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Review

Phytoalexin transgenics in crop protection—Fairy tale with a happy end?

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ABSTRACT

Phytoalexins are pathogen induced low molecular weight compounds with antimicrobial activities derived from secondary metabolism. Following their identification, phytoalexins were directly incorporated into the network of plant defense responses. Due to their heterogeneity, the metabolic pathways involved in phytoalexin formation and in particular the regulatory mechanisms remained elusive. Consequently, research focus shifted to the characterization of other components of plant immunity such as defense signaling and resistance mechanisms, including components of systemic acquired and induced systemic resistance, effector and pathogen-associated molecular pattern triggered immunity as well as R-gene resistance. Despite the obtained knowledge on these immunity mechanisms, genetic engineering employing these mechanisms and classical breeding reached too low improvements in crop protection, probably because classical breeding focused on yield performance and taste, rather than pathogen resistance. The increasing demand for disease resistant crop species and the aim to reduce pesticide application therefore requires alternative approaches. Recent advances in the understanding of phytoalexin function, biosynthesis and regulation, in combination with novel methods of molecular engineering and advances in instrumental analysis, returned attention to phytoalexins as a potent target for improving crop protection. Based on this, the advantages as well as potential bottlenecks for molecular approaches of modulating inducible phytoalexins to improve crop protection are discussed.

Contents

Abbreviations: CHS, chalcone synthase; HR, hypersensitive response; IAA, indole-3-acetic acid; MAPK, mitogen activated protein kinase; PAL, phenylalanine ammonia-lyase; PR, pathogenesis related; PAMP, pathogen- associated molecular patterns; ROS, reactive oxygen species; STS, stilbene synthase; TMV, tobacco mosaic virus; TOGT, tobacco glucosyltransferase.

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1. Introduction

1.1. Plant defense mechanisms

Crop plants have to cope with a multitude of abiotic and biotic stresses. Especially infections by pathogens represent a major threat. However, classical breeding selected for traits related to yield parameters and taste, rather than stress tolerance. Considering the variety of pathogens, the necessity of efficient and versatile defense strategies to avoid diseases is evident. The first line of defense is based on physical barriers such as cuticles that hamper pathogen penetration into the host. On cellular level, cell walls form strong and flexible barriers that mainly consist of cellulose fibers, but also glycans, pectins, callose, cutin, lignin, suberin and waxes can be incorporated for stabilization [1]. Further, some preformed secondary metabolites called phytoanticipins occur in plants. These are also involved in the defense against herbivores and pathogens [2]. These chemical compounds are mainly stored as inactive (non-toxic) molecules that can be released and activated (toxic) following biotic attacks [1].

The majority of defense mechanisms are however activated in response to the invading pathogen as second line of defense. During the initial contact, plant cells recognize pathogens in particular by specific pathogen-associated molecular patterns (PAMPs). The perception of these PAMPs by pattern recognition receptors is essential for initiating downstream signaling and basal resistance, the so-called PAMP triggered immunity. Pathogens acquired effectors that suppress this resistance in the host and promote virulence (effector triggered susceptibility). These effectors have various functions affecting the host defense signaling cascades [3]. Plants evolved counteracting resistance (R) proteins that detect pathogen specific effectors, which cause a secondary defense response, the so-called effector triggered immunity. A specific feature of this triggered immunity is induction of the hypersensitive response (HR), leading to programmed cell death that limits pathogen spread. This effect is mainly mediated by the accumulation of reactive oxygen species (ROS), which can also function as important signaling molecules during pathogen defense responses and directly attack the invading pathogen. The initial stimulus is transduced by central signaling cascades that maintain the coordinated defense responses. These signaling pathways are predominantly based on phosphorylation cascades, involving mitogen activated protein kinases (MAPKs) that modulate the activity of transcriptional regulators [3,4], and phytohormones. Phytohormone signaling is mainly based on salicylate, jasmonate and ethylene, depending on the type of pathogen attacking the plant [3]. Other phytohormones, e.g. abscisic acid and cytokinins, also act in plant defense and can partially interact with the salicylate–jasmonate–ethylene signaling backbone. Pathogen specific as well as general defense responses are mediated via these signaling pathways and associated transcriptional regulation. These defense responses include systemic acquired resistance and induced systemic resistance. The salicylate-dependent systemic acquired resistance is induced as a long lasting and broad spectrum response, which is also triggered in distal healthy plant tissue. In contrast to a direct activation of defense, which can be triggered by nonpathogenic bacteria, induced systemic resistance is mediated via jasmonate/ethylene and is characterized as priming enhanced defense [3].

In addition to general pathogen responses such as cell wall fortification (callose deposition, lignification) and stomatal closure [1], which prevent the entrance of the pathogen into the host, specific modulations of plant primary and secondary metabolism are induced by pathogens. These comprise the parallel regulation of sugar levels and distribution through changes in photosynthesis rates and sugar related enzyme activities [5], and induced synthesis of antimicrobial peptides or enzymes. Antimicrobial compounds mainly belong to two classes of substances of either proteinogenic origin or derived from secondary metabolism. So-called pathogenesis related (PR) proteins act specifically against certain (classes of) pathogens. These include enzymes such as chitinases and glucanases, proteinase inhibitors and antimicrobial peptides (thionins and defensins) [6]. Additionally, invertases involved in responses to biotic and abiotic stress [5,7] are considered as PR-proteins. PR-proteins are not spatiotemporally restricted to the infection sites, but can also spread systemically for a relative long time post infection. In contrast, accumulation of phytoalexins is restricted to the infection site and they are rapidly catabolized after successful pathogen defense [8]. Phytoalexins form a heterogenous group of low molecular secondary metabolites from various interlinked biosynthesis pathways. The spectrum of phytoalexins produced is specific to species or families [8,9]. Despite their diverse biosynthesis, phytoalexins commonly mediate a relatively unspecific and thus broad antimicrobial activity against a wide range of pathogens. The increasing knowledge on the biosynthesis genes involved and their regulation, render phytoalexins a promising target for use to improve plant protection.

1.2. History of phytoalexin research

Originally, the concept of phytoalexins as antimicrobial compounds was first postulated in the 1940s [10], based on observations regarding the infection of *Solanum tuberosum* with the oomycete *Phytophthora infestans*. Without knowing distinct effective substances, phytoalexins were postulated as inhibitory chemicals that are activated only in the presence of the pathogen in living host cells close to the infection site. Phytoalexin formation occurs de novo following pathogen infection and differs between compatible and incompatible interactions in the dynamics of their biosynthesis rather than the presence/absence in resistant/susceptible host plants [2,8,11]. Consequently, the general success of this defense reaction is based on its dynamics, which is determined by the genetic repertoire of the host [12]. Furthermore, toxicity to pathogens was described as non-specific. However, pathogens vary in sensitivity to phytoalexins. This phytoalexin model was the first postulated plant resistance mechanism, creating a working concept for future studies. Subsequent research was focused on the identification of phytoalexins and their induction. Many different secondary metabolites were associated with

the phytoalexin phenomenon, including simple phenolics, but also compounds such as tannins or caffeic acid [12]. The first phytoalexin, pisatin, was isolated from *Pisum sativum* post infection with *Sclerotinia fruticola* and seven additional facultative and obligate phytopathogenic fungi in the 1960s [13]. The post infection accumulation of pisatin and its function as an antifungal compound correlated with the originally postulated characteristics of phytoalexins. The formation of antifungal phytoalexins was later observed in other host plants (*Phaseolus vulgaris* and *Capsicum frutescens*). Pisatin was excluded as the effective compound in these species, indicating species specific differences in phytoalexin accumulation. Many subsequent physiological and biochemical analyses of plant–pathogen interactions revealed that the spectrum of accumulated phytoalexins in response to various fungal as well as bacterial pathogens strongly depends on the plant species/families [8,9]. These studies also contributed to the identification of metabolic pathways involved in phytoalexin biosynthesis. Initially, phytoalexin biosynthesis was linked to malonate, mevalonate and shikimate metabolism [8]. Further analyses of plant–pathogen interactions, incorporating new techniques, showed that the most important classes of phytoalexins are derived from the shikimate (phenylpropanoid) pathway (e.g. stilbenes and iso/flavonoids), the isoprenoid (terpenoid) pathway (e.g. diterpenes and sesquiterpenes) and from different alkaloid forming pathways (e.g. camalexin) [2].

1.3. Plant protection

Although the phytoalexin concept was the first theory postulated for pathogen resistance mechanism, the molecular and genetic revolution changed scientific views with focus towards molecular mechanisms of the different plant defense responses described above. Due to this, the main focus to develop tools for plant protection was for a long time on the generation of pesticides or the genetic engineering of other defense mechanisms than phytoalexins [14]. This may also be caused by the extreme diversity of phytoalexins and complexity of the biosynthesis for many of them, which made it difficult to unequivocally identify general regulatory mechanisms suited for genetic engineering. However, the progress made in the last decades on molecular methods including analysis of mutants and transgenic plants also greatly contributed to the understanding of phytoalexin biosynthesis and their regulation. This knowledge together with the characteristics of phytoalexins as broad spectrum antimicrobial substances makes them a promising target for improving crop species, which was first discussed in the 1990s [15,16]. Considering the aim to reduce the use of pesticides and the limitations of classic breeding and genetic manipulation of other defense components, the genetic modulation of phytoalexins or secondary metabolites in general is again regarded as a potent target for improving crop protection [14,17–20]. An additional focus in phytoalexin research is the potential positive effect on food quality as antioxidants and the application in human medicine [21].

The main focus of this review is to discuss the advances made on phytoalexin research using mutant and transgenic studies and how this knowledge currently is and in the future can be applied for the improvement of pathogen resistance.

2. Phytoalexin biosynthesis and regulation

Plants do not only rely on preformed compounds such as phytoanticipins, but also specifically activate the synthesis of additional antimicrobial substances upon pathogen infection, saving resources. Phytoalexin production as part of specific inducible plant responses to stress is spatiotemporally restricted, depending on the stress conditions. The biosynthesis of phytoalexins is part of the central plant secondary metabolism pathways, from which also other important compounds are derived such as phytohormones [22] or cell wall components [2]. Exogenous elicitors and endogenous regulators of phytoalexin biosynthesis are summarized in Table 1. Since the precursors for the different secondary metabolism pathways are derived from primary metabolism (Fig. 1), phytoalexin induction may lead to shifts from primary to secondary metabolism [23] as well as within the respective secondary metabolism pathways. Thus, phytoalexin biosynthesis needs to be strongly regulated to prevent metabolic imbalances that limit crop growth and consequently yield.

2.1. Metabolic pathways

Phytoalexins are derived from central interconnected metabolic pathways [24], which can be roughly separated into alkaloid, isoprenoid (terpenoid) and phenylpropanoid metabolism (Fig. 1) [2]. These metabolic pathways form different phytoalexin classes depending on either the basal precursors or subsequent enzymatic conversions along specific metabolic branches. Consequently, although the phytoalexins produced by different plant species within the same family are in general closely related, both phytoalexin spectrum and quantity can vary strongly among plant species.

2.1.1. Alkaloids

Alkaloid metabolites are of very different origin, mainly derived from different aliphatic and aromatic amino acids, which are formed via the citric acid cycle or the shikimate pathway, respectively. Thus, alkaloid biosynthesis does not follow a common pathway, but nevertheless alkaloids are ubiquitous in plants. Based on their precursors and chemical structure, alkaloid phytoalexins can be divided into several classes [2]. Toxic and/or medicinal (e.g. anti-cancer) properties are described for many alkaloids, which include alkaloid phytoalexins such as brassinin or camalexin, both derived from tryptophan [25]. Camalexin is the key phytoalexin of *Arabidopsis* and was intensely studied for its physiological relevance. Its biosynthesis involves several P450 enzymes [26] and is directly connected to the formation of glucosinolate phytoanticipins and the phytohormone IAA (auxin) via the intermediate metabolite indole-3-acetaldoxime [2]. Thus, indole-3-acetaldoxime is the key branch point in the synthesis of these compounds, indicating a molecular switch between different metabolites of diverse function and connecting signaling compounds (phytohormones) with bioactive substances (phytoalexins, phytoanticipins).

2.1.2. Isoprenoids

Isoprenoids (alternatively terpenoids) are derived from a central metabolic pathway in which isopentenyl diphosphate (IPP) and its isomer dimethylallyl diphosphate (DMAPP) are formed as basal C_5-building blocks for the different terpene classes. IPP and DMAPP are synthesized by two distinct pathways via methylerythritol phosphate in plastids or via mevalonic acid in the cytosol [27]. The formation of IPP and DMAPP in plastids is based on pyruvate and glyceraldehyde 3-phosphate.The initial fusion of these molecules by 1-deoxy-D-xylulose-5-phosphate synthase is the rate limiting step. In the cytosol, IPP and DMAPP derive from acetyl-CoA as precursor. The rate limiting conversion of β-hydroxy-β-methyl-glutaryl-CoA (HMG-CoA) into the intermediate mevalonate is catalyzed by the enzyme HMG-CoA reductase. Depending on the number of fused building blocks, hemiterpenes (C_5), monoterpenes (C_{10}), sesquiterpenes (C_{15}), diterpenes (C_{20}), sesterpenes (C_{25}) triterpenes (C_{30}) or tetraterpenes (C_{40}) are formed [24]. Terpenoid phytoalexins [28] belong to the groups of diterpenes [27] and sesquiterpenes [29], which form the major

Table 1
Examples of phytoalexin biosynthesis regulators, including exogenous elicitors as well as endogenous regulatory and signaling components.

Plant species	Regulators	Phytoalexin(s)	Genes regulated	Refs.
Arabidopsis thaliana	Acifluorfen, $AgNO_3$, Amino acid starvation, α-amino butyric acid, Auxin (2,4-D), *Botrytis cinerea*, $CuCl_2$, Hydrogen peroxide, miR393, MPK3, MPK4, MPK6, Oligogalacturonides, *Pseudomonas syringae*, UV light, Wounding, WRKY18, WRKY33, WRKY40	Camalexin, Scopoletin	*CYP71A13*, *CYP79B2*, *F6′H1*, *ICS*, *PAD3*	[34–36,44,45,47,48,65,68,74,77,78,82]
Arachis hypogaea	*Botryodiplodia theobromae*, Ethephon, *Ganoderma lucidum*, Jasmonate, Paraquat, Salicylate, Sucrose, UV light, Wounding	Arachidins, Resveratrol	*RS*	[54,55,75]
Cucumis sativus	*Pseudomonas putida*	Phenylpropanoid phytoalexins		[104]
Daucus carota	Elicitor of *Aspergillus niger* and *Fusarium moniliforme*, $HgCl_2$	6-Methoxymellein		[72]
Glycine max	$AgNO_3$, $CdCl_2$, $CuSO_4$, Elicitor of *Phytophthora megasperma*, $HgCl_2$, $K_2Cr_2O_7$, Lactofen, Nonident P-40, Triton X-100	Glyceollin		[71,73,79]
Gossypium arboretum	Elicitor of *Verticillium dahlia*, GaWRKY1	Gossypol	*CAD1*	[31]
Nicotiana tabacum, *N. plumbaginifolia*	Abscisic acid, Cytokinin, Fungal elicitors, Hydrogen peroxide, Methyl-jasmonate, pathogenic *Pseudomonas*, Phytoprostanes, Salicylate, Sulfated fucans, Tobacco mosaic virus, Wounding	Capsidiol, Scopoletin	*C4H*, *EAH*, *EAS*, *HMGR*, *PAL*, *TOGT*	[30,50–53,76,87–91,95]
Oryza sativa	$CuCl_2$, OsACDR1, OsCIPK14/15, OsMPK3/6, OsRAC1	Momilactones, Phytocassanes, Sakuranetin		[69,154–158]
Phaseolus vulgaris	*Colletotrichum lindemuthianum*, Ozone	Isoflavonoid phytoalexins (e.g. Phaseollin, Kievitone)	*CHI*, *CHS*, *PAL*	[2,61]
Pisum sativum	Electric currents, *Sclerotinia fruticola* and other pathogenic fungi	Pisatin		[13,80]
Solanum tuberosum	*Glomus etunicatum*, *Rhizoctonia solani*	Rishitin, Solavetivone		[100]
Sorghum bicolor	*Colletotrichum sublineolum*	Apigenin, Luteolin	*CYP93G3*	[19]
Thellungiella halophila, *T. salsunginea*	*Albugo candida*, $CuCl_2$, *Leptosphaeria maculans*, NaCl, UV light	Methoxybrassenin B, Rapalexin A, Wasalexins A & B		[62,68]
Trigonella foenum-graecum	$AlCl_3$, $CdCl_2$, $CuCl_2$, Electric currents, Na_2SeO_3, KCN, Sodium orthovanadate	Medicarpin	*CHS*, *IFR*, *GST*, *VR*	[70,80]
Vitis vinifera	*Acinetobacter lwoffii*, *Bacillus subtilis*, *Botrytis cinerea*, Ethylen, Methyl-jasmonate, Ozone, *Pantoea agglomerans*, *Pseudomonas fluorescens*, Sucrose	Resveratrol, Viniferin	*CHS*, *PAL*, *STS*, *UFGT*, *VST1*	[56,57,105]
Zea mays	*Aspergillus flavus*, *Aspergillus sojae*, *Cochliobolus heterostrophus*, *Colletotrichum sublineolum*, Ethephon + Jasmonate, *Fusarium graminearum*, *Ostrinia nubilalis*, *Rhizopus microspores*, *Ustilago maydis*	Kauralexins, Zealexins	*AN2*, *TPS6*, *TPS11*	[58,173]

AN2, ent-copalyl diphosphate synthase; CAD1, (1)-d-CADINENE SYNTHASE; CHS, chalcone synthase; CYP, cytochrome P450; C4H, cinnamate-4-hydroxylase; EAH, epi-aristolochene dihydroxylase; EAS, epi-aristolochene synthase; F6′H1, 2-oxoglutarate and Fe(II)-dependent oxygenase; GST, glutathione-S-transferase; HMGR, 3-hydroxy-3-methyl-glutaryl-CoA reductase; ICS, Isochorismate synthase; IFR, isoflavone reductase; PAD3, phytoalexin deficient 3; PAL, phenylalanine ammonia-lyase; RS, resveratrol synthase; STS, stilbene synthase; TOGT, tobacco glucosyltransferase; TPS, terpene synthase; UFGT, UDP-glucose:flavonoid-O-glucosyltransferase; VR, vestitone reductase; VST1, grapevine resveratrol synthase.

phytoalexin groups in rice and *Solanaceae*, respectively. In rice, several genes are described that are coding for enzymes catalyzing biosynthesis of diterpenoid momilactone, oryzalexin and phytocassane phytoalexins [27]. In tobacco, several sesquiterpenoid phytoalexins are known [24], of which capsidiol is the most important. The formation of 5-*epi*-aristolochene as important precursor for capsidiol synthesis has been shown to be catalyzed by *epi*-aristolochene synthase [30]. In cotton, the *(1)-d-CADINENE SYNTHASE* gene, which encodes a sesquiterpene cyclase was shown to be involved in sesquiterpene phytoalexin synthesis [31]. Via

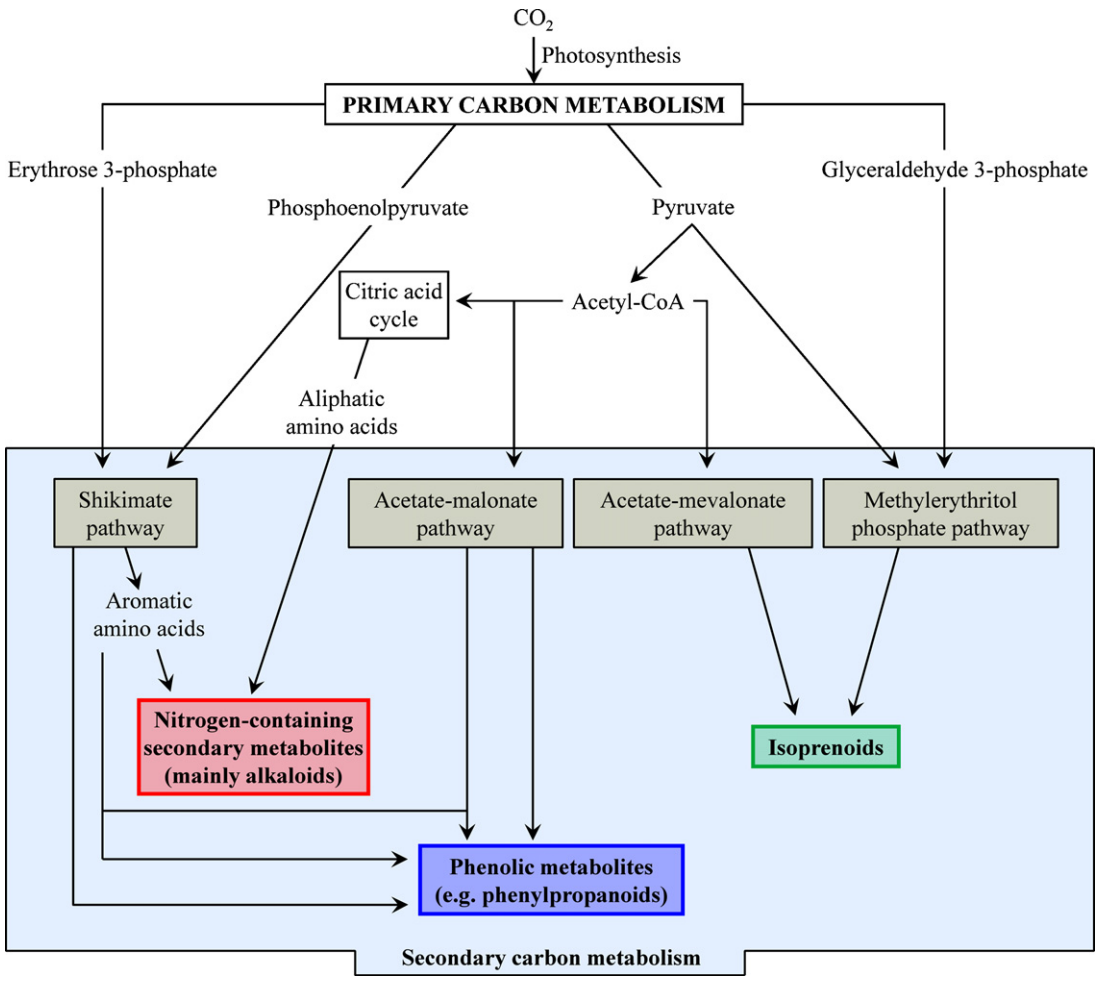

Fig. 1. Network of biosynthetic pathways and metabolites leading to the formation of phytoalexins. Intermediates derived from primary carbon metabolism are used as precursors for specific synthesis of secondary metabolite classes (bold).

the isoprenoid pathway, the biosynthesis of these phytoalexins is closely related to the formation of photosynthetic pigments, vitamins and sterols. Furthermore, the phytohormones abscisic acid, cytokinins, brassinosteroids and giberellins [2] are derived from this pathway, which is also indicated for strigolactones [32]. Therefore, enzymatic conversions in this biosynthesis pathway can be regarded as potential metabolic switches, which may be used for genetic engineering of various groups of metabolites.

2.1.3. Phenylpropanoids

Phenylpropanoid metabolites are based on modifications of the aromatic amino acid phenylalanine and thus are derived from the shikimate pathway. The initial conversion from phenylalanine to the intermediate cinnamic acid is catalyzed by phenylalanine ammonia-lyase (PAL). Depending on subsequent conversions, functionally diverse metabolites can be formed, including (hydroxy)benzoic acids such as salicylate, which however can also directly be derived from chorismate. Alternatively, cinnamic acid can be converted to coumaric acid by cinnamate-4-hydroxylase. Cinnamic acid can be further modified to caffeic acid, ferulic acid and sinapic acid (hydroxycinnamates), which can ultimately (via the aldehyde intermediates) be transformed to their alcohols (monolignols). Together, they are basal precursors of the antioxidant lignan and structural components such as lignin, suberin and cutin [2]. However, ferulic acid can be also transformed into the phytoalexin scopoletin. Besides the sesquiterpenoid capsidiol, scopoletin is the main phytoalexin in tobacco, where it can be reversibly glucosylated to scopolin by tobacco glucosyltransferases (TOGT) [33]. Scopoletin was detected in *Arabidopsis* [34–36], although camalexin is considered the major phytoalexin in this plant species. Phenylpropanoid phytoalexins can also be derived from simple phenols by addition of another benzene ring (cyclization of three malonic acid residues) via stilbene synthase (STS) or chalcone synthase (CHS) leading to stilbenes or flavonoids, respectively [2]. The formation of flavonoid compounds results from several subsequent enzymatic reactions involving e.g. chalcone isomerase, chalcone reductase, flavone 3-hydroxylase, flavone synthase, flavonol synthase and 2-hydroxyisoflavonone synthase [37]. Stilbenes are the main phytoalexin group in *Vitaceae* and peanut and are intensively studied for medicinal applications (e.g. anticancer and anti-inflammatory functions) with resveratrol and piceids as best studied examples [38]. Flavonoid phytoalexins occur in several plant species such as alfalfa (medicarpin, sativan), bean (kievitone, phaseollin), pea (maackiain, pisatin) rice (sakuranetin) and soybean (glyceollins) [39–41]. The strong connection of phytoalexins and other essential compounds to the phenylpropanoid pathway as well as to alkaloids (via aromatic amino acids), underlines the necessity of strict regulatory mechanisms that maintain metabolic balances.

2.2. Endogenous regulation of phytoalexins

Arabidopsis is intensively studied for the regulation of phytoalexin biosynthesis as model organism in plant research.

Arabidopsis secondary metabolites were a long time not considered to be as diverse as those from other species [42]. Nevertheless, the endogenous regulation of its major phytoalexin camalexin is still not completely understood, especially regarding its regulation by phytohormones where studies draw conflicting conclusions [41]. There is evidence for salicylate–jasmonate–ethylene dependent as well as independent regulation of camalexin biosynthesis. However, these studies involved different *Arabidopsis* mutants and partially deploying poorly described pathogen isolates [43]. The studies could have been flawed by pathogen specific differences in camalexin regulation by phytohormones. Strong camalexin regulation via MAPK signaling is also known, mainly involving MPK3, MPK4 and MPK6 converging on the activation of transcription regulators such as WRKY33 [41,44]. Further, WRKY18 and WRKY40 suppress camalexin accumulation, since double loss-of-function mutants have enhanced camalexin biosynthesis in untreated plants as well as upon infection [45]. MicroRNAs also interfere with secondary metabolism in *Arabidopsis*. For example, *miR163* and its target genes are involved in the natural variation of secondary metabolites in related *Arabidopsis* species and in defense responses [46]. Interestingly, *miR393* directly acts as a metabolic switch between camalexin and glucosinolates, where it suppresses camalexin accumulation [47]. Although scopoletin is not considered as an important phytoalexin in *Arabidopsis*, its levels are increased post auxin (2,4-D) treatment, while treatment with salicylate, methyl-jasmonate or kinetin had no impact [35]. The increased accumulation of scopoletin post 2,4-D treatment is in agreement with enhanced transcript levels of its key biosynthetic gene *F6′H1* [34]. Strikingly, both phytoalexins seem to be regulated by hydrogen peroxide since the *Arabidopsis cat2* mutant, which overaccumulates hydrogen peroxide, has increased camalexin and scopoletin levels [48]. Hydroxygen peroxide is important in the oxidative burst (ROS), which has to be tightly controlled by antioxidant activity to avoid (premature) host cell death.

Much additional insight in the regulatory mechanisms for different phytoalexins was obtained from other plant species. Phytohormones are important regulators of phytoalexin biosynthesis in tobacco. Elevated cytokinin levels enhanced accumulation of different phenolic acids as potential phytoalexin precursors [49] and induced scopoletin and capsidiol biosynthesis independently of salicylate, jasmonate and ethylene [50]. Concomitantly, transcription of the capsidiol (*3-HYDROXY-3-METHYLGLUTARYL-CoA REDUCTASE* and *epi-ARISTOLOCHENE SYNTHASE*) and scopoletin/scopolin biosynthesis genes (*CINNAMATE-4-HYDROXYLASE* and *TOGT*) was induced, whereas *FLAVONOL SYNTHASE* seemed to be repressed [50]. In contrast, abscisic acid suppressed capsidiol synthesis [51]. The promoter of *epi-ARISTOLOCHENE SYNTHASE*, the key biosynthesis gene for capsidiol, is only slightly activated by salicylate and even repressed by methyl-jasmonate treatment [52]. Interestingly, this promoter is induced by hydrogen peroxide [52], which indicates similarities to phytoalexin regulation in *Arabidopsis* [48]. The promoter of the *TOGT* gene, responsible for the reversible conversion from scopoletin to scopolin, is induced by salicylate [53]. Peanut callus cultures responded to sucrose with increased resveratrol and piceatannol synthesis [54]. In peanut leaves, the accumulation of resveratrol and expression of its biosynthesis gene was enhanced by ethylene, salicylate and jasmonate, but not by abscisic acid or hydrogen peroxide [55]. Similarly, the promoter of the grape resveratrol synthase gene *VST1* is induced by ethylene [56]. In grape cell cultures, resveratrol accumulation could be induced by methyl-jasmonate and sucrose. Interestingly, the combination of methyl-jasmonate and sucrose caused a faster and stronger resveratrol accumulation. This not only identified sucrose as positive regulator of resveratrol biosynthesis, but also its synergistic effect with methyl-jasmonate. However, on transcriptional level this effect was only observed for *CHS3*, whereas transcript levels for *PAL*, the initial key gene in the phenylpropanoid pathway, and the specific biosynthesis gene *STS* were not altered by sucrose [57]. Comparably, *(1)-d-CADINENE SYNTHASE* expression in cotton and accordingly the synthesis of sesquiterpene phytoalexins such as gossypol were enhanced by methyl-jasmonate, but not by salicylate or hydrogen peroxide. The *(1)-d-CADINENE SYNTHASE* promoter was identified as direct target of the GaWRKY1 transcription factor [31]. Recently, it was shown that combined ethylene (ethephon) and jasmonate treatment induces maize kauralexins [58], whereas single treatments were not effective.

Together, these findings indicate a strong regulation of phytoalexins via phytohormones, although species and/or phytoalexin specific effects have to be considered. The regulatory functions of especially hydrogen peroxide and sugars in relation to hormones are important to understand phytoalexin regulation. Because of the strong regulation by phytohormones, also developmental [2] and organ specific regulation of phytoalexins have to be considered. This is indicated by differential accumulation of phytoalexins and/or transcripts of biosynthesis genes in various tissues [31,35,52,55,58].

2.3. Phytoalexins modulated by stress conditions

2.3.1. Abiotic stress

Originally, phytoalexins were thought of as induced defense responses to infection. Many phytoalexins also quench ROS (antioxidants), which could explain the broad range of stimuli inducing the same secondary metabolites, including abiotic stress conditions [2,8,59]. The importance of phytoalexins and other secondary metabolites in the regulation of ROS levels was substantiated using the catalase deficient mutant *cat2* [36]. The interaction between phytoalexins and ROS is complex, since ROS are important as signaling molecules during pathogen defense responses as well as in removal of the pathogen by directly attacking the pathogen and/or the host cell (HR). Therefore, ROS levels must be tightly controlled, either to prevent or to induce host cell death depending on the type of pathogen, respectively necrotrophic or biotrophic pathogens.

One major abiotic factor interfering with plant health is ozone, which leads to ROS production. Ozone has a strong impact on secondary metabolism, inducing changes in the alkaloid, isoprenoid and phenylpropanoid pathways [2]. For example, in *Arabidopsis PAL* expression is induced by ozone independently of salicylate [60]. Genes specific for certain subclasses of secondary metabolites including phytoalexins derived from phenylpropanoids were induced by ozone. These include *CHS* and *CHALCONE ISOMERASE* for flavonoid biosynthesis in bean [61] or *VST1* for resveratrol biosynthesis in grape [56]. Many additional regulations in the different metabolic pathways in response to ozone have been described [2]. Plants have to cope with many other environmental stress factors such as UV exposure. UV elicits ozone formation and induces anthocyanins and phytoalexins in different plant species [55,62–65]. UV treatment enhanced resistance against *Penicillium digitatum* in citrus fruits [63]. Wasalexin phytoalexins were strongly elevated by UV treatment in *Thellungiella salsuginea* as well as by salt and heavy metal stress, although the increase of phytoalexins was not as strong following post UV treatment [62].

Heavy metals are often strong inducers of phytoalexin accumulation. $CuCl_2$ induce phytoalexins in several dicotyledonous plant species [66–68], as well as in the monocot rice, where the phytoalexins sakuranetin and momilactone A were jasmonate-dependently induced [69]. In addition, $AlCl_3$, $CdCl_2$ or Na_2SeO_3 [70], $HgCl_2$ [71–73] and $AgNO_3$ [73,74] were shown to induce phytoalexins. Phytoalexins were induced in peanut [55,75] and in tobacco [76] after wounding. In addition, wounding is priming camalexin synthesis in *Arabidopsis*, which is increased upon *Botrytis cinerea* infection [77]. Furthermore, phytoalexin biosynthesis

can be increased in response to other abiotic stresses such as amino acid starvation, treatment with the herbicide acifluorfen or α-amino butyric acid [78]. Similarly, glyceollin was induced in soybean by the herbicide lactofen [79], resveratrol in peanut by the DNA damaging agent paraquat [55] and different phytoalexins in various plants by electric currents [80]. In contrast, information on the impact of salt, drought or cold stress on phytoalexin accumulation is very limited, which could be explained by the antioxidant function of phytoalexins. Whereas these different stress conditions strongly induce ROS production (oxidative burst) and cell death, salt, drought or cold stress conditions, despite their severe impact on plant performance, initially have little effect on these parameters. Thus, phytoalexin accumulation following abiotic stress conditions appears to be correlated with the degree of ROS accumulation, although their role and regulation in these responses remains largely unclear.

2.3.2. Biotic stress

A wide spectrum of phytoalexins has been described in various plant species, induced by diverse pathogens including bacteria and fungi of different life style [2,41]. Although phytoalexins are of very different origin, their antimicrobial effect is in general rather unspecific and thus efficient against a broad range of pathogens. Most studies in *Arabidopsis* focused on camalexin and its induction by a wide range of necro- and biotrophic pathogens is well established [9,41,81,82]. Recently, the specific spatiotemporal accumulation of scopoletin in parallel to camalexin was reported in response to an avirulent strain of the hemibiotrophic bacterial pathogen *Pseudomonas syringae*. The accumulation of both phytoalexins was accompanied by increased transcript levels of their biosynthetic genes, suggesting coordinated transcriptional regulation [36]. Despite its accumulation post infectioncamalexin was considered not essential for the resistance against *P. syringae*, since the camalexin deficient *pad3* mutant showed no effect on the growth of this pathogen [83]. However, *P. syringae* was shown to be sensitive to camalexin in vitro by destruction of its membrane integrity [84]. In contrast, depending on the used strain, *B. cinerea* infection of *Arabidopsis* is strongly affected by camalexin, since elevated levels increased resistance [77] and camalexin deficient mutants showed increased susceptibility [82,85].

In tobacco, a minimum of 17 phytoalexins have been described to accumulate in response to pathogen infections [24,86], while the hydroxycoumarin scopoletin and the sesquiterpene capsidiol are considered to be the most important. Both phytoalexins accumulated in response to infection with tobacco mosaic virus (TMV) [87,88] and after challenge with pathogenic *Pseudomonas* strains [50,89]. The synthesis of capsidiol was also induced after treatment with fungal elicitors [90,91], which is in agreement with the induction of *epi-ARISTOLOCHENE SYNTHASE* by cellulase [30]. Scopoletin and capsidiol are induced by cytokinins independent of salicylate, jasmonate and ethylene signaling in tobacco, conferring resistance against virulent *P. syringae* infection, but not against necrotrophic fungi [50]. Furthermore, high scopoletin levels correlated with resistance against TMV [92] and *B. cinerea* [93]. Various phytoprostanes are induced following the infection of tobacco cell cultures by *B. cinerea*, which trigger scopoletin biosynthesis [94]. Additionally, scopoletin is induced in parallel with salicylate accumulation and the transcriptional activation of *PAL* and PR-protein encoding genes by sulfated fucans. Treatment of tobacco with these cell wall components of brown algae increased the resistance against TMV [95]. Scopoletin accumulation is impaired in tobacco plants overexpressing *TOGT* antisense constructs, and TMV susceptibility is increased [33]. TOGT has also been shown to have a critical role in compartmentalization of scopoletin/scopolin and other phenolics as antioxidants [96]. Thus, phytoalexins can also contribute to compartment specific accumulation of antioxidative activities which is observed during pathogen infections [97,98]. Interestingly, *TOGT* genes in tobacco are inducible by salicylate, but its induction by elicitors was also observed in salicylate deficient plants [33,53]. Phytoalexins are induced by several pathogens in many other plant species, as described in several excellent reviews [2,38,41,62,99], but the molecular mechanisms involved and their regulations during infection are still largely unclear.

Secondary metabolism and specifically phytoalexin synthesis can also be triggered or primed by beneficial (antagonistic) microbes, which contributes to so-called biocontrol effects. The levels of the phytoalexins rishitin and solavetivone were increased in potato upon pathogen infection when colonized by the mycorrhizal fungus *Glomus etunicatum* [100]. Also different rhizobacteria systemically induce monoterpene synthesis in Italian oregano [101] and isoflavone metabolism in soybean [102]. The accumulation of phytoalexins in carnation paralleled induced resistance against *Fusarium oxysporum* when plants were colonized by the antagonist *Pseudomonas* strain WCS417r [103]. Root-colonization with *Pseudomonas putida* systemically induces phytoalexins in cucumber [104], which contributes to the resistance against *Pythium aphanidermatum*. The synthesis of the phytoalexins resveratrol and viniferin in grape was strongly induced by several rhizobacteria that increased resistance against *B. cinerea* [105]. Expression of phytoalexin biosynthesis genes was proposed to prime the plants [82], enabling faster and stronger pathogen defense reactions following pathogen infection without yield penalties, which could contribute to the similarly proposed effect of such beneficial microbes.

Phytoalexins are potent and essential for successful defense against pathogens, but they can also have negative impact on the plant itself. Besides energetic costs that have to be spent for their biosynthesis, high levels of phytoalexins can damage the tissue. Although this could contribute to programmed cell death during HR, reliable detoxification mechanisms or transformation into lignin are necessary that control HR spread and to maintain tissue integrity. Successful defense against biotrophic pathogens, which need a living host cell, and necrotrophic pathogens, which thrive on deceased host cells, requires different strategies. Phytoalexins as antimicrobial compounds could form the first line of defense for induced resistance mechanisms, after which other pathogen specific mechanisms could step in and attack the already weakened pathogen. The phytoalexins could not only kill the invading pathogen, but also delay their propagation, thereby enabling the plant to launch more sophisticated yet slower dynamic defense responses [50].

PAL and other enzymes involved in phytoalexin synthesis were shown to be induced not only by pathogen infections but also metabolically by sugars [106]. Soluble carbohydrates are known for long to be involved in pathogen response since high sugar levels were shown to increase resistance (high sugar resistance) [107]. The induction of phytoalexin biosynthesis in response to stress related stimuli is also coordinately regulated with changes in source–sink relations. Extracellular invertase is the central part of this mechanism for co-regulation of photosynthesis, sink metabolism and defense responses in response to both sugars and stress related stimuli. The original findings obtained with suspension cultures [106,108] were extended to plants infected by necrotrophic fungi and biotrophic bacteria [109], respectively. Sugars and stress-related stimuli independently activate different signal transduction pathways. Extracellular invertase could have a dual function, providing carbohydrates to maintain sink metabolism and to also channel carbohydrates into secondary metabolism, while simultaneously keeping the system induced by elevated sugar concentrations [110]. Thus, in addition for long distance transport of assimilates to the sink tissue, this regulatory mechanism will contribute to phytoalexins synthesis by partitioning carbohydrates from primary to secondary metabolism.

In contrast to the induced formation of phytoalexins as defense against pathogens, secondary metabolites involved in the defense against insects such as herbivores are rather preformed/constitutively formed (e.g. glucosinolates, cyanogenic and iridoid glycosides) [111].

2.4. Phytoalexin catabolism

Pathogenic fungi evolved several mechanisms undermining plant defense responses allowing successful infection of host plants, including detoxification of phytoalexins. The necrotrophic fungus *Fusarium solani* efficiently detoxifies the bean kievitone by its own kievitone hydratase [112]. Brassinins are detoxified by *Leptosphaeria* sp. via oxidation [113] and in *Sclerotinia sclerotiorum* by brassinin glycosyltransferase [114]. Medicarpin and maackiain, two major phytoalexins of *Leguminosae*, are detoxified by *Nectria haematococca* via an FAD-dependent mono-oxygenase [115]. *N. haematococca* demethylates pisatin overcoming resistance in pea [116]. The rice diterpenoid phytoalexin momilactone A is degraded by blast fungus [117]. Some *B. cinerea* strains degrade stilbenoid phytoalexins by laccases [43,118]. Similar detoxification mechanisms may exist for other fungal- and bacterial pathogens. This could be a major determinant for successful establishment of infection for virulent pathogens. Expression of such detoxifying enzymes in plants could functionally prove the involvement of phytoalexins in plant defense responses in vivo. This might function more efficient than antisense or RNAi suppression of biosynthetic pathways, since these enzymes are specifically directed against the active phytoalexins targeting the respective pathogens. In addition to the enzymatic detoxification of phytoalexins, microorganisms evolved other mechanisms conferring resistance such as cell membrane [119] or cell wall modification [120] and the activity of multidrug efflux pumps [65,121]. Thus, the development of the appropriate transgenic strategy to introduce pathogen resistance through altered phytoalexin production needs to consider the occurrence of possible pathogen resistance strategies.

3. Transgenes and mutants

Because of the specificity in host–pathogen interactions and the ability of successful pathogens to detoxify phytoalexins [43], one strategy to produce resistant plants is to introduce a new type of phytoalexins in host plants using transgenic expression, rather than simply elevating the levels of the "naturally" produced phytoalexins [11,40]. In addition to their antimicrobial activity and thus importance for plant protection, phytoalexins have beneficial effects on human health, either by prevention of cardiovascular diseases [40,59,122], as anticancer drugs [123,124], or by inhibiting human pathogens [99]. Consequently, another approach is the high level production of such beneficial or toxic phytoalexins in plants for use in human medicine, which requires different strategies than the development of resistant plant species [59]. The development of tools to achieve these goals greatly depends on our knowledge of the enzymes involved in phytoalexin biosynthesis, regulatory mechanisms and interaction with primary metabolism [20,23]. Some approaches to genetically modify phytoalexin accumulation are summarized in Table 2.

3.1. Mutants in pathogen defense responses

The use of mutants defective in pathogen resistance, is a powerful approach to study the signaling pathways involved in plant disease resistance. *Arabidopsis* mutant screens following ethyl methanesulfonate mutagenesis or T-DNA tagging, and the simultaneous rapid development of new molecular and genetic tools, enabled the directed identification and characterization of many additional genes involved in plant defense pathways. This resulted in the isolation of several phytoalexin deficient (*pad*) mutants in *Arabidopsis* [83], which were either disturbed in the signaling leading to phytoalexin biosynthesis [125] or enzymes involved in the actual biosynthesis. The cloning of the *PAD3* gene, showed the involvement of P450 enzymes (CYP71B15) in camalexin biosynthesis [126]. Subsequently, the majority of the genes directly involved in camalexin biosynthesis and their regulators were identified employing a combination of *Arabidopsis* mutant and transgenic approaches [9,41].

The vast amount of *Arabidopsis* mutants defective in defense responses and the ease to study *Arabidopsis* mutant combinations, further substantiated the independence of camalexin biosynthesis from salicylate signaling [127] and enabled the identification of pathways essential for defense responses towards different pathogens [128,129]. This revealed that depending on the pathogen, besides camalexin (phytoalexin) also aliphatic glucosinolate (phytoanticipin) biosynthesis is important for defense responses [129]. Two tobacco mutants defective in abscisic acid biosynthesis revealed the involvement of abscisic acid in the regulation of capsidiol biosynthesis and the subsequent regulation of abscisic acid levels by capsidiol, by inducing the abscidic acid 8′-hydroxylase enzyme responsible for abscisic acid degradation [51]. The effect of abscisic acid on phytoalexin biosynthesis in general is complex and can also result in stimulation of phytoalexin biosynthesis [8], which could explain the conflicting data for the role of abscisic acid in biotic stress responses.

3.2. Direct modulation of phytoalexin biosynthesis

The genes for phytoalexin biosynthesis identified by different mutant approaches and their regulatory factors opened the way for directed manipulation of phytoalexin levels employing targeted ectopic expression of the genes acting in phytoalexin biosynthesis (rate limiting steps) and/or upstream signaling pathways (coordinated overexpression).

3.2.1. Stilbene biosynthesis

Considering the important role of resveratrol in both human health and plant resistance, the initial focus was on the use of the *STS* gene, especially from grape [118], to either boast endogenous resveratrol biosynthesis or introduce resveratrol as new phytoalexin in other species. The natural promoter was employed, which retained the inducibility by pathogens and abiotic stress conditions in a wide range of di- and monocotyledonous plant species, or constitutive promoters such as the *UBIQUITIN* or *CaMV* 35S promoters [59,118]. In both cases, strong resveratrol production was achieved (up to 400 μg/g fresh weight) and an increased resistance following infection with fungal pathogens. However, the transgenic plants responded differently in resistance depending on the fungal pathogen, although similar resveratrol levels accumulated following infection [130]. Thus, the host–pathogen relationship plays a decisive part in plant resistance to a certain pathogen, since several pathogens degrade phytoalexins, including resveratrol [43,59,118]. This shows the necessity to perform infection experiments employing several isolates for one specific pathogen species. Only few studies analyzed the resistance towards bacterial pathogens. Increased resveratrol synthesis conferred resistance to the fungal pathogen *Pyricularia oryzae*, but also the bacterial pathogen *Xanthomonas oryzae* in rice [131]. Although resveratrol is not a natural product in the different host plants, it was further modified by hydroxylation, O-methylation, and O-glucosylation by endogenous enzymes in these species [132]. Increased pathogen resistance was not achieved in each study, despite high phytoalexin levels. In one case, resveratrol or its derivatives were not detected in *STS* transgenics and flavonol levels were decreased.

Table 2
Examples of genetic modifications affecting phytoalexin accumulation.

Metabolic pathway *Class*	Phytoalexin(s)	Plant species	Genetic modification	Result(s)	Refs.
Alkaloid pathways					
Indole	Camalexin	*Arabidopsis thaliana*	Knock out of biosynthesis gene (*PAD3*)	Decreased camalexin biosynthesis, no effect on *Pseudomonas syringae* infection	[83]
			EtOH inducible oomycete elicitor (PaNie)	Time-dependent induction of tryptophan and camalexin biosynthesis genes by PaNie	[172]
			Overexpression of *miR393*	Re-direction of secondary metabolism from camalexin to glucosinolates, increased resistance against *Pseudomonas syringae* and *Hyaloperonospora parasitica*, increased susceptibility against *Alternaria brassicicola*	[47]
			Dexamethasone inducible overexpression of *MEK2*; *mpk3*/*mpk6* knock out	Camalexin accumulation after *MEK2* induction, reduced/delayed camalexin biosynthesis in *mpk3* and *mpk6* mutant lines, increased susceptibility against *Botrytis cinerea* of *mpk3* mutant line	[44]
			Knock out of *WRKY33*	Reduced camalexin levels in *wrky33* mutant lines	[41]
Isoprenoid pathway					
Diterpenes	Momilactones	*Oryza sativa*	Overexpression of transcription factor (e.g. *OsTGAP1*) and biosynthesis genes Knock down of biosynthesis genes	Increased momilactone levels in overexpression lines, constitutive momilactone accumulation in *OsTGAP1* overexpression lines, reduced momilactone levels in knock down lines	[27]
			CIPK14/15 RNAi and overexpression of *CIPK15*	Decreased momilatone accumulation in *CIPK14/15* RNAi-line, increased momilactone accumulation in *CIPK15* overexpression line	[157]
	Momilactone A		Overexpression of *ACDR1*	Increased momilactone A accumulation and increased resistance against *Magnaporthe grisea* in *ACDR1* overexpression lines	[155]
			Spl18 mutant	Increased momilactone A accumulation and increased resistance against *Magnaporthe grisea* in *spl18* mutant	[161]
			Overexpression of Rac GTPAse *RAC1*	Increased momilactone A accumulation and increased resistance against *Xanthomonas oryzae* in *RAC1* overexpression lines	[154]
			Overexpression of *SBP*	Increased momilactone A accumulation an increased resistance against *Magnaporthe grisea* and *Xanthomonas oryzae* in *SBP* overexpression lines	[162]
	Phytocassanes		*CIPK14/15* RNAi and overexpression of *CIPK15*	Decreased phytocassane accumulation in *CIPK14/15* RNAi-line, increased phytocassane accumulation in *CIPK15* overexpression line	[157]
Sesquiterpenes	Capsidiol	*Nicotiana tabacum*	Overexpression of *ipt*	Induction of capsidiol biosynthesis and increased resistance against *Pseudomonas syringae*, increased susceptibility to *Botrytis cinerea*	[50]
		Nicotiana plumbaginifolia	ABA deficient mutants *Npaba1* & *2*	Increased capsidiol accumulation in *Npaba1* and *Npaba2* mutant lines	[51]
	Gossypol	*Gossypium hirsutum*	Overexpression of *NPR1*	Increased gossypol accumulation and increased resistance against *Rhizoctonia solani* and *Alternaria alternata* in *NPR1* overexpression lines	[142]

Table 2 (*Continued*)

Metabolic pathway *Class*	Phytoalexin(s)	Plant species	Genetic modification	Result(s)	Refs.
Phenylpropanoid pathway					
Cinnamates/Coumarins	Scopoletin	*Nicotiana tabacum*	Overexpression of *ipt*	Induction of capsidiol biosynthesis and increased resistance against *Pseudomonas syringae*, increased susceptibility to *Botrytis cinerea*	[50]
			TOGT antisense	Reduced scopoletin accumulation and increased TMV susceptibility in *TOGT* antisense lines	[33]
Flavones/Isoflavones	Medicarpin	*Arabidopsis thaliana*, *Solanum lycopersicum*	Overexpression of *CHI*	Increased flavonol levels in *CHI* overexpression lines	[86]
		Medicago sativa	Overexpression of *IOMT*	Increased medicarpin accumulation and increased resistance against *Phoma medicaginis* in *IOMT* overexpression lines	
	e.g. Coumestrol, Glyceollins, Medicarpin	Various	Various modulations of biosynthesis genes and regulatory elements	Diverse	[40]
	Vestitol	*Lotus corniculatus*	*CHS* antisense	Reduced vestitol accumulation in *CHS* antisense lines	[138]
	Formononetin, Medicarpin	*Medicago truncatula*	Overexpression of *IFS*	Increased formononetin and medicarpin accumulation in *IFS* overexpression lines	[37]
	Pisatin	*Pisum sativum*	*HMM*, *IFR* and *SOR* RNAi	Reduced pisatin accumulation in *HMM*, *IFR* and *SOR* RNAi lines	[137]
			HMM antisense, overexpression of *PDA*	Reduced pisatin accumulation in *HMM* antisense and *PDA* overexpression lines	[116]
	Sakuranetin	*Oryza sativa*	Overexpression of *ACDR1*	Increased sakuranetin accumulation and increased resistance against *Magnaporthe grisea* in *ACDR1* overexpression lines	[155]
			Spl18 mutant	Increased sakuranetin accumulation and increased resistance against *Magnaporthe grisea* in *spl18* mutant	[161]
Stilbenes	e.g. Resveratrol	Various	Various overexpression of *STS*	Increased stilbene accumulation and increased resistance to various pathogens in *STS* overexpression lines	[59,118]
	Resveratrol	*Lactuca sativa*	Overexpression of *STS*	Transfer of resveratrol accumulation	[124]
		Oryza sativa, *Solanum lycopersicum*	Overexpression of *STS*	Increased resistance against *Magnaporthe grisea* and *Phytophthora infestans*	[79]

ACDR1, accelerated cell death and resistance 1; CHI, chalcone isomerase; CHS, chalcone synthase; HMM, (+)6a-hydroxymaackiain 3-O-methyltransferase; IFR, isoflavone reductase; IFS, isoflavone synthase; IOMT, isoflavone o-methyltransferase; ipt, isopentenyl transferase, NPR1, nonexpresser of pathogenesis related genes 1; PAD3, phytoalexin deficient 3; PDA, pisatin demethylating activity; SBP, selenium-binding protein; SOR, sophorol reductase; spl, spottet leaf; STS, stilbene synthase; TOGT, tobacco glucosyltransferase.

This altered phenolic compound profile was caused by decreased *CHS* transcript levels and led to increased pathogen susceptibility [133].

Therefore, a complete knowledge of the different biosynthetic pathways involved and possible cross-regulation and competition between different enzymatic branches is important to design transgenic approaches for the modulation of phytoalexin levels. STS and CHS compete for the same metabolic precursors *p*-coumaroyl-CoA and malonyl-CoA, which could explain the inverse correlation between *STS* and *CHS* transcript levels in transgenic alfalfa [134] and the decreased *CHS* transcript levels in transgenic strawberry, both engineered for *STS* overexpression [133]. Further, this substrate competition could explain the lack of correspondence between STS levels and resveratrol content in leaves of transgenic white poplar [135]. To avoid such complications due to substrate competition, *STS* overexpression in seeds to achieve seed specific resveratrol production was combined with suppression of sinapate ester biosynthesis, which also competes for the same substrate, by an *UDP-GLUCOSE:SINAPATE GLUCOSYLTRANERASE* ds-RNA-interference construct. Resveratrol glucoside (piceid) was produced up to 616 μg/g fresh weight in the seeds of homozygous rapeseed plants, whereas sinapate ester content was reduced 3-fold, without affecting other seed characteristics [136]. The observed inverse correlation between resveratrol and sinapate ester levels showed that this double transgenic approach is promising. However, no analysis of resveratrol levels was reported in *STS* transgenics without this *BnSGT1* ds-RNA-interference construct to substantiate the added value of such combined approaches.

3.2.2. Biosynthesis of other phytoalexins

The introduction and/or enhancement of (iso)flavonoid levels in crop species has become one major focus in crop biotechnology [40], because of the potential beneficial effects of isoflavonoids on human health, and the function of flavone and isoflavone phytoalexins in pathogen defense. The role of the isoflavonoid phytoalexin pisatin was studied in resistance towards the pathogen

N. haematococca, employing sense and antisense overexpression of *ISOFLAVONE REDUCTASE* and *(+)6a-HYDROXYMAACKIAIN 3-O-METHYLTRANSFERASE* in pea. Reduced pisatin levels correlated with reduced pathogen resistance, functionally showing the role of phytoalexin production in pathogen defense [116]. Likewise, RNAi suppression of several genes proposed to be involved in pisatin production identified the involvement of O-methyltransferases in pisatin biosynthesis [137]. A pathogen inducible gene encoding a cytochrome P450 protein (CYP93G3) was identified in sorghum. Heterologous overexpression in *Arabidopsis* showed that *CYP93G3* encodes a functional flavone synthase [19], responsible for the formation of sorghum phytoalexins.

Other approaches for direct manipulation employed genes encoding the first enzymes involved (iso)flavonoid biosynthesis. Constitutive *2-HYDROXYISOFLAVANONE SYNTHASE1* expression in *Medicago truncatula* resulted in a strong production of genistein glucoside and additional isoflavones, in the absence of major changes in global gene expression [40]. Suppression of *2-HYDROXYISOFLAVANONE SYNTHASE* or *CHALCONE REDUCTASE/CHS* in soybean resulted in decreased 5-deoxyisoflavonoid levels and reduced resistance to pathogen infection [40]. However, antisense *CHS* expression in *Lotus* resulted in unexpected biochemical changes caused by differential regulation of the multigene *CHS* family [138]. Pharmacological experiments proposed feedback regulation in the phenylpropanoid pathway. This was genetically confirmed by *CINNAMATE-4-HYDROXYLASE* overexpression in tobacco, which caused down-regulated *PAL* expression [139]. Overexpression of *ISOFLAVONE O-METHYLTRANSFERASE* in alfalfa conferred resistance towards *Phoma medicaginis*, but resulted in different effects on gene transcripts and isoflavone levels between control and $CuCl_2$ stressed or *P. medicaginis* infected plants [140]. Thus, despite the direct manipulation of these defined pathways, unexpected results occurred on the biochemical spectrum affected by transgenic expression from feedback regulation, complex regulation of multigene families and influences from growth conditions.

According to the role of phytoalexins as ROS scavenger, ROS levels were increased following antisense *TOGT* expression in tobacco [33] and concomitantly resistance towards TMV infection was reduced. The comparison of transgenic tobacco lines that express either *TRYPTOPHAN DECARBOXYLASE* or *TYROSINE DECARBOXYLASE* showed that tyrosine decarboxylase controls the rate-limiting step in the production of wound inducible biosynthesis of phytoalexin hydroxycinnamic acid amides of tyramine, which are important during suberisation of wounded potato tubers [76].

GaWRKY1 was identified as transcriptional regulator of the cotton *(1)-d-CADINENE SYNTHASE* gene, which endcodes a sesquiterpene cyclase involved in the biosynthesis of sesquiterpene phytoalexins. The activation of *(1)-d-CADINENE SYNTHASE* expression by GaWRKY1 was validated employing heterologous expression in both *Arabidopsis* and tobacco [31]. The identification of transcriptional regulators for phytoalexin biosynthesis could allow the coordinated regulation of several genes participating in the same pathway to achieve a more robust increase in phytoalexin levels.

3.3. Indirect modulation of phytoalexin biosynthesis

In addition to the direct manipulation of phytoalexin levels by employing biosynthetic genes or their transcriptional regulators, also indirect regulation is possible by affecting upstream signaling pathways.

Despite the importance of salicylate in pathogen defense pathways, constitutively elevated salicylate levels did not result in increased sesquiterpenoid phytoalexin levels in the absence of pathogen infection [141]. This is in agreement with the finding that the cytokinin mediated phytoalexin production in tobacco is initially independent from salicylate levels [50]. Constitutive expression of the important defense regulator *NPR1* (salicylate signaling) in cotton resulted in similar defense related gene expression and biochemical accumulation under control conditions as wild-type plants. However, the transgenic plants had stronger defense responses, including the accumulation of phytoalexins, following infection with different pathogens [142], thereby improving pathogen resistance without noticeable negative effects on plant growth. NPR1 is an important preformed component of plant defence responses and its activity tightly regulated post-translationally [143].

Reduced glutathione is the precursor of the thiazole ring required for camalexin biosynthesis as elucidated using *GLUTATHIONE S-TRANSFERASE F6* (*GSTF6*) gain- and loss-of-function analysis. In addition, *γ-GLUTAMYL TRANSPEPTIDASES* and *PHYTOCHELATIN SYNTHASES* were coordinated upregulated with *GSTF6* during camalexin biosynthesis, and are involved in camalexin biosynthesis [144]. This agrees with the fact that the *Arabidopsis pad2* mutant has lower glutathione levels and is defective in the gene encoding γ-glutamylcysteine synthetase (γ-ECS, GSH1), which catalyzes the first committed step of glutathione biosynthesis [145]. Accordingly, overexpression of *γ-ECS* in tobacco increased glutathione levels and resistance towards *P. syringae*, but not *Alternaria alternata* [146]. The increased glutathione accumulation mainly affected NPR1-dependent salicylate signaling, but induction of a putative *ISOPRENE SYNTHASE* could be correlated with an increased phytoalexin production, according to the importance of glutathione as precursor for phytoalexin biosynthesis in *Arabidopsis*.

Transgenic tobacco plants expressing the bacterial *isopentenyl transferase* (*ipt*) gene involved in cytokinin biosynthesis, overaccumulated phenolic compounds and PR-proteins [49]. Although the function of the *rolC* gene from *Agrobacterium rhizogenes* is still not elucidated, transgenic expression results in cytokinin related phenoytpes. *rolC* expression in *Rubia cordifolia* resulted in reduced steady-state and stress-induced ROS levels, as well as increased phytoalexin levels [147]. In line with these findings, a cytokinin mediated pathogen resistance mechanism was identified in tobacco against *P. syringae*, involving the production of the phytoalexins scopoletin and capsidiol [50]. A novel transgenic approach employing a synthetic pathogen inducible promoter [148] for *ipt* expression enabled autoregulated cytokinin synthesis in response to pathogen infection and mediated enhanced resistance through increased levels of scopoletin and capsidiol. This was confirmed by two additional independent transgenic approaches to increase endogenous cytokinin production. Exogenous supply of adenine and phenylurea derived cytokinins prior to bacterial infection also conferred pathogen resistance [50]. Importantly, these approaches resulted in rapidly elevated phytoalexin levels early after pathogen infection, resulting in high phytoalexin/pathogen ratios compared to control treatments (Fig. 2A and B). The absolute phytoalexin levels in the late infection phase were much higher in control infections, confirming that the dynamics of phytoalexin biosynthesis are decisive for resistance, rather than absolute levels [8,11]. Leaves were infiltrated with scopoletin and capsidiol in parallel to the bacterial infection, and the phytoalexin levels rapidly decreased *in planta* to physiological levels similar to the control infection around 24 h post infection (Fig. 2C). Infiltrated phytoalexins conferred resistance, but the pathogen induced phytoalexin accumulation occurred too late to be effective (Fig. 2D). The latter approach further highlights the importance of rapid phytoalexin dynamics for successful defense. The application of phytoalexins themselves could be of little value in agriculture, due to the apparently rapid degradation. Together, these studies identified cytokinins as important regulators of phytoalexin production and thus pathogen defense responses in some species.

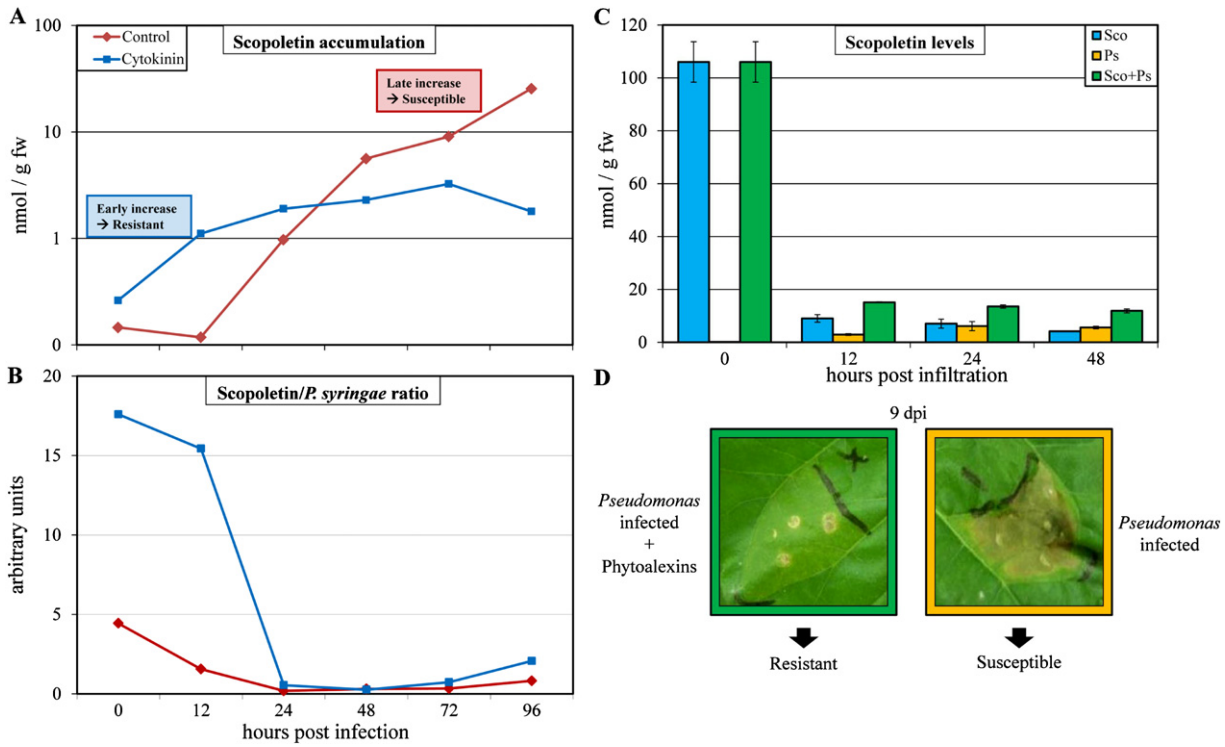

Fig. 2. Dynamics of phytoalexin accumulation in tobacco determining resistance effects against *Pseudomonas syringae*. (A) Dynamics of scopoletin accumulation after infection (up to 96 h post infection) with *P. syringae* in control (red) and leaves with increased cytokinin levels (blue). Early increase of phytoalexins post cytokinin treatment mediates resistance against the pathogen, whereas late (and stronger) increase in controls is not sufficient to restrict infection. (B) Scopoletin/*P. syringae* ratio during infections with (blue) and without (red) cytokinin treatment. The increased ratio post cytokinin treatment during the initial phase of infection leads to resistance against the pathogen. At later time points, scopoletin/*P. syringae* ratios are similar with and without cytokinin treatment. (C) Scopoletin levels *in planta* post infiltration of 200 μM scopoletin (Sco; blue), 10^7 cfu/ml *P. syringae* (Ps; yellow) and combination of both (Sco + Ps; green). Infiltrated scopoletin levels decrease rapidly and reach similar levels at 24 h post infiltration compared to physiological levels accumulating after Ps infection. Scopoletin levels after the combined treatment are equal to the sum of both single treatments. (D) Resistance against *Pseudomonas* infection is determined by the dynamics of scopoletin accumulation rather than absolute levels. Whereas scopoletin infiltrated in parallel to the infection restricts symptom development and confers resistance (green frame), scopoletin formation triggered by the infection is not sufficient, thus the host is susceptible and symptoms develop (yellow frame), although both treatments reached similar levels around 24 h post infiltration.

The cytokinin induced pathogen resistance via endogenous phytoalexins offers the advantage of a fine-tuned regulation, allowing gentle increases in phytoalexin levels, which caused efficient pathogen resistance without the induction of HR [50]. Avoiding HR formation protects tissue integrity and thus limits the ecological costs for the plant resulting from losses in photosynthetic tissue [149], thereby maintaining plant performance. In addition, cytokinins are also important in promoting the tolerance towards abiotic stress conditions [150,151] and generally promoting plant growth and counteracting senescence, which could further contribute to crop performance. However, excessive increases for general plant regulators such as phytohormones can also result in negative side-effects, for example cytokinin induced ethylene production that impairs plant growth [152].

The elicitation of phytoalexin (benzophenanthridine) biosynthesis was shown in California poppy (*Eschscholzia californica*) cell cultures. The involvement of phospholipase A2 signaling and the efflux of vacuolar protons were demonstrated using both *Gα* antisense expression and anti-Gα single-chain antibodies. This identified a second pathway inducing phytoalexin production, independent from the jasmonate regulated HR [153]. Rice Rac GTPAse (OsRAC1) is a positive mediator of phytoalexin production, HR responses and pathogen resistance, as shown through constitutively active and dominant-negative OsRAC1 mutations [154]. The rice putative Raf-like MAPKKK OsACDR1 promoted phytoalexin production, expression of defense-related marker genes and resistance to the fungal pathogen *Magnaporthe grisea*. This was demonstrated by biochemical, gain- and loss-of-function approaches [155]. Rice CBL-Interacting Protein Kinases OsCIPK14 and OsCIPK15 are positive regulators in calcium mediated PAMP signaling leading to increased defense responses, including phytoalexin accumulation [156,157]. In addition, diterpenoid phytoalexin production in rice is regulated by a MAPK cascade [158]. Thus, in addition to hormone signaling, phosphorylation relays and cascades are also potentially important regulators for the modulation of phytoalexin production and pathogen resistance.

Transgenic *Arabidopsis* plants overexpressing the gene *PATHOGENESIS RELATED*5 (*PR5*) had increased resistance to *Alternaria brassicicola* and higher camalexin levels. This showed that PR5 functions both as an anti-fungal protein and as positive regulator for genes in other defense pathways [159]. Transgenic potato lines with an antisense *INVERTASE* gene were exposed to a range of biotic and abiotic stresses to ascertain the production of toxic secondary metabolites, which are divided into two groups: sesquiterpenes (phytoalexins) and glycoalkaloids. Whereas phytoalexins confer pathogen resistance and in general are not harmful for human health, glycoalkaloids can be toxic to humans. The antisense *INVERTASE* plants had increased levels of sesquiterpenes but reduced glycoalkaloids levels in tubers, which offers a tool to combine enhanced pathogen resistance to improvement in food quality [160]. The *Spotted leaf 18* mutant in which the overexpression of an *ACYLTRANSFERASE* (*OsAT1*) promoted *PR* gene expression was identified in screening of activation-tagged rice lines. This mutant had enhanced accumulation of phytoalexins (momilactone A and sakuranetin) and was resistant to fungal blast (*M. grisea*) disease [161]. Overexpression of the elictor induced selenium binding protein *OsSBP* in rice also resulted in elevated phytoalexin levels and

PR gene expression. The plants had increased resistance towards blast fungus and bacterial blight. This represents one of the rare studies addressing resistance towards bacterial pathogens. Despite the function of some phytoalexins as antioxidants, hydrogen peroxide production was increased, probably because the activity of scavenging enzymes was reduced [162].

These studies revealed that the regulation of different phytoalexin levels is very complex, involving G-protein mediated signaling, phosphorylation cascades, hormonal regulation and glutathione biosynthesis, varying with both plant species and phytoalexins. Further, several other unexpected regulatory mechanisms are involved, including invertase activities for which an important function in both sugar mediated, coordinated regulation of source–sink relations and defense responses [163], and cytokinin function [164] act in pathogen defense responses [165]. Such indirect mechanisms of phytoalexin regulation offer the possibility for coordinated regulation of several pathways leading to stronger, more efficient pathogen defense. Further, the cytokinin regulated pathogen resistance allows priming of phytoalexin production in the host plant [50], resulting in better pathogen defense without the cost of reduced growth accompanying full scale constitutive defense responses.

4. Outlook

4.1. Knowledge is power

Following the molecular/genetic revolution, the initial biochemical research focus on phytoalexin detection has moved to the biosynthetic genes, their transcriptional regulators and upstream signaling pathways. The different transgenic studies described in this review show that directed manipulation of phytoalexin levels requires a complete knowledge about the involved mechanisms, avoiding potential negative side-effects on plant productivity. These include the interconnection of primary and secondary metabolic pathways [23], transcriptional and post-transcriptional regulation of enzymatic activities and competition between enzymatic branches using the same precursors [20,40]. Extensive network analysis between transcriptomics, metabolomics and proteomics is required, which shifts the focus of phytoalexin research back to biochemical analysis. Consequently, improvements are required in the high-throughput detection of secondary metabolites, allowing a complete categorization of phytoalexins and concurrently enhanced non anti-microbial metabolites in the studied plant species [42]. Another important aspect is to assess phytoalexin production in transgenics in field studies in parallel to well established experimental systems, because the varying environmental conditions in the field could strongly influence basal phytoalexin levels [79]. The available strategies for the modulation of phytoalexin levels employing transgenics are discussed, considering the advantages, bottlenecks and necessary improvements.

4.2. Phytoalexins and phenomics

Automated high throughput phenotyping is receiving increasing attention within basic plant research and also by breeding companies. Therefore “phenomics” technologies are currently developed and phenotyping facilities are established by various consortia in different countries. Within these approaches for non-invasive characterization of plants, special attention should also be given to the non-invasive detection of phytoalexins within the context of stress responses by the measurement of fluorescence and reflectance. A number of secondary metabolites including various phytoalexins emit either specific fluorescence patterns or are characterized by specific absorption signatures [166]. For example soluble plant phenolics including flavones, flavonols, cinnamic acids, coumarins and stilbenes show characteristic blue and/or green fluorescence [167]. These signals are increased through degradation of chlorophyll and carotenoids in response to stress stimuli. The use of fluorescence as analytic tool has been fuelled to a large degree by the introduction of a number of highly user friendly fluorometers. The drawback in the past for using fluorescence with small sampling areas has been overcome by the development of imaging systems that allowed the application of this technique for high throughput screening. This high resolution method is more precise and provides stress information at an earlier stage than point data fluorescence measurements. Applications of fluorescence and reflectance imaging in screening for disease and stress resistance have a clear potential for quantitative assessment and even discriminating between individual components such as salicylate and the phytoalexin scopoletin is possible [168]. Thus, application of multi- or hyper-spectral fluorescence and reflectance imaging [169,170] in high-throughput phenotyping screens of transgenic plants, ecotype tilling and mutant analyses should provide further insight in the function and regulation of phytoalexins.

4.3. Requirements of transgenic phytoalexin modulation

The modulation of phytoalexin levels can be of importance for the production of (1) phytoalexins for direct medicinal application, (2) phytoalexins to improve the antioxidant capacity and thereby food quality (indirect medicinal application) and (3) phytoalexins to improve crop resistance, each requiring different transgenic strategies. The production of specific phytoalexins for direct medicinal application requires constitutively high levels to facilitate purification of the desired compound for which organ specific promoters could be employed. Cost/benefit factors such as reduced plant productivity are only of secondary importance and even could be avoided by employing prokaryotic and eukaryotic cell culture based systems [40]. Phytoalexin production could be optimized by suppression of competing enzymatic branches that prevent substrate competition, and/or accumulation of toxic side products. To achieve this, antisense or miRNA suppression is possible, but also mutant approaches such as tilling [171] are promising since transgenic suppression could be unstable due to transgenic silencing. The improvement of nutrient quality, i.e. antioxidant capacity of crop species, requires enhanced production in the harvested organ only and therefore organ/stage specific promoters are the method of choice. This can avoid negative side effects such as reduced plant productivity and consequently yield. The possible formation of toxic compounds should carefully be assessed, requiring extensive biochemical analysis (metabolomics) and animal testing. It is important to understand the infection strategies of pathogens and occurrence of insensitive isolates attacking the respective crop species, before the proper strategy can be designed for the improvement of crop resistance. Since host–pathogen interactions are frequently very specific, the transgenic introduction of “new” phytoalexins could be the weapon of choice to prevent infections.

A basal increase in phytoalexin levels could provide an efficient protection against pathogens and (constitutively) high levels of phytoalexins did not result in strong pleiotropic effects [37,136] in the few cases studied. Nevertheless, the possible consequences need to be investigated carefully for the respective plant species to avoid negative side effects like reduced productivity. The cytokinin mediated pathogen resistance in tobacco showed that induced expression systems also provide effective protection [50], even after pathogen infection took place. Therefore, inducible expression from pathogen inducible (artificial) promoter sequences [148] creates a potentially powerful tool for generating resistant crop species by providing protection on demand, which could avoid much of the possible negative side effects related to increased phytoalexin

production. Further optimization of available pathogen inducible promoter sequences could improve the efficiency (reduced background expression) and reaction time of such systems and in combination with organ specificity reduce the danger of accumulating potentially toxic chemicals in the organs used for food production. Another concern is that phytoalexins can result in host cell death. Therefore, such HR formation should be avoided or at least as much as possible restricted to a very small diameter to limit the ecological costs [149].

4.4. Direct or indirect modulation of phytoalexin levels?

The direct manipulation of phytoalexin levels by transgenic expression of genes involved in biosynthesis was successful for improved crop resistance, which in part could result from the introduction of "foreign" phytoalexins to overcome the natural resistance of pathogens evolved during specialization on specific host species. With the current knowledge from several plant species on phytoalexin biosynthesis, enough genes should be available for this strategy. However, phytoalexins are only a part of the complex pathogen defense responses [2,8], and as such the achieved resistance could be too weak and/or too limited for effective protection against a variety of pathogens. Therefore, indirect "broad based" approaches employing transcriptional regulators or components of upstream regulatory pathways, like NPR1 [142], are more suited for transgenic manipulation. Such approaches could confer coordinated regulation for several enzymes to ensure higher phytoalexin production or several defense pathways for stronger and more durable defense response [40], which also would make it more difficult for pathogens to evolve escape mechanisms/resistance. Inducible expression systems could have the disadvantage that the induction is not fast and/or strong enough to build up an efficient protection. In this case a self-amplifying mechanism could be created by expressing a PAMP or elicitor (flagellin) from a pathogen responsive promoter in plants, which could amplify the initial pathogen response and thus create a very strong defense. For example, following induced accumulation of the Nep1 protein, an elicitor of the oomycete *Pythium aphanidermatum*, camalexin biosynthesis as well as other defense responses were activated [172]. Thus, such an inducible expression system could serve as trigger to enhance the natural defense system of the host, strongly involving phytoalexin synthesis as one of the components.

4.5. Transgene stacking to create combi-weapons

Since phytoalexins are rapidly degraded after relief from the stress responses [8], care has to be taken to prevent premature degradation of the desired product. Further, phytoalexins are only a part of the complex pathogen defense responses [2,8]. Therefore, combining several different but complementing approaches [11] might create crop species with "superior" protection against a wide variety of pathogens. This could also ensure resistance towards pathogens that are insensitive to- or even feed on phytoalexins and thus could endanger crop yield. The advantages made in the ability to transform crop species and binary vector construction, allow the strategy of transgene stacking, which enables such combined approaches. To this means, a wide variety of weapons can be chosen [14], including genes encoding proteins/enzymes that prevent phytoalexin degradation by either the host or the pathogen.

Concluding, to design sophisticated strategies combining effective protection and optimal plant productivity, we need to deepen our knowledge on the extensive cross-regulation between the different metabolic pathways involved. However, the transgenic studies discussed in this review clearly show that the ability to modulate phytoalexin levels using transgenic approaches offers a strong potential to generate crops species with improved pathogen resistance as well as improved food quality. Thus, the use of phytoalexin transgenics in crop protection currently looks like a fairy tale, but the prospect is that the increased knowledge on transgenic approaches may bring it to a happy end.

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