively straightforward, the total mixture of different AS structures found in a single extract is enormous (Liu et al. 2017; Ghosh and Jones 2017). As both the sugar backbone, the length, the number and the position of the acylchains can vary, the acylsugar composition of a plant can be complex and diverse. Genetic divergence among biosynthetic genes led to changes in enzymatic activity, enzyme efficiency and substrate affinity, which in turn resulted in the plethora of AS found in different plant species today (Fan et al. 2017; Kroumova and Wagner 2003; Kandra et al. 1990; Walters and Steffens 1990; Ning et al. 2015; Schillmiller et al. 2015; Nadakuduti et al. 2017; Smeda et al. 2016).

The different AS compositions between and within plant species raises questions about the selection pressure and about specificity of the plant-herbivore interactions. Bioassays with synthetic AS showed that the backbone, the number and the length of the acylchains all affect the mortality rate of *B. tabaci* and aphid *Myzus persicae* (Chortyk et al. 1996; Puterka et al. 2003). Bioassays using fractionated AS extracts from wild tomato indicated a clear difference between acylglucoses with C5

acylchains and fractions with acylsucroses with a mixture of C5/C10/C12 acylchains on mortality of *B. tabaci* and thrips *F. occidentalis* (Leckie et al. 2016).

Cyanogenic glucosides

Cyanogenic glucosides (CNglc) are the stable and relatively non-toxic, sugar-linked cyanide groups derived from amino acid catabolism that are stored inside the vacuole (Gleadow and Møller 2014). However, upon cell disruption i.e. by herbivory, they congregate with β -glycosidases that cleave off the sugar moiety creating instable cyanohydrins. Subsequent hydrolysis, either spontaneous or by α -hydroxynitrilases, releases toxic hydrogen cyanides (HCN; (Gleadow and Møller 2014). Although all plants produce minute amounts of HCN as a by-product of ethylene biosynthesis, it is estimated that approximately 5 to 11% of all plant species actively produce CNglc as part of their defence system (Gleadow and Møller 2014). In a similar fashion, *Brassica* species produce isothiocyanates (ICN) via the hydrolysis of glucosinolates by myrosinases, also known as the mustard-oil bomb (Mithen 2001). The role of CNglc in defence are primarily as insect feeding deterrents, and not so much as toxins (Compton and Jones 1985). Insects that are able to prevent tissue rupture such as phloem-feeders, are able to avoid the release of these metabolites (Gleadow and Woodrow 2002).

Though HCN was already isolated in 1802, CNglc were first proclaimed as a target for breeding programmes against herbivore resistance in the early 1990's (Vetter 2000). This may in part be because some insects are able to detoxify CNglc and either use it as a carbon source or sequester HCN as a defence against predators (Hopkins et al. 2009; Jeschke et al. 2016). Various specialist, but also generalist insect herbivores appear to be able to tolerate cyanogenic plants (Pentzold et al. 2015). The crucifer-specialist *Plutella xylostella* is able to detoxify glucosinolates and moreover, even relies on CNglc as host-cues for oviposition (Sun et al. 2009). Nevertheless, CNglc could have potential in crop breeding. For example, elevated ICN content in cabbage (*Brassica olerace*) resulted in delayed development of its pest herbivore *Pieris rapae* (Agrawal and Kurashige 2003). Also, ectopical expression of *Sorghum* CNglc biosynthetic genes in

Arabidopsis resulted in high levels of dhurrin, a cyanogenic monoglycoside that caused flea beetle (*Phyllotreta nemorum*) larvae survival to drop to 2% compared to the non-transgenic control (Tattersall et al. 2001). Poplar trees induce the expression of CNglc-related *P450s* upon herbivory its natural enemy the gypsy moth caterpillar *Lymantria dispar* (Irmisch et al. 2014). The authors showed that this leads to the production of 2-methylbutyronitrile and 3-methylbutyronitrile that then deters the gypsy moth caterpillar.

Regulation and secretion

Secretory structures

The production and storage of defence metabolites often, though not always, takes place in specialised structures on the plant body. Physical separation into predisposed tissues enables the plant to produce and store metabolites at high concentrations with minimal phytotoxic effect on the primary plant tissues. Different structures can be found on and within the plant body, depending on the species. These include glandular trichomes (GTs), peltate glands, secretory cavities and glandular ducts (Lange 2015; Tissier et al. 2017). It is noteworthy that herbivory (and JA-application) can not only affect specialised-metabolite biosynthesis but also induce the formation of the secretory structures in which this takes place (Martin et al. 2002; Boughton et al. 2005; van Schie et al. 2007).

GTs are hairy structures on the outer surface of the plant body. Though they appear in many shapes and sizes, GTs can be defined as one-or more glandular-head cells on top of a single- or multicellular stalk connected to the epidermis (Glas et al. 2012). Peltate glands resemble GTs in many ways, though their glandular cells appear as stalk-less oil-bodies directly appearing from the epidermis (Gang et al. 2001). According to labelling studies, gland cells are actively metabolising the sucrose imported from the leaf while exploiting photosynthesis for the reducing power (Balcke et al. 2017). The final product is subsequently stored into subcuticular spaces or intracellular cavities, though the secretion system is largely unknown (Tissier et al. 2017). Nevertheless, GTs and peltates serve as "oil bombs" for insects that encounter physical contact and subsequently get in contact with a high dose of sticky and/or toxic exudates.

Secretory cavities are extracellular spaces below the epidermal cells, surrounded by a complex of metabolically active secretory cells (Russin et al. 1992; Kalachanis and Psaras 2005). The cavity develops by programmed cell death (lysigenous cavities), the delamination of cells (schizogenous cavities) or a combined event (schizolysigenous cavities). These cavities are rich in essential oils of which the constituents, as described so far, are mainly volatile terpenoids, non-volatile terpenoid derivatives and phenolics. Although occurring in the foliar tissue of many plant species, their secretion to the plant surface and the role in plant defence is not well understood (Fahn 1988; Goodger et al. 2009; Schneider and Hölscher 2007; Lange 2015). Glandular ducts are vein-like structures running through the plant body that transport and secrete defence compound to the outside environment. Examples of such ducts are laticifers and secretory ducts (Pickard 2008). Laticifers are known for their role in rubber formation- and storage (Castelblanque et al. 2017; Wang et al. 2017). They are extremely elongated cells (up to tens of centimetres), or cell files, that grow tightly through the extracellular space between neighbouring cells. By importing resources from adjacent cells they synthesise an, often milk-like, emulsion called latex. Diverse metabolic classes are represented in the chemical composition of latex; sesquiterpenes, triterpenes, phenolics and alkaloids are frequently found (Sessa et al. 2000). Upon damage by herbivory, the laticifers break and release their deterrent or toxic latex. Secretory ducts are extracellular channels, also formed by schizogeny or lysigeny, from which the lumen is loaded with resin components via the epithelial cells (Pickard 2008). Although omnipresent in vascular land plants most literature is based on coniferous plants, likely because of their high resin content. The ducts are formed in many vegetative tissues and increase in size and number upon induction by insect or pathogen attack (i.e. traumatic ducts). Resin usually consists of a viscous mixture of phenolics, monoterpenoids, sesquiterpenoids and diterpene acids and has multiple functions in defence (Keeling and Bohlmann 2006; Erbilgin et al. 2006; Langenheim 2003). First, resin is sticky and traps the insect. Second, it clogs and seals the wound formed by herbivore attack, as the resin hardens after evaporation of the mono- and sesquiterpenoids. Third, as described before, the resin may contain repellent and/or toxic compounds that reduce insect feeding, development and/or reproduction.

Transcriptional regulation

The production of specialised metabolites comes at a cost and continuous production can thereby negatively affect plant growth and development, especially under conditions of low environmental resources, e.g. low photosynthetic capacity (Koricheva 2002; Strauss et al. 2002; Massad et al. 2012). Therefore, regulation of the flux through metabolic pathways is imperative. The regulation of specialised metabolite biosynthesis is complex and dynamic, responding to changes in the environment and hormonal signalling e.g. upon herbivory, in a spatial and temporal manner (Zhou and Memelink 2016; Wasternack and Strnad 2017). The production of nicotine, for instance, is induced by the plant hormone jasmonic acid (JA) in response to herbivore attack (Afrin et al. 2015). JA is a central signal in the defence against herbivores (Howe and Jander 2008) and the downstream responses are regulated by MYC- (Dombrecht et al. 2007) and ERF-type transcription factors (Pré et al. 2008; Zhu et al. 2011). Prior exposure to herbivory can prime the plant defence responses to react rapidly when it is attacked a consecutive time (Ton et al. 2007). Moreover, the regulation of gene expression can take place at different layers; transcriptional and post-transcriptional. The two key players in these processes will be discussed here; transcription factors and regulation via small RNAs.

Transcription factors (TFs) are regulatory proteins that can transactivate the promoters of biosynthetic genes through which they influence the metabolic flux. They exert this effect often specifically in the secretory structures. Several families of TFs have been found to play a role in the accumulation of specialised metabolites, either through repression or via activation of gene expression as a response to internal and/or external cues (Grotewold 2008; Iwase et al. 2009; Schluttenhofer and Yuan 2015; Chezem and Clay 2016). Among these TFs many belong to the WRKY-family (Schluttenhofer and Yuan 2015; Phukan et al. 2016; Pandey and Somssich 2009). For example, *S. lycopersicum* SIWRKY7 was shown to be involved in the activation of three monoterpene synthase genes (Spyropoulou et al. 2014) and *N. attenuata* WRKY3 and WRKY7 affect diterpene-glycoside production in response to herbivory (Skibbe et al. 2008). In *Arabidopsis*, nematode infection induces the expression of *WRKY23*, which is involved in flavonol biosynthesis (Grunewald et al. 2012; Grunewald et al. 2008) and WRKY33 binds the promoter of the

camalexin biosynthesis gene PAD4 (Qiu et al. 2008). Other TFs implicated in specialised metabolism are MYBs and bHLHs (Liu et al. 2015; Chezem and Clay 2016; Scully et al. 2018), with family members controlling the phenylpropanoid pathway leading to anthocyanins (Chu et al. 2013; Cavallini et al. 2015) and other flavonoids (Mitsunami et al. 2014). For example, *AtMYB75* modulates flavonoid biosynthesis and is implicated in caterpillar resistance (Onkokesung et al. 2014). Exerting their effect, R2R3-type MYBs often engage in a complex with bHLH TFs and a WD40 domain (Ramsay and Glover 2005). Outchkourov et al. (2014) describe such a complex that not only regulates the anthocyanin production in *Nicotiana benthamiana* as expected, but additionally affects the accumulation of phenolamides and nornicotine conjugates, compounds with a role in defence against herbivores (Laue et al. 2000; Gaquerel et al. 2014).

Another dimension of regulation; small RNAs

Over the past decade it has become clear that many transcription factors themselves are under control of yet another layer of regulation that involves endogenous small RNAs (sRNAs; Karlova et al. 2013; Borges and Martienssen 2015; Samad et al. 2017). These 20–24 nt sized non-coding RNAs proved important in biotic stress response via coordination and resetting of stress-responsive gene expression (Sunkar et al. 2007). sRNAs have been divided into subgroups depending on their source and mode of processing (Vazquez et al. 2010). MicroRNAs (miRNAs) are the most well-known and studied, however the majority of sRNAs consists of so-called small interfering RNAs (siRNAs; Liu et al. 2014). miRNAs originate from actual, evolutionary conserved, genes named MIR-genes. In contrast, most siRNAs do not originate from autonomous genes, but rather derive from double stranded RNA of different sources including transposons or transcripts with complementarity to each other (Kozomara and Griffiths-Jones 2010; Carthew and Sontheimer 2009). Though they differ in origin, processing pathway, targeting and mechanism of action, miRNA and siRNA are both excised from their precursor by Dicer-like endoribonucleases and associate with the Argonaute protein into RNA-induced silencing complexes (RISC). In general miRNAs act in post-transcriptional gene silencing through mRNA degradation or translational arrest, whereas siRNAs are mainly involved in repression of transcription or transcriptional-

gene silencing e.g. via RNA-directed DNA methylation (RdDM; Matzke and Mosher 2014). In plants in particular, degradation and target regulation is based on (near) perfect sequence complementarity of the seed region of the miRNA to the mRNA target (Brodersen et al. 2008).

Despite having been discovered decades ago, research into sRNAs thus far focused mostly on their role in plant development (Chitwood et al. 2007; Djami-Tchatchou et al. 2017). However, evidence is accumulating that also specialised metabolite biosynthesis is a target for sRNA-based regulation. Although the involvement of various sRNAs in specialised metabolism in multiples species has been predicted computationally (Bulgakov and Avramenko 2015; Gupta et al. 2017), only a few of them have actually been experimentally validated (Table 3). miRNA156b appears to be a master regulator of specialised metabolism, involved in terpene, flavonol and anthocyanin biosynthesis in *Arabidopsis* through targeting SPL9-type TFs (Yu et al. 2015; Gou et al. 2011). Yu et al. (2015) showed that sesquiterpene biosynthesis in both *Arabidopsis* and *Patchouli* is regulated via miRNA156b-targeting of TFs. In both *Arabidopsis* and tomato miRNA858a targets R2R3-MYB TFs that regulate the phenylpropanoid pathway and eventually the flavonoid content (Jia et al. 2015; Sharma et al. 2016). Also, the production of camalexin, a metabolite that affects aphid resistance in *Arabidopsis*, was shown to be under miRNA-mediated regulation (Kettles et al. 2013). WRKY TFs, although extensively shown to be involved in specialised metabolite regulation, have not yet been identified as targets of sRNAs. However, miRNAs that target WRKYs controlling pathogenesis-related (PR) protein expression have recently been described (Zhang et al. b). Besides TFs, miRNAs predicted to target key enzymes in specialised metabolite synthesis have also been discovered (Ng et al. 2011; Li et al. 2015). When a miRNA targets a so-called *PHAS* locus, it results in the production of secondary-phased siRNAs (Komiya 2017; Zhai et al. 2011). Yang et al. (2013) first identified this type of trans-acting phased siRNAs regulating anthocyanin biosynthesis. Chalcone synthase, an enzyme central in anthocyanin- and terpene synthesis (Kang et al. 2014), appears to be targeted by this type of siRNAs (Tuteja et al. 2009; Cho et al. 2013). Finally, miRNA activity itself can also be fine-tuned through a mechanism called endogenous target mimicry (eTM; Franco-Zorrilla et al. 2007). The mechanism is based on the sequence-complementarity principle where a

Table 3 List of small RNAs experimentally validated to be involved in plant specialised metabolism.

miRNA	length	target	type	affected gene	affected metabolite	reference
I -containing IR	21	CHS cluster	enzyme	-	- anthocyanin	Tuteja et al. 2009
miR156b	20	SPL9	TF	TPS21/14/24	- sesquiterpenes	Yu et al. 2015; Franco-Zorrilla et al. 2007
miR156b	20	SPL9-type	TF	ANS, F3'H, DFR, UGT75)	+ anthocyanin/flavonols	Schwab et al. 2005; Gou et al. 2011
miR163	24	FAMT	enzyme	NR	+ farnesoic acid	Ng et al. 2011
miR393a	22	TIR1, AFB2 and AFB3	receptor	ARF1, ARF9	+ glucosinolates	Robert-Seilaniantz et al. 2011; Navarro et al. 2006
miR397a	20	Laccases	enzyme	NR	- camalexin - lignin down, - uronic acid down, + xylan	Lu et al. 2008; Lu et al. 2013
miR828	22	TAS4/MYB1	non-coding	PAP1, PAP2	- anthocyanin	Rajagopalan et al. 2006; Hsieh et al. 2009; Yang et al. 2013
miR858	21	MYB7/48-like	TF	PAL, CHS, DFR, ANS, 3GT	- anthocyanin	Jia et al. 2015
miR858a	21	MYB11/11/12 other flavonol-MYBs	TF	CHS, CHI, F3'H, FLS1	- phenylpropanoid pathway	Sharma et al. 2016; Fahlgren et al. 2007
miRX13	24	PMT2	enzyme		NA	Li et al. 2015
miRX17	24	QPT1	enzyme		NA	Li et al. 2015
miRX19	21	NAC_148	TF		NA	Li et al. 2015
miRX20	21	CYP82E4	enzyme		NA	Li et al. 2015
miRX27	21	QPT2	enzyme		- nicotine	Li et al. 2015

The miRNA length, target, target type, the affected genes and the downstream effect on metabolites (+ / - : increasing / decreasing levels respectively) are indicated

decoy non-coding mRNA interferes with the cleavage of the target mRNA by competing for binding to the miRNA. Li et al. (2015) showed that nicotine biosynthesis is additionally regulated by such a (non-cleavable) decoy molecule. To this end, nta-eTMX27 interacts with the miRNA, miRX27, whereby the original target *QPT2*, encoding a key enzyme for nicotine biosynthesis, is safeguarded from getting processed and nicotine levels increase.

Towards breeding for endogenous insect defence metabolites

As reviewed, different classes of plant-produced specialised metabolites can be effective in defence against insects, and these could be the basis of an approach where we shift from using high concentrations of synthetic pesticides to plants producing their own defensive chemistry. Breeding for metabolite-based insect-resistance in crop plants, however, comes with multiple challenges. To establish biologically relevant metabolite levels, biosynthetic enzymes, their regulators, sites/structures of biosynthesis and storage should all be available in the crop germplasm and successfully introduced towards a resistant variety. This may mean introgression of multiple QTLs which makes breeding applications complex, especially in crops with higher ploidy. Also, even if insect resistance is found in a crop germplasm, making an interspecific cross with a more distantly related relative can be a major challenge, and linkage-drag i.e. introduction of undesired traits of which the underlying genes are located in the same genomic region as the metabolic QTL, is a common confounding factor. Successful examples towards breeding for self-defence compounds are the increased acylsugar levels in glandular trichomes of tomato lines by introgression of multiple, unlinked, wild tomato QTLs (Leckie et al. 2012; Smeda et al. 2018). Underlying the quantitative production of metabolites in that material are numerous metabolic QTLs, each with their own positive or negative influence and individual inheritance characteristics (Schilmiller et al. 2010; Schilmiller et al. 2012; Alseekh et al. 2015). To assess which of these loci need to be introgressed (or excluded) to achieve an effective concentration of a desired bioactive compound, whilst maintaining crop quality, the underlying genes should be determined.

A genetic engineering, or GMO, approach might be a faster, more efficient and comprehensive way to reach the goal of improved herbivore resistance in crops. Various genetic technologies including the production of insecticidal Bt in transgenic crops, the use of RNA interference designed to target insect-specific sequences, and modern genome editing tools (e.g. CRISPR-Cas, of which the GMO-status for now remains elusive in the EU) have been introduced (Douglas 2017). However, especially in the EU, there are not many examples of application in the field due to regulatory issues and the public debate around the use of GMOs in agriculture (Yu et al. 2012; Raman 2017). Feasibility of a GMO approach for specialised metabolite production is exemplified by e.g. the induction of monoterpene biosynthesis in tomato fruits and consequent improved resistance against larvae of the corn earworm through overexpression of neryl-diphosphate synthase 1 (NDPS1) and the phellandrene synthase 1 (PHS1) (Gutensohn et al. 2014). Also, in rice, overexpression of the endogenous terpene synthase 46 (*OsTPS46*) leads to enhanced concentrations of predominantly (*E*)- β -farnesene and limonene in the headspace, by which the bird cherry-oat aphid is deterred (Sun et al. 2017).

The fact that the biological activity of allelochemicals is not always straightforward can lead to inconsistent results. More than three decades ago, Gibson and Pickett (1983) first described the repellent effect of (*E*)- β -farnesene emission by wild potato (*S. berthaultii*) on the green peach aphid *M. persicae*. Follow-up work using transgenic *Arabidopsis* plants overexpressing a (*E*)- β -farnesene synthase showed a deterrent response of *M. persicae* to the alarm pheromone volatile (Beale et al. 2006). Unfortunately, later work could no longer confirm host-plant preference nor the effect on insect performance (Kunert et al. 2010; Joachim and Weisser 2015) and also wheat plants engineered for (*E*)- β -farnesene synthesis that initially repelled multiple cereal aphid species under laboratory conditions, displayed a loss of the phenotype in field trials (Bruce et al. 2015). This example illustrates the complex response of insects to volatiles and shows that herbivore behaviour does not solely depend on a single signal but rather involves a balanced combination of various volatile cues, effective concentrations and visual cues from the environment (Carrasco et al. 2015).

The environment itself is a major complicating factor that influences the quality and quantity of specialised-

metabolite production. Since a plant is unable to escape its environment, it needs to integrate physiology and metabolism, constantly responding to rapidly changing biotic and abiotic factors such as light quality (i.e. photosynthetic carbon fixation and biomass accumulation), temperature, osmotic-, nutrient- and water status (Holopainen and Gershenzon 2010; Akula and Ravishankar 2011; Selmar and Kleinwächter 2013; Borges et al. 2017).

Allocation of energy and nutrients from primary-towards specialised metabolism might come at a cost, for example in the form of a yield penalty (Koricheva 2002; Strauss et al. 2002). Züst et al. (2015) showed a negative correlation between the level of cardenolide, a toxin found in milkweed latex and the growth rate of the plants. However, Massad et al. (2012) argued that trade-off between plant growth and defence depends on the origin of the compound and the environmental conditions. The cost of producing phenolics is carbon-dependent, whereas triterpenoid saponin levels are photosynthesis dependent due to competition for nitrogen. The allocation cost of specialised metabolites may be alleviated when their potential turnover and re-allocation to primary processes is taken into account (Neilson et al. 2013). Recent studies show that there is no intrinsic trade-off of resources but rather a regulatory growth-defence network which can be uncoupled and thus, in theory, could be manipulated (Campos et al. 2016; Kliebenstein 2016; Guo et al. 2018).

Key players in the regulation of metabolic networks are signalling hormones and transcriptional regulators (Pieterse and Dicke 2007). Targeting a transcription factor that operates as a regulatory hub could be an attractive way to manipulate a part or a complete biosynthetic pathway, possibly avoiding flux bottlenecks and feedback inhibition (Grotewold 2008). With increased knowledge about the role of transcription factors and recently developing insights into their co-regulating small RNAs, one can envisage possibilities to predict and manipulate the production of a specific specialised metabolite. One such an example is the enhanced nicotine production by overexpression of a miRNA decoy (Li et al. 2015). Another possibility would be disturbing the perfect pairing between a miRNA and its metabolic target transcript (e.g. transcription factors) through introgression of an insensitive allelic variant, if available in the germplasm, or through (non-synonymous) mutagenesis of the target.

Production of specialised metabolites in specific tissues like glandular trichomes is not only effective as such glands are geared to be “metabolic factories”. It is also a way to produce compounds away from the parts of the plant to be consumed, and therefore to avoid interference with taste or palatability. Though, this approach might work for fruit-crops like tomato, pepper and cucumber, but might not in all cases be suitable for leaf-crops that are eaten without cooking, such as lettuce. Moreover, the off-target effect of botanical defence compounds on beneficial insects like predators and pollinators should be evaluated and considered prior to implementation. Also, manipulation of specific specialised metabolites aimed at one insect, can in theory result in unexpected and possibly unwanted newly introduced pest who are insensitive or even attracted to the compound.

Finally, in the same way that insects developed resistance to conventional insecticides (Simon-Delso et al. 2015; Brevik et al. 2018), insects can counter the plant strategy of natural anti-insect metabolite production. Not only does this happen by behavioural adaptation (i.e. learning), but the plasticity of the genepool in an insect population also allows for co-adaptation to toxins e.g. by metabolite sequestration (Bramer et al. 2015; Bramer et al. 2017) or product detoxification (Kliot et al. 2014; Crossley et al. 2017; Hardy et al. 2017). Whiteflies show increased survival on nicotine producing tobacco already after 10 generations, reflected in the adaptation of genes involved in muscle contraction and carbohydrate metabolism (Xia et al. 2017). Moreover, they appear to adjust their body volume and muscle strength to withstand being physically trapped in glandular trichome exudates. Engineering the defence trait in an inducible fashion, i.e. upon insect attack, may prolong the time it takes before adaptation occurs. Known mechanism to achieve this are the JA-responsive TFs eliciting specialized metabolic pathways (Zhou and Memelink 2016). Overall, high resistance can be a high risk, with strong selection pressure leading to the development of resistance in the insect, regardless of the chemical origin. Like for synthetic insecticides, plant-based antibiosis and antixenosis can also provide selection pressure enhancing the evolution of pest-resistance (Akhtar et al. 2003). High levels of resistance might however not be necessary and partial resistance may actually be more durable. The extent to which a crop variety can compensate or endure herbivore injury whilst still maintaining acceptable yield compared to

susceptible relatives, is a resistance mechanism still largely unexplored (Peterson et al. 2017). Crop cultivation in a system where other means of pest-control are integrated might be the most sustainable approach. Integrated pest-management includes sanitary hygiene, restricting the entry of the insect, crop rotation and intercropping and a targeted employment of natural enemies. Furthermore, stacking of more than one metabolic resistance, such as a repellent volatile compound in combination with a feeding deterrent, a toxin, or a disruptor of insect-development, despite making for a complex breeding approach, will likely make for a more durable solution.

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