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Review article

Review: Potential biotechnological assets related to plant immunity modulation applicable in engineering disease-resistant crops

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ABSTRACT

This review emphasizes the biotechnological potential of molecules implicated in the different layers of plant immunity, including, pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI), effector-triggered susceptibility (ETS), and effector-triggered immunity (ETI) that can be applied in the development of disease-resistant genetically modified (GM) plants. These biomolecules are produced by pathogens (viruses, bacteria, fungi, oomycetes) or plants during their mutual interactions. Biomolecules involved in the first layers of plant immunity, PTI and ETS, include inhibitors of pathogen cell-wall-degrading enzymes (CWDEs), plant pattern recognition receptors (PRRs) and susceptibility (S) proteins, while the ETI-related biomolecules include plant resistance (R) proteins. The biomolecules involved in plant defense PTI/ETI responses described herein also include antimicrobial peptides (AMPs), pathogenesis-related (PR) proteins and ribosome-inhibiting proteins (RIPs), as well as enzymes involved in plant defensive secondary metabolite biosynthesis (phytoanticipins and phytoalexins). Moreover, the regulation of immunity by RNA interference (RNAi) in GM disease-resistant plants is also considered. Therefore, the present review does not cover all the classes of biomolecules involved in plant innate immunity that may be applied in the development of disease-resistant GM crops but instead highlights the most common strategies in the literature, as well as their advantages and disadvantages.

1. Introduction

Plant pathogens, including viruses, bacteria, fungi, and oomycetes are a primary concern in agribusiness [1–3]. The diseases caused by these organisms in plants represent an important and persistent threat to food supplies worldwide [4]. The development of disease-resistant plants through biotechnological approaches aims to obtain economically important crops through elite genetically modified (GM) lines that not only display durable and broad-spectrum resistance to multiple phytopathogens, but that are also biosafe to the environment and consumers. To achieve this goal, several challenges related to transgene must be overcome, such as fine-tuning the choice, origin (i.e., heterologous species and/or non-host plant) and the number of genes to be employed and stacked, as well as gene expression control (e.g., by signal peptides, gene silencing and gene promoters). The current knowledge of the molecular mechanisms involved in plant-pathogen interactions has now provided a large set of biomolecules that can be applied in the development of GM disease-resistant/less susceptible crops.

Plant-pathogen interactions involve a two-way communication process, whereby plants can recognize and induce defense strategies against pathogens, while pathogens can threaten plant functional

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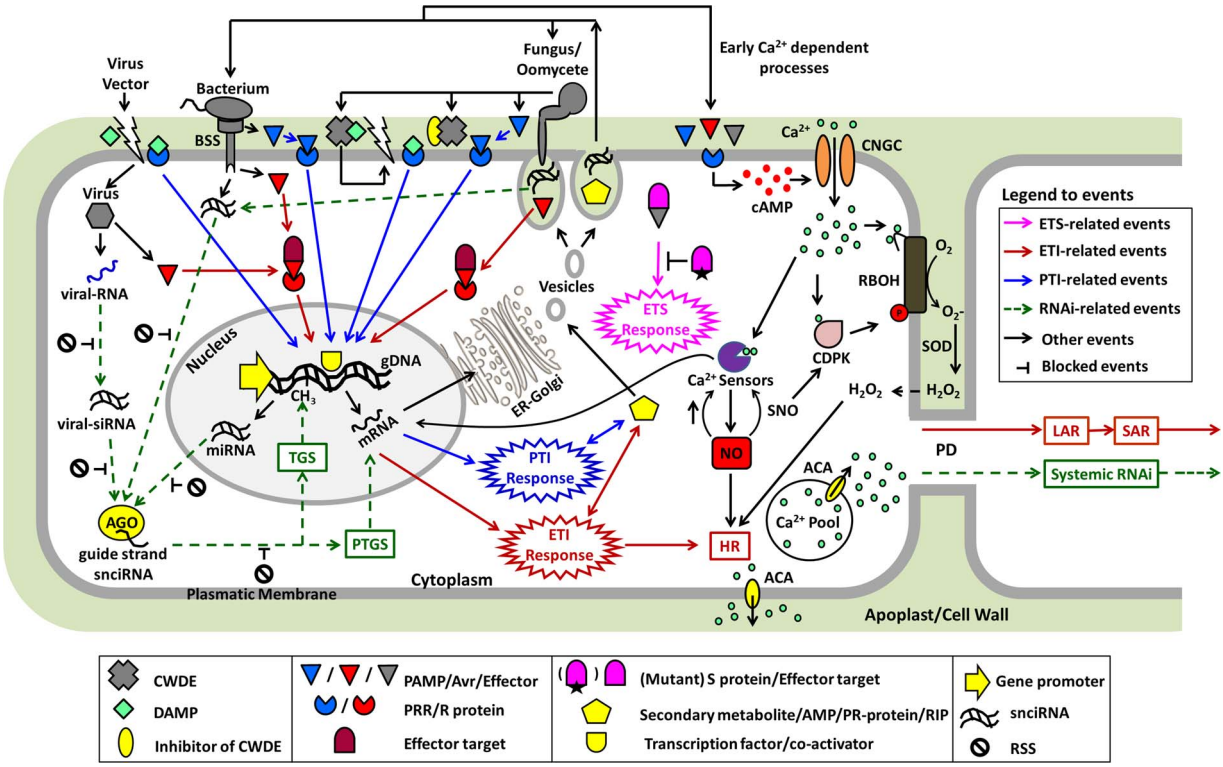

Fig. 1. General plant immune pathways and potential biotechnological assets found in plant-pathogen interactions used to engineering disease-resistant crops. The schematic figure illustrates the intricate relations between plant innate immunity (PTI, ETI), ETS-related resistance response and the regulation of the genes involved in plant-pathogen interactions by RNAi-mediated TGS and PTGS. Summary of manly early calcium (Ca^{2+}) dependent processes are also illustrated. The interaction of PAMP/Avr/Effectors with plasma membrane receptor induces cAMP production and stimulates the rapid influx of Ca^{2+} into the cell through CNGC. Free Ca^{2+} from apoplast and/or intracellular Ca^{2+} pools can stimulate H_2O_2 production by RBOH in two ways: (i) directly activation by Ca^{2+} interaction with RBOH N-terminal; (ii) and indirectly activation with RBOH phosphorylation by Ca^{2+} activated CDPK. Activated Ca^{2+} sensors (for example calmodulin/calmodulin-like) increase NO production, which regulates Ca^{2+} sensors by positive feedback and stimulates HR. Both Ca^{2+} sensors and CDPK are S-nitrosylated (SNO − a post-translational regulatory mechanism during which NO is covalently and reversibly bonded to the sulfahydryl groups of rare, low pKa cysteine residues). Ca^{2+} sensors can induce PTI/ETI through transcription regulation of genes related with stress responses. Intracellular Ca^{2+} levels can be regulated by the efflux of the second messenger through the ACA protein. For details and discussion, see text. Abbreviations (in alphabetical order): **ACA**: autoinhibited Ca^{2+} ATPase; **AGO**: argonaute; **AMP**: antimicrobial peptide; **BSS**: bacterial secretion systems; **cAMP**: cyclic AMP; **CDPK**: calcium-dependent protein kinase; **CH_3**: methyl; **CNGC**: cyclic nucleotide-gated channel; **CWDE**: cell wall-degrading enzymes; **DAMP**: damage-associated molecular pattern; **ER-Golgi**: endoplasmic reticulum-Golgi complex; **ETI**: effector-triggered immunity; **ETS**: effector-triggered susceptibility; **gDNA**: plant genomic DNA; **HR**: hypersensitivity response; **LAR**: local acquired resistance; **miRNA**: micro RNA; **mRNA**: messenger RNA; **NO**: nitric oxide; **PAMP**: pathogen associated molecular pattern; **PD**: plasmodesma; **PRR**: pattern recognition receptor; **PR protein**: pathogenesis-related protein; **PTGS**: post-transcriptional gene silencing; **PTI**: PAMP-triggered immunity; **R**: resistance protein; **RBOH**: NAPDH oxidase; **RIP**: ribosome-inhibiting proteins; **RSS**: RNA silencing suppressor; **S protein**: susceptibility protein; **SAR**: systemic acquired resistance; **siRNA**: small interfering RNA; **sncRNA**: small non-coding interfering RNA; **SNO**: S-nitrosylation; **SOD**: superoxide dismutase; **TGS**: transcriptional gene silencing.

physiology and counterattack plant defense mechanisms. The intricate plant-pathogen exchange of interactions among biomolecules involve specific characteristics depending upon whether the pathogen is a virus, bacterium, nematode or filamentous microbe: (i) viruses are directly introduced, either by mechanical damage or by a biological vector (i.e., insect, nematode, fungus) into the plant cell cytosol, where they expose their genome, structural proteins and lipids (in the rare case of enveloped viruses); (ii) bacteria biomolecules related to virulence are secreted by type II, III and IV secretion systems to interact with the host plant cell [5–7]; and (iii) filamentous pathogens (herein referred as Eumycota true fungi and oomycetes with fungal-like growth, also known as water molds) release a range of biomolecules into the plant apoplast and cytosol (Fig. 1). In opposition to the first barrier to plant invasion, filamentous pathogens secrete cell-wall-degrading enzymes (CWDEs) [8], and plants, in turn, respond to the cell wall damage by strengthening/reprogramming the cell wall and by secreting CWDE inhibitors.

Invasion by most pathogens is perceived through transmembrane plant proteins called pattern recognition receptors (PRRs), which detect microbe-derived molecules termed pathogen-associated molecular patterns (PAMPs). In addition to PAMPs, PAMP-triggered immunity (PTI) is also activated by endogenous plant signals released during pathogen invasion termed damage-associated molecular patterns (DAMPs). The first active line of plant immunity is triggered upon the specific detection of PAMPs by PRRs [9–11]. Well-adapted pathogens secrete a plethora of effectors (i.e., molecules secreted by pathogens that modulate host cell mechanisms and physiology) that suppress PTI through susceptibility (S) proteins (effector targets), allowing host cell infection and resulting in effector-triggered susceptibility (ETS) [12–15] (Fig. 1).

In response to effectors, plants developed a second line of receptors, encoded by resistance (*R*) genes, that are activated via specific recognition of the cognate effector or pathogen avirulence (Avr) proteins, yielding effector-triggered immunity (ETI) [16] (Fig. 1). PTI involves PAMPs that are evolutionarily conserved across a class of organisms, while ETI is highly specific to certain pathogens that secrete a unique effector or Avr product. ETI frequently involves localized programmed cell death, known as the hypersensitive response (HR) that restricts pathogen spread at the infection site [17]. To restrain infection, both PTI and ETI induce the expression of a range of antimicrobial peptides (AMPs), pathogenesis-related (PR) proteins, ribosome-inhibiting proteins (RIPs) and defensive secondary metabolites, among other plant physiological defense biomolecules [18–21]. The HR in infected cells is associated with the transfer of defense signals to neighboring uninfected cells within the same organ. This transfer is performed through plasmodesmata and to other uninfected organs through the phloem, which results in induced distal resistance responses called local acquired resistance (LAR) and systemic acquired resistance (SAR),

respectively [22–25].

Furthermore, the pathogen recognition in plants can increase the production of reactive oxygen species (ROS) and nitric oxide (NO), triggered by calcium (Ca^{2+}), a ubiquitous intracellular second messenger. Early increase in cytosolic Ca^{2+} concentration is induced by several extracellular stimuli, such as exogenous H_2O_2, microbe-associated molecular patterns (MAMPs), effectors or DAMPs. These changes are detected by Ca^{2+} sensors, such as calmodulin or calmodulin-like protein (CaM/CML) and calcium-dependent protein kinase (CDPK), and transduced into a signal, which led to HR, transcription regulation of genes related with stress responses, as well as rapid production of H_2O_2 and NO. Thus, Ca^{2+} signaling is intrinsically linked with early PTI/ETI perception mechanisms [26–31] (Fig. 1).

Thus, PTI, ETS, and ETI are layers of the plant innate immune system that may induce plant resistance to pathogens [9,13]. Several studies use a concept of plant resistance to pathogens expressed as a gray scale ranging from total susceptibility (white value) to total resistance (black value) [32]. On this scale, the HR is considered as an absolute/qualitative level of resistance, while the remainder of the reduced susceptibility levels are regarded as quantitative levels of resistance.

In addition, both plants and pathogens produce small-non-coding interfering RNA molecules (snciRNAs), which fine-tune the plant immunity layers (PTI, ETS, and ETI) through distinct, yet overlapping, genetic and epigenetic RNA interference (RNAi) silencing pathways to silence different genes involved in complex plant-pathogen interactions [33,34] (Fig. 1). RNAi, also called RNA-mediated gene silencing or simply RNA silencing, comprises a sequence-specific mechanism in which snciRNAs recognize and suppress complementary undesirable nucleic acids, such as genes, transposons, overexpressed transcripts, and RNA viruses. In both plants and some microbes, RNAi regulates the expression of genes involved in countless physiological processes through either post-transcriptional gene silencing (PTGS) or transcriptional gene silencing (TGS), thereby modulating the expression of various genes that are involved in the intricate plant-pathogen interactions and plant immunity [33,34]. In plants, snciRNAs are double-stranded RNA (dsRNA) molecules of approximately 21–24 nucleotides in length that originate from the processing of long dsRNAs. This cellular process is mediated by an RNA III-like endonuclease called Dicer-like (DCL) or by the amplification/synthesis of long dsRNAs from long single-stranded RNAs (ssRNAs) by RNA-dependent RNA polymerases (RdRPs) [35,36]. Endogenous plant snciRNAs can be divided into two main classes, microRNAs (miRNAs) and small interfering RNAs (siRNAs), according to their biogenesis [37,38]. miRNAs originate from imperfect stem-loop precursor RNAs transcribed by RNA polymerase II, while siRNAs are derived from perfectly complementary dsRNAs transcribed by various RNA polymerase types [33]. After its biogenesis, one strand (the so-called guide strand) of the snciRNA (either miRNA or siRNA) is loaded into an Argonaute (AGO) protein. The loaded AGO recognizes the target coding ssRNA by sequence complementarity with the guide strand and either (i) degrades or represses the translation of the target ssRNA, thus triggering PTGS, or (ii) recruits proteins related to epigenetic regulation by RNA-dependent DNA methylation (RdDM) of the target DNA (e.g., gene, transgene, transposable element) corresponding to the guide strand/target ssRNA, thus triggering TGS [39]. Though many small RNAs are involved in PTGS, the majority of snciRNAs in plants are correlated with RdDM and TGS. Within the plant host, snciRNAs move locally through plasmodesmata and long-distance through the phloem to induce local and systemic RNAi, respectively. As an evolutionary counteraction, upon viral, bacterial, fungal and oomycetal infection, pathogen-derived RNA silencing suppressors (RSSs) repress the plant innate immunity RNAi mechanisms, resulting in resistance to plant disease [40,41].

This review focuses on the potential of biomolecules involved in the layers of plant immunity (PTI, ETS, and ETI) that can be applied in the development of disease-resistant GM plants. The biomolecules presented herein involved in the first layers of plant immunity, PTI and ETS, include plant inhibitors of pathogen CWDEs, plant PRRs, and S proteins, while the ETI-related biomolecules include plant R proteins and pathogen effectors. Biomolecules involved in plant defense PTI/ETI responses described herein include AMPs, PR proteins, RIPs and enzymes involved in plant defensive secondary metabolite (phytoanticipins and phytoalexins) biosynthesis. The applicability of these biomolecules in the successful development of GM crops with durable and broad disease resistance is reviewed. Moreover, the regulation of immunity by RNAi in GM disease-resistant plants is considered. The present review does not cover all the classes of biomolecules involved in plant innate immunity that may be applied in the development of disease-resistant GM crops but instead highlights the most common strategies in the literature. Therefore, biomolecules such as plant hormones, among others, are not herein addressed in the context of the development of disease-resistant transgenic plants.

2. Biomolecules involved in the first layers of plant immunity: PTI and ETS

2.1. Plant inhibitors of pathogen-encoded cell wall degrading enzymes

Filamentous pathogens secrete CWDEs, such as cellulases, polygalacturonases, xylanases, xyloglucan-endoglucanase, chitinases and protease inhibitors, as an early attack against the plant cell wall [8,42]. Plants, in turn, respond to the cell wall damage by strengthening/reprogramming the cell wall and by secreting inhibitors of CWDEs. Potent plant inhibitors of microbial CWDEs with potential for biotechnological application in agriculture include polygalacturonase-inhibiting proteins (PGIPs), xylanase-inhibiting proteins (XIPs) and xyloglucan-specific endoglucanase-inhibiting proteins (XEGIPs) [43–47].

Transgenic plants overexpressing PGIPs, which degrade microbial polygalacturonases, results in a delay in plant cell pectin hydrolysis and consequently restricts fungal infection [48–50]. Reduced susceptibility to the necrotrophic fungus *Botrytis cinerea* has already been established in GM tomato [51], GM grapevine [52], GM tobacco [53] and GM *Arabidopsis thaliana* (hereafter Arabidopsis) [54] expressing plant PGIPs. More recently, GM *Nicotiana tabacum* expressing a *Phaseolus vulgaris* PGIP also presented reduced susceptibility to *R. solani*, *Phytophthora parasitica* var. *nicotianae* and *Peronospora hyoscyami* f. sp. *tabacina* [55]. Similarly, expression of a pepper PGIP in GM tobacco plants reduced susceptibility to *Phytophthora capsici*, and overexpressing the protein GhPGIP1 increases resistance to *Verticillium* and *Fusarium* wilts in Arabidopsis and cotton (Table 1) [50,56].

Among CWDEs, xylanases are key enzymes in the degradation of xylan, a main component of plant cell walls. The activity of microbial xylanases can be controlled by XIPs localized at the plant cell wall [57]. The potential of XIPs in plant protection was demonstrated by the constitutive expression of the *Triticum aestivum* xylanase inhibitor III (TAXI-III) in GM wheat. TAXI-III delayed symptoms caused by *Fusarium graminearum* in GM wheat by direct competitive inhibition and counteraction of the *F. graminearum* necrotic xylanase activity [47,57,58].

Transgenic soybean hairy roots expressing elevated levels of a *Glycine max* XEGIP (GmGIP1), an inhibitor of a *Phytophthora sojae* xyloglucan-specific endoglucanase (PsXEG1), decreased *P. sojae* biomass and oospore production [59]. Recently, studies demonstrated that the expression of pectin methylesterase (PME) inhibitors in Arabidopsis could prevent damage to the plant cell wall during *Botrytis cinerea* infection [60]. The Arabidopsis PME inhibitors were strictly regulated by jasmonic acid and ethylene signaling as well as PME-related DAMPs, such as oligogalacturonides and methanol.

Therefore, the development of GM plants expressing CWDE inhibitors can be a promising strategy to obtain durable and broad reduced susceptibility to pathogens, as cell wall components represent an evolutionary conserved defense mechanism for pathogen CWDEs to overcome.

Table 1
Examples of biomolecules (proteins, secondary metabolites and snciRNAs) arising from plant-pathogen interactions applied to developing disease-resistant genetically modified (GM) plants.

Biomolecule[a]		Biomolecule gene[a][reference]	Less susceptible/Resistant GM plant (gene[a]) target pathogen [reference]
Type	Sub-type		
Plant inhibitors of pathogen-encoded CWDEs	PGIPs	pepper *PGIP* [56]	GM tobacco (pepper *PGIP*)/ *Phytophthora capsici* [56]
	XIPs	*Triticum aestivum* xylanase inhibitor III (*TAXI-III*) [57]	GM durum wheat (*TAXI-III*)/ *Fusarium graminearum* [57,58]
Plant innate immunity receptors	Plant PRRs (RLKs)	*Nicotiana benthamiana FLS2* (*NbFLS2*) [68]	GM Hamlin sweet orange and GM Carrizo citrange (*NbFLS2*)/ *Xanthomonas citri* [68]
	Plant PRRs (RLPs)	*N. benthamiana NbCSPR* [69]	GM Arabidopsis (*NbCSPR*)/ *Pseudomonas syringae* [69]
	R proteins (NB-LRR)	pepper *Bs2* [105]	GM tomato (pepper *Bs2*)/ *Xanthomonas perforans* [105]
Plant S proteins	Susceptibility factors	tomato *eIF4E* [79]	GM Arabidopsis (*Arabidopsis* mutant *eIF4*)/ *Cucumber mosaic virus* and *Turnip crinkle virus* [82]
	Negative defense regulators	Apple *MdMLO19* [91]	GM apple (loss-of-function of *MdMLO19*)/ powdery mildew (*Podosphaera leucotricha*) [91]
Plant antimicrobial defense proteins	AMPs	various plant encoded *defensins* (*PR-12*), *thionins* (*PR-13*), *lipid transfer proteins* (*PR-14*), *snakins*, *cyclotides*, *knottins* and *hevein-like proteins* [113]	GM tomato (tomato *snakin-2*)/ *Clavibacter michiganensis* [116]
	PR proteins	17 families of plant-encoded PR protein genes named *PR-1* to *PR-17* [117]	GM potato (tobacco *PR-5 osmotin*)/ *Phytophthora infestans*, *Fusarium solani* and *Rhizoctonia solani* [122].
	RIPs	maize modified RIP *MOD1* [140]	GM rice (maize modified RIP *MOD1*)/ *Rhizoctonia solani* [140]
Pathogen effector proteins	Non-RSSs	*Phytophthora* spp *CRN* [112]	GM *Nicotiana benthamiana* (*Phytophthora sojae CRN*)/ *Phytophthora capsici* and *Phytophthora parasitica* [112]
Plant defensive secondary metabolites	Phytoanticipins	various *Brassicae* plants *saponins* that require the enzyme *beta-amyrin synthase* for biosynthesis [159]	GM grass plants (*Avena strigosa beta-amyrin synthase*)/ *Gaeumannomyces graminis* vars *tritici* and *avenae*, *Fusarium culmorus*, *F. avanaceum*, *Stagonospora nodorum* and *S. avenae* [156]
	Phytoalexins	various plant stilbene-derived resveratrol that requires a single enzyme (*stilbene synthase*) for biosynthesis [163]	GM tobacco (grapevine *stilbene synthase*)/ *Botrytis cinerea* [163]
RNAi-related biomolecules	siRNAs	*Bean golden mosaic virus AC1* [174]	VIGS GM common bean (*Bean golden mosaic virus AC1*)/ *Bean golden mosaic virus* [174]
	miRNAs	*artificial miRNAs* based on the *Arabidopsis miR159* precursor that target both *Turnip yellow mosaic virus* (TYMV) and *Turnip mosaic virus* (TuMV) RSSs [176]	HIGS GM Arabidopsis (*artificial miRNAs* based on the *Arabidopsis miR159* precursor)/ TYMV and TuMV [176]
	RSSs	potyviral *HC-Pro* [182]	GM tobacco carrying the *R* gene *N* (potyviral *HC-Pro*)/ *Tobacco mosaic virus, Tomato black ring nepovirus* and *Peronospora tabacina* [182]

[a] Abbreviations (in alphabetical order): **AC**: replication-associated; **AMPs**: antimicrobial peptides; **Bs**: bacterial spot; **CRN**: crinkling and necrosis; **eIF**: eukaryotic translation initiation factor; **FLS**: flagellin-sensitive; **GM**: genetically modified; **HC-Pro**: helper component-protease; **HIGS**: host-induced gene silencing; **MdMLO**: *Malus domestica* mildew resistance locus O; **miRNAs**: microRNAs; **MOD**: modulation of locomotion defective; **NbCSPR**: *Nicotiana benthamiana* cold shock protein; **NbFLS**: *Nicotiana benthamiana* flagellin-sensitive; **NB-LRR**: nucleotide binding site-leucine-rich repeats; **PGIP**: polygalacturonase-inhibiting protein; **PR proteins**: pathogenesis-related proteins; **PRRs**: pattern recognition receptors; **R genes/proteins**: resistance genes/proteins; **RSSs**: RNA silencing suppressors; **RIPs**: ribosome-inhibiting proteins; **RLKs**: receptor-like kinases; **RLPs**: receptor-like proteins; **siRNAs**: small-interfering RNAs; **TAX**: *Triticum aestivum* xylanase inhibitor; **TuMV**: Turnip mosaic virus; **TYMV**: Turnip yellow mosaic virus; **VIGS**: virus-induced gene silencing; **XIPs**: xylanase inhibitor proteins.

2.2. Plant pattern recognition receptors

Unlike mammalian cells, which use both surface-localized and intracellular PRRs to perceive PAMPs or DAMPs, all plant PRRs are surface-localized and translocate to the plasma membrane via vesicles. Recognition of self-derived signals (i.e., DAMPs) or non-self-derived signals (i.e., PAMPs) by PRRs results in PTI resistance. PRRs comprise both the families of receptor-like kinases (RLKs) and receptor-like proteins (RLPs). They can contain an extracellular leucine-rich repeat (LRR), a lysine (LysM) domain, a lectin motif, or an epidermal growth factor (EGF)-like domain, which all recognize PAMPs and DAMPs. Although both receptor families have similar structures, RLPs lack the intracellular kinase domain present in RLKs [61,62]. Many of the plant PRRs identified are known to be involved in innate immunity [63,64].

A well-characterized PRR-ligand pair is the LysM-containing RLK known as chitin elicitor receptor kinase (CERK1) from Arabidopsis, which perceives fungal chitin oligomers [65]. Long-length chitin oligomers act as bivalent ligands, leading to chitin-triggered CERK1 homodimerization, which initiates chitin-induced PTI [65]. Another well-studied PRR is the Arabidopsis extracellular LRR-containing RLK flagellin sensing 2 (FLS2), which recognizes and directly binds the 22-amino acid epitope of the N-terminus of bacterial flagellin (flg22). Upon recognition, a heterodimer is formed between FLS2 and the regulatory LRR-containing RLK BAK1/SERK3 to induce downstream PTI [66]. In rice, the LysM-containing RLP chitin-elicitor binding protein (CEBiP) homodimerizes and hetero-oligomerizes with CERK1 to bind long chitin oligomers and activate PTI [67].

The genetic engineering of disease-resistant plants overexpressing PRRs is a promising strategy to generate GM crops with broad and durable resistance [10]. For instance, the *Nicotiana benthamiana FLS2* (*NbFLS2*) gene was transgenically expressed in Hamlin sweet orange and Carrizo citrange [68]. Transgenic lines overexpressing *NbFLS2* were resistant to *Xanthomonas citri*. The *N. benthamiana* RLP required for the recognition of the Csp22 peptide derived from bacterial cold shock protein was isolated and termed receptor-like protein required for Csp22 responsiveness (NbCSPR) [69]. GM Arabidopsis expressing NbCSPR presented responsiveness to Csp22 and resistance to *Pseudomonas syringae* [69].

The use of PRR transgenes from unrelated botanic species represents a promising approach to enhance the durability of GM resistance to fungal and bacterial pathogens. For example, the Arabidopsis PRR *AtEFR* transgenically expressed in tomato [70], the tomato PRR *Ve1* transgenically expressed in Arabidopsis [71] and the Arabidopsis PRR *AtEFR-Tu* transgenically expressed in wheat [72] are expected to increase the durability of disease resistance, as the transgene is from an unrelated heterologous plant species source.

Since PAMP molecule structures are conserved across different microbe species, individual PRR genes are thought to confer broad resistance against multiple pathogens [73]. However, adapted pathogens may evolve PAMPs that circumvent the plant PRR-mediated perception by altering key residues involved in the PRR-PAMP interactions [74]. In addition, the interfamily transfer of a new PRR to a particular plant species may boost PTI responses via additional PTI activation signaling from the new PAMP/PRR recognition system [75]. Therefore, PRR genetic engineering has emerged as a promising tool in engineering durable plant disease resistance, since the new non-host PRR signaling resistance pathways are more difficult to overcome by pathogen PAMPs that are specialized to a particular host [70].

2.3. Plant susceptibility proteins

Bacterial, virus and filamentous pathogens effectors modulate and suppress PTI by binding to S proteins and triggering ETS. Plant S proteins are effector targets (also known as virulence targets) that play positive or negative roles in plant immunity depending on the presence or absence of the cognate R protein [76]. Thus, S proteins function either as susceptibility factors that promote infection and disease development (positive regulator of plant immunity) or as negative defense regulators of the plant immune system that suppress plant defense (negative regulator of plant immunity). In a sense, any plant gene required for plant host susceptibility to pathogens and ETS establishment, supporting compatibility, may be called an *S* gene.

Functionally, *S* genes encode proteins required by pathogens either for their growth in the host plant or for the negative regulation of the plant immune system, which has been found occur during three different stages of the infection: (i) early pathogen establishment, allowing basic compatibility during pre-penetration; (ii) modulation of host defenses, including negative regulators of immune signaling; and (iii) pathogen sustenance, allowing sustained compatibility during post-penetration [15,76]. Many examples of *S* genes that have the potential to be used in resistance breeding are reviewed in [15].

When a wild-type dominant *S* gene, which *in planta* normally enhances susceptibility through ETS, suffers natural/directed loss-of-function mutations, the resulting mutant *S* gene may contribute to reduced susceptibility or recessive resistance [76].

2.3.1. Susceptibility factors

Some plant effector targets function as susceptibility factors, which are required for pathogen growth and development, such as plant translation factors used for potyviral replication *in planta*. Loss of function of susceptibility factors does not alter normal plant development [76]. Natural occurrences of mutant recessive *S* genes that encode *translation initiation factors 4E* and *4G* (eIF4E/eIF4G) have been reported to be effective against *Potyviridae* viruses [15,77–81]. These eIF4E/eIF4G factors interact with the RNA cap structure of transcripts, either from the plant or from the virus, to allow translation. Because potyvirus transcripts do not present a cap structure but instead possess the viral-encoded protein *Virus-protein genome linked* (VPg), upon potyviral infection, the plants shut off cap-dependent translation and only allow the cap-independent translation of potyviral transcripts through the factors eIF4E/eIF4G. Therefore, mutations in the plant *S* genes encoding eIF4E/eIF4G can lead to recessive resistance to some plant virus genera that encode VPg. *Arabidopsis thaliana eIF4* genes with loss-of-function mutations resulted in deficient eIF4 factors that, once transgenically expressed in GM Arabidopsis, conferred resistance to *Cucumber mosaic virus* (CMV) and *Turnip crinkle virus* (TCV) due to impairment of viral movement within the plant (Table 1) [82]. Another example of a plant recessive resistance *S* gene is the Arabidopsis *rwm1* gene, which confers resistance to several isolates of *Watermelon mosaic potyvirus* (WMV) [83]. The rwm1 factor is a nucleus-encoded chloroplast phosphoglycerate kinase that impairs viral accumulation in infected leaf tissues.

Xanthomonas oryzae causes devastating disease in rice by exploiting the plant *S* gene *OsSWEET14* through the *transcription activator-like* (TAL) effector protein AvrXa7. AvrXa7 binds to the OsSWEET14 promoter region and activates transcription of the sucrose efflux transporter, facilitating nutrient availability for bacterial cells and promoting disease [84]. Genome editing-mediated gene knockout was successfully used to edit the promoter region of the *OsSWEET14S* gene. Hence, the TAL effector AvrXa7 could neither bind to the *S* gene promoter nor induce OsSWEET14 expression, which resulted in reduced plant susceptibility [85,86]. However, restriction of pathogen growth caused by sugar limitation in the apoplast can affect plant growth as well. This study demonstrates that effector genes can activate the expression of susceptibility factors and that TAL effector technology seems to be effective for *S* gene identification as well as for genome editing.

2.3.2. Negative defense regulators

Plant immune responses are suppressed in the absence of a pathogen threat. However, some effector targets that are activated by pathogen effectors to promote disease play a role as negative regulators of plant immunity [15]. The activity of these *S* genes increases susceptibility. Loss of function of negative regulators is sometimes accompanied by pleiotropic effects associated with constitutive defense activation [76].

The most well-characterized examples of such negative defense regulators are the mildew resistance locus O (*MLO*) alleles, which encode plasma membrane S proteins that negatively regulate disease resistance to powdery mildew infections from *Erysiphales* spp [87,88]. Naturally, occurring recessive mutations in barley or tomato *MLO* genes confer pre-invasion resistance in these plants against a variety of powdery mildews, with no deleterious pleiotropic effects for the plants [89]. Apparently, loss-of-function mutations in *MLO* genes convert a compatible interaction between the host plant and powdery mildew into a non-host resistance-like incompatible interaction. This new non-host-pathogen interaction was able to confer broad and durable resistance against many powdery mildews in the field for decades [90]. Because MLO protein families are present in several crops, the engineered inactivation of the *MLO* gene by gene silencing and targeted or nontargeted gene knockout can potentially confer immunity to several powdery mildews [88]. Knockdown of the *MdMLO19* (*Malus domestica MLO 19*) *S* gene in GM apple reduced the disease severity of powdery mildew (*Podosphaera leucotricha*) by 75%, with no obvious negative phenotype observed in *mlo* knockdown plants (Table 1) [91]. Another example of successful cross-species resistance to powdery mildew was demonstrated by the antisense expression of the peach mildew resistance locus O (*PpMLO1*) gene in *Fragaria X ananassa* [92]. However, a tradeoff between *MLO* resistance to one pathogen and increased susceptibility to another has been observed, depending on the plant genetic background and environmental conditions [93,94].

Notably, for a mutant *S* gene to confer actual recessive resistance in GM plant contexts, some aspects must be considered: (i) it is necessary that the mutation of *S* gene does not cause serious pleiotropic effects (e.g., plant dwarfing); (ii) the level of recessive resistance ought to be quantitatively/qualitatively improved; and (iii) in case the S protein is encoded by multiple (redundant) *S* genes, it must be feasible to target multiple genes and combine multiple alleles [15,93].

Since the recessive *S* gene-mediated resistance is horizontal (i.e., it acts against various strains/pathovars/races of a specific pathogen species), it tends to be broader and more durable than the dominant *R* gene-mediated resistance (which is strain/pathovar/race-specific) [15].

3. Biomolecules involved in the ultimate layer of plant immunity: ETI

3.1. Plant resistance proteins

ETI is triggered following the perception of pathogen Avr proteins/

effectors by the cognate plant R protein, resulting in the expression of plant physiological defense reactions, which frequently culminates in HR and in induced distal resistance responses called LAR and SAR [22–25].

Functional domains that are commonly present in R proteins include Toll interleukin-1 receptor (TIR), nucleotide-binding site (NBS), leucine-rich repeat (LRR), LRR trans-membrane domain (TrD), coiled-coil (CC), nuclear localization signal (NLS), mitogen-activated protein kinase (MAPK) and MAPK kinase (MAPKK) domains. Based on the arrangement of these functional domains, R proteins have been classified into eight major groups: (i) the TIR-NBS-LRR or TNL group; (ii) the CC-NBS-LRR or CNL group; (iii) the LRR-TrD group; (iv) the LRR-TrD-kinase group; (v) the LRR-TrD protein degradation domain or LRR-TrD-PEST group; (vi) the TrD-CC group; (vii) the TNL-NLS group; and (viii) enzymatic groups such as the MAPK and MAPKK groups [95]. The largest R protein subset comprises the NBS-LRR proteins (from the TNL and CNL groups) [96]. The LRR C-terminal domain is highly variable among the different R proteins, functioning as the main region that determines the specificity for different Avr molecules. The R-Avr interaction rarely occurs through direct contact of the R receptor with the Avr factor but instead occurs indirectly through other host-specific proteins that play a role as effector targets/decoys/guards [13,97]. Due to structural similarities, *R* genes may be adapted from more ancient *PRR* genes [98].

Although *R* genes are pathogen/pest species-specific, some *R* genes have been demonstrated to function against different types of pathogens. For instance, the tomato *Mi* gene (from the CNL group) confers resistance to root-knot nematodes (*Meloidogyne* spp.), potato aphids (*Macrosiphum euphorbiae*), and sweet potato whitefly (*Bemisia tabaci*) by using common components of defense signaling against diverse pests [99]. Moreover, Arabidopsis *RRS1* and *RPS4* genes act as a dual *R* gene system against fungal and bacterial pathogens, including *Ralstonia solanacearum* and *Colletotrichum higginsianum*. In addition, *RPS4-Ws* is a well-characterized *R* gene that confers resistance to the *P. syringae* pv. *tomato* strain DC3000. The report that RRS1 and RPS4 function together to confer resistance to bacterial wilt in GM Brassica crops provides insights into a novel strategy for the development of a plant species resistant to multiple pathogens through the expression of heterologous plant genes [100].

Some plant R proteins that act against viruses have an identified corresponding Avr molecule, which is usually a viral *coat protein* (CP), replicase, movement protein (MP) or another viral-encoded factor. For instance, the tomato Sw5 receptor perceives the *Tomato spotted wilt virus* NSm MP [101], the Arabidopsis HRT receptor recognizes the TCV CP [102], and the kidney bean RT4-4 receptor senses the CMV 2a RSS [103]. Among the hundreds of *R* gene-Avr pairs studied to date, only a few tens of *R* genes have been cloned and are appropriate for testing in plant genetic engineering for protection against phytoviruses [20].

Several plant *R* genes conferring resistance against pathogenic bacteria have been identified [14,95,104], some of which have been validated in the context of GM plants. For instance, successful field trials of GM tomato plants expressing *Bs2*, an *R* gene from pepper that recognizes AvrBs2 present in some *Xanthomonas campestris* pathovars, demonstrated robust resistance against *Xanthomonas perforans* [105]. This example reinforces the efficiency of heterologous gene expression systems using related solanaceous plants and supports the use of the *Bs2* gene as a source of resistance to different *Xanthomonas* species and *X. campestris* pathovars in multiple solanaceous species.

Numerous plant *R* genes and their cognate fungal Avr factors have already been elucidated in various pathosystems [95,106], including *Hordeum vulgare-Blumeria graminis; Solanum lycopersicum-Fusarium oxysporum; S. lycopersicum-Cladosporium fulvum*; *Oryza sativa-Magnaporthe grisea;* and *Zea mays-Puccinia sorghi*.

The engineering of GM plants that overexpress R proteins is a plausible strategy for crop protection. Nevertheless, R protein-based GM resistance may not be durable due to the strain/race level of specificity against the pathogen. However, durable and broad-spectrum disease resistance against different species and subspecies of pathogens may be achieved in GM plants by the heterologous expression of an *R* gene in phylogenetically related plant species, for example, a *Bs2* gene from pepper expressed in GM tomato, both solanaceous plants [105]. Alternatively, *R* genes may successfully function in non-related plant species by co-transformation with the cognate *Avr* factor. Moreover, once individual *R* genes can be overcome by phytopathogens, *R* gene stacking strategies may potentially confer more durable and broad resistance against pathogens by mimicking the non-host status of a plant species to a non-adapted pathogen species [107,108].

The generation and expression of mutant host receptors is a feasible strategy for engineering resistance. The extremely high specificity of R protein-Avr factor interactions tremendously limit the range of action against different pathogen species and even against populations within the same pathogen species. In this sense, induced mutation mechanisms in NBS-LRR protein domains, resulting in new binding specificities and fine-tuning the strength of defense response, have already been successfully demonstrated and may be adapted to engineer novel resistance genes that can be deployed in agriculture [109]. For instance, artificial evolution was achieved for the *Solanum tuberosum R3a* gene (from the CNL group), which confers resistance to the *P. infestans* Avr3a^{KI} isolate but not to the Avr3a^{EM} isolate. Thus, *N. benthamiana* expressing R3a* mutant variants presented significantly improved recognition of AVR3a^{EM} [110]. Such application of biotechnological plant protection strategies based on mutant *R* genes may therefore increase the possibilities of developing durable plant resistance to phytopathogens under field conditions, with *R* genes recognizing pathogens and/or strains that overcame the original *R* gene.

3.2. Pathogen effector proteins

In addition to plant-derived proteins, plant-pathogen interactions involve a plethora of pathogen-encoded proteins called effectors, which are molecules that modulate plant metabolism. Effectors act as virulence factors sustaining pathogen growth in a susceptible host or inducing HR in a resistant host plant. Effectors from filamentous phytopathogens are classified as secreted effectors that target sites in the host plant apoplasts and as cytosolic effectors that are delivered inside the host cell [8,111]. It has recently been reported that the *Phytophthora* spp. *crinkling* and *necrosis* (CRN) cytosolic effectors are targeted to plant nuclei, where they disturb host nuclear processes. The expression of the *P. sojae* CRN effector PsCRN115 in *N. benthamiana* GM plants significantly up-regulated defense responses, such as ABC transporters, Cytochrome P450 and PRRs, and increased the level of plant resistance to *P. capsici* and *P. parasitica* oomycetes. In addition, PsCRN115 improved plant tolerance to salt and drought stresses [112]. Hence, CRN effectors could be directly used as functional genes in GM plant strategies to enhance tolerance to both biotic and abiotic stresses.

4. Biomolecules involved in plant defense PTI/ETI responses

The synthesis of plant antimicrobial defense proteins may be constitutive or induced by PAMP-PRR recognition (PTI) or by the interactions of the Avr-R proteins (ETI). Plants produce antimicrobial defense proteins of various classes, such as antimicrobial peptides (AMPs), pathogenesis-related proteins (PR proteins), and RIPs. Plant antimicrobial defense proteins that present activity in response to phytopathogens have been commonly used to develop disease-resistant GM plants, as these defense proteins usually confer broad-spectrum resistance.

Plant AMPs are promising antibiotic molecules for biotechnological applications, in particular against bacterial and filamentous phytopathogens, though more rarely against phytoviruses. Importantly, signal peptides that drive the delivery of antimicrobial plant defense proteins toward the site of action must be considered in GM plant

engineering strategies.

4.1. Antimicrobial peptides

AMPs are plant defense peptides that are up to 100 amino acids in length, with the majority ranging from 10 to 50 amino acids. AMPs exhibit structural and functional diversity, exerting antimicrobial activity against different phytopathogens through various mechanisms. The main families of plant AMPs include defensins, thionins, lipid transfer proteins, snakins, cyclotides, knottins and hevein-like proteins (Table 1) [113].

Various plant AMPs have been used in the development of GM plants with resistance to various phytopathogenic bacteria [114]. Wheat thionin expression in Arabidopsis was shown to reduce a *P. syringae* population when compared with wild-type control plants [115]. Similarly, overexpression of oat thionin in *Japonica* rice plants reduced susceptibility to infections caused by *Burkholderia plantarii* and *B. glumae*. The snakin-2 protein expressed in GM tomato (*S. lycopersicum*) conferred resistance to *Clavibacter michiganensis* [116].

4.2. Pathogenesis-related proteins

PR proteins accumulate in diseased plants and directly or indirectly participate in plant defense against pathogens [117]. To date, a total of 17 PR protein families have been described (PR-1 to PR-17), of which three are also AMPs, namely, PR-12 (defensins), PR-13 (thionins) and PR-14 (lipid transfer proteins) [117]. A total of 10 PR proteins act directly on filamentous phytopathogens (PR-1, PR-2, PR-3, PR-4, PR-5, PR-8, PR-11, PR-12, PR-13, and PR-14), and the PR-1 and PR-5 families are also active against oomycetes [117,118].

The PR-5 proteins, called osmotins, act as membrane permeabilizers and glucan-binding proteins that participate in programmed cell death and glucan hydrolysis, respectively [117,119–121]. GM potato plants expressing a tobacco PR-5 osmotin were resistant to the oomycete *Phytophthora infestans* and both *Fusarium solani* and *Rhizoctonia solani* fungi, [122]. GM soybean plants expressing the tobacco osmotin Tbosm were tolerant to salinity stress and to *Microsphaera diffusa, Septoria glycines* and *P. pachyrhizi* infections [123]. Similarly, the *Prunus domestica* PR-5 (PdPR5-1) protein, ectopically expressed in Arabidopsis GM lines, activated defense response pathways and triggered phytoalexin production and resistance to the fungal pathogens *Monilinia fructicola* and *Alternaria brassicicola* [124].

Members of the PR-2 protein family are glucanases that hydrolyze glucans present in the cell wall of filamentous phytopathogens. Members of the PR-3*R*-3, PR-4, PR-8 and PR-11 protein families are endo-chitinases that hydrolyze chitin from fungal cell walls [119,125,126]. The heterologous overexpression of a rice chitinase gene in GM peanut conferred a high level of resistance to *Cercospora arachidicola* and *Aspergillus flavus* [127].

AMPs from the PR-12, PR-13, and PR-14 protein families, classified as defensins, thionins and lipid transfer proteins, respectively, act as putative membrane permeabilizers with antifungal and antibacterial activities [126,128–131]. When the AFP2 defensin from *Raphanus sativus* was expressed in GM rice, it conferred resistance to *Magnaporthe oryzae* and *R. solani* fungi [132].

On the other hand, fungi and oomycetes can neutralize plant resistance by secreting proteins that inhibit plant antimicrobial defense factors [133]. For instance, *Phytophthora sojae* secretes chitinase- and glucanase-inhibiting proteins (GIP1 and GIP2) that inhibit the soybean glucanase EGaseA (a PR-2 protein) [134].

Since secreted plant antimicrobial defense proteins participate in the first constitutive and inducible lines of defense, the expression of more than one antimicrobial plant protein within the same GM plant may also contribute to durable and high disease resistance. In this context, antimicrobial gene pyramiding was engineered for the co-expression of a bacterial chitinase from *Streptomyces griseus* and a plant defensin (AMP and PR-12 protein) from *Wasabia japonica* in re-transformed *S. tuberosum* plants presenting a high level of resistance against the fungal pathogens *F. oxysporum* and *Alternaria solani* [135]. Similarly, GM tobacco expressing pyramided protease inhibitor genes (PR-6 protein) exhibited dual resistance against insects and phytopathogens [136].

4.3. Ribosome-inhibiting proteins

RIPs are proteins that display N-glycosidase activity on RNA, hydrolyzing a glycosidic bond and removing an adenine residue in a highly conserved sequence at the 3′ terminal region of the ribosomal RNA (rRNA) in the 23/25/28S ribosomal subunits. As such, these proteins may irreversibly inactivate ribosomes and hamper protein synthesis in targeted phytopathogens [137]. The RIP-targeted adenine is responsible for the interaction of the ribosome with translation elongation factor 2 in eukaryotes and factor G in prokaryotes. The specificity of RIP interactions varies depending on their substrates: certain RIPs remove a single adenine, while others depurinate both RNAs (including poly-adenine tail-containing RNA and viral RNA) and DNA. Therefore, RIPs are also termed as polynucleotide:adenosine glycosidases [138].

Based on structural diversity, plant RIPs are grouped into three types: (i) type I RIPs have a single RNA N-glycosidase domain; (ii) type II RIPs present an RNA N-glycosidase domain (A chain) linked via a disulfide bond to a D-galactose-binding lectin domain (B chain); and (iii) type III RIPs possess an N-terminal domain similar to the type I RIPs linked through a disulfide bond to a C-terminal domain of unknown function. RIPs exhibit potent *in vitro* and *in vivo* (in GM plants) antifungal and antiviral activities [139]. The maize modified RIP *MOD1* gene was used for the successful development of GM rice resistant to *R. solani* (Table 1) [140], indicating the potential of RIPs for engineered crop disease protection. Moreover, ectopic expression of the well-characterized maize endosperm RIP1 (b-32), classified as type III, in GM maize lines diminished *Fusarium verticillioides* symptoms in leaf tissue assays. RIP1 (b-32) was also shown to reduce head blight caused by *Fusarium culmorum* in GM wheat [141,142].

4.4. Enzymes involved in plant defensive secondary metabolite biosynthesis

Plants are collectively able to produce more than 100,000 secondary metabolites, which constitute a conserved plant innate immunity framework [143–145]. Plant secondary metabolites, also referred as phytochemicals, comprise a large group of structurally diverse compounds that are produced from various primary metabolites or their biosynthetic intermediates and are either constitutively produced or induced by various environmental stimuli [146].

For the efficient contribution of plant secondary metabolites to immunity, it is necessary that active biomolecules accumulate at appropriate times and concentrations in the correct subcellular site. Moreover, as several secondary metabolites that are involved in plant immunity can be toxic, the generation of such phytochemicals in tissues that are not challenged by pathogens would certainly be biologically detrimental to the plant. Thus, constitutive defensive secondary metabolites are usually separated from their activating hydrolytic enzymes. For instance, benzoxazinone glucosides accumulate in vacuoles, whereas their cognate activating glucosidases are housed in plastids [147]. Certain defensive secondary metabolites, instead of being constitutively expressed, are induced and activated by the recognition of PAMPs through PRRs [148]. Therefore, the subcellular site, concentration, timing and activation of plant defensive secondary metabolites represent major challenges to engineering GM plant disease resistance.

Although *in vitro* antimicrobial activity has been demonstrated for numerous plant defensive secondary metabolites, antimicrobial modes of action are far less understood. Saponins, however, represent an

exception, as their membranolytic activity on pathogenic fungi is relatively well understood [146]. Interestingly, in addition to the antimicrobial activity of defensive phytochemicals, some secondary metabolites have been demonstrated to modulate plant immunity. For example, products of indolic glucosinolate metabolism are signaling molecules that trigger callose deposition [149]. Other examples are the aliphatic glucosinolate-derived isothiocyanates(ITCs), which are required for ETI HR cell death [150]. Moreover, ITCs also induce stomatal closure, which probably hampers the pathogen infection of plant tissue [151]. For these reasons, engineered production of defensive secondary metabolites in GM plants, by transgenic expression of a key enzyme for the synthesis/activation of the cognate metabolite, is a promising strategy to confer broad and durable disease resistance. Conversely, expression of defensive secondary metabolites that involve multiple synthesis enzymes is a challenge to engineering GM disease-resistant plants.

Defensive secondary metabolites are mainly divided into phytoanticipins, which are constitutively produced and stored in plants, and phytoalexins, which are induced in response to pathogen infection [146]. Glucosinolates, cyanogenic glucosides, benzoxazinone glucosides and saponins are among the phytoanticipins that have been investigated in greater detail. Phytoalexins that have been highlighted in the literature include terpenoids and phenylalanine-derived phytoalexins.

4.4.1. Phytoanticipin biosynthesis

Glucosinolates are amino acid-derived phytoanticipins mainly produced by Brassica plants, which accumulate mostly methionine-derived aliphatic glucosinolates and tryptophan-derived indolic glucosinolates [152]. These biomolecules are biologically inactive until some loss of cell integrity initiates their hydrolysis into chemically unstable aglycones that decompose into various molecules, including ITCs [152]. The chemical reactivity of ITCs is associated with their antimicrobial activity, as demonstrated against the bacterial pathogen *P. syringae* [153]. Pathogen infection in Arabidopsis triggers the CYP81F2/PEN2-dependent hydrolysis of indolic glucosinolates into active antimicrobial biomolecules, resulting in broad resistance to several fungal and oomycetal pathogens [146]. Cyanogenic glucosides, such as glucosinolates, are constitutively stored as inactive biomolecules that must be hydrolyzed to exert their antimicrobial activity [154]. Benzoxazinone glucosides are mostly produced by grass families (*Poaceae* plants), including maize, wheat and barley [147]. In grass plants, benzoxazinone glucosides are hydrolyzed into aglycones to exert their antifungal activity against a broad range of species [146].

Saponin phytoanticipins are glycosides whose contribution to plant immunity against three fungal species, namely, *Gaeumannomyces graminis*, *F. culmorum* and *Fusarium avenaceum*, has been validated in oat using genetic data [155–160]. Beta-amyrin synthase is involved in saponin biosynthesis in plants. The full genomic sequence of a beta-amyrin synthase was cloned from *Avena strigosa* and transgenically expressed in grass plants, resulting in resistance to fungal pathogens with sterol-containing membranes, namely, *G. graminis* vars *tritici* and *avenae*, *Fusarium culmorum*, *F. avenaceum*, *Stagonospora nodorum* and *Stagonospora avenae* (Table 1) [156].

On the other hand, some fungi have enzymes that can detoxify the saponins produced by plants. *Gaeumannomyces graminis* mutants generated by targeted gene disruption of a saponin detoxifying enzyme no longer able to infect the saponin-containing host oats but retained full pathogenicity to wheat (which does not contain saponins) [161]. This study was able to prove that these enzymes may be a source of resistance of certain fungal populations to this secondary metabolite. Thus, it is possible to engineer GM crops that can produce high levels of saponin and concomitantly silencing the saponin detoxifying enzyme gene by RNA interference.

4.4.2. Phytoalexin biosynthesis

Terpenoid phytoalexins are produced at high levels by rice and maize, but their biosynthesis involves complex pathways, with more than 20 associated genes identified to date [162], which makes the engineering of GM plants to express terpenoids a challenge. A remarkable representative of the phenylalanine-derived phytoalexins is resveratrol. Resveratrol biosynthesis requires a single enzyme (stilbene synthase) to make metabolic engineering feasible: the heterologous expression of stilbene synthase in tobacco plants led to pathogen-inducible biosynthesis of resveratrol and resistance to *B. cinerea*. This study was the first report of disease resistance resulting from foreign phytoalexin expression in a heterologous plant [163,164]. The expression of a stilbene synthase allele from a Chinese wild grapevine in Arabidopsis as a heterologous system could confer resistance to the powdery mildew *Golovinomyces* [165].

5. Regulation of plant immunity by RNA interference (RNAi)

5.1. Virus-induced gene silencing (VIGS) and host-induced gene silencing (HIGS)

In the past decade, researchers have developed an exceptional interest in RNAi technology. RNAi has been extensively used as a key strategy for functional genomics through gene silencing. RNAi pathways regulate PTGS and TGS during the complex plant-pathogen interactions through the production of snciRNAs by both plants and pathogens [33,34,166]. Most plant viruses possess an RNA genome and generate long dsRNA molecules upon the production of replication intermediates in the plant cell by virus-encoded RdRPs. These viral replication intermediates are recognized by the plant DCLs that generate virus-derived primary snciRNAs. Secondary viral snciRNAs are generated upon amplification directed by plant-encoded RdRPs, and dicing is subsequently performed by plant-encoded DCLs. Both primary and secondary viral snciRNAs are loaded into plant AGOs, which promote antiviral defense through RNA-mediated PTGS of the cognate virus [167,168]. This process is called virus-induced gene silencing (VIGS) [169]. Several studies show that VIGS is a widely applied RNAi strategy in the development of GM plants resistant to viruses, based on the engineered production of siRNAs that silence the viral RNA genome [170]. To use VIGS as an RNAi-based alternative to engineering GM plants resistant to viruses, a cassette encoding a self-complementary small hairpin dsRNA (intron-spliced) is designed to form inverted repeats of the viral RNA genome, mimicking viral siRNA. Similar to VIGS, which involves the delivery of virus-encoded snciRNAs to plant hosts, the transfer of snciRNAs from filamentous phytopathogens to plant hosts is called filamentous pathogen-induced gene silencing (FIGS) [169,171]. VIGS has been successfully applied to generate GM crops resistant to viruses, for instance, by silencing viral coat transcripts, such as GM cantaloupe melon resistant to *Papaya ring spot virus* [172] and GM plum resistant to *Plum pox virus* [173]. RNAi has also been successfully used against DNA viruses, such as in GM common bean resistant to *Bean golden mosaic geminivirus* (Table 1) [174].

Not only do pathogens deliver siRNAs into the plant host, but GM plants may be engineered to express artificial miRNAs (amiRNAs) targeting specific pathogen genes [175]. This biotechnological approach, termed host-induced gene silencing (HIGS), has emerged as a promising strategy for plant protection as it combines high selectivity for the target pathogen with minimal side effects compared with chemical treatments. [169]. HIGS has been used to confer plant resistance to viruses by generating miRNAs targeting viral RSSs. For example, GM Arabidopsis expressing an amiRNA based on the Arabidopsis miR159 precursor was resistant to *Turnip yellow mosaic virus* (TYMV) and *Turnip mosaic virus* (TuMV), as the amiRNA targeted both TYMV P69 RSS and TuMV HC-Pro RSS (Table 1) [176]. HIGS strategies using amiRNAs targeting viral genes involved in replication, transmission and virulence are equally promising RNAi-based strategies for GM plant protection.

For instance, an amiRNA targeting the coat protein (AV1) transcripts of the geminivirus *Tomato leaf curl virus* (ToLCV) conferred high tolerance in GM tomato plants [177].

The phytopathogenic bacteria *Agrobacterium tumefaciens* transfers oncogenes to plants, leading to the formation of tumors (also called galls) in the host and resulting in crown gall disease symptoms. Targeting of such agrobacterial oncogenes via HIGS has been successfully used to control *A. tumefaciens*: tomato plants expressing two self-complementary RNA constructs designed to silence two agrobacterial oncogenes, denoted iaaM and IPT, were resistant to crown gall disease [178].

There are reports of HIGS controlling filamentous pathogen infection. For instance, HIGS of the essential ergosterol biosynthetic genes from the sterol demethylase CYP51 family was successfully used to protect GM plants against Fusarium species [179]. Similarly, HIGS of the fungal glucanosyltransferase genes from *B. graminis* resulted in reduced symptoms in GM barley [180]. HIGS of the *B. graminis* effector Avra10 gene decreased the number of functional fungal haustoria in a barley genotype lacking the Avra10 cognate *Mla10 R* gene [180].

Notably, several miRNAs naturally suppress plant innate immunity receptors, such as PRRs and R proteins, when the plant is not challenged by pathogens. For example, the plant miRNA miR472 and the RdRP6 protein control the expression of plant host *PRR* and *R* genes: in non-infected plants, the expression of *PRR* and *R* genes is repressed by miR472/RdRP6-guided PTGS [181]. Upon plant infection with *P. syringae*, bacterial, PAMP and Avr molecules suppress the miR472/RdRP6 repression of their cognate PRRs and R proteins, resulting in promotion of plant resistance to the bacteria through PTI and ETI [181]. Thus, HIGS may be engineered in GM plants to promote PTI and ETI by overexpression of miRNAs that revert the repression of PRRs and R proteins into activation.

5.2. RNA silencing suppressors

In addition to mutual interference by exchange of snciRNAs (siRNAs and miRNAs) during plant-pathogen interactions [166], pathogens evolved to counteract plant RNAi-mediated gene silencing immunity mechanisms by expressing pathogen-encoded RNA silencing suppressors (RSSs) [40,41]. RSSs are pathogen effector proteins that promote disease caused by the pathogen of origin, while they may decrease susceptibility to unrelated heterologous pathogens [182]. Several viral RSSs, isolated from nearly all phytovirus families, target RNAi components at diverse points (e.g., AGO, DCL, RdRP, siRNAs) through various mechanisms [40,183,184]; for instance, P38 from TCV inhibits AGO1 [185], and V2 from *Tomato yellow leaf curl virus* binds to the suppressor of gene silencing 3 (SGS3; cofactor of the RdRP6), thereby hampering *de novo* dsRNA synthesis and RNAi amplification [186]. Similar to plant viruses, phytopathogenic bacteria also encode RSSs. Some of the bacterial effectors that are injected by *P. syringae* pv. tomato into plant cells have evolved to become RSSs: for example, the bacterial avirulence protein AvrPtoB suppresses the transcriptional activation of plant miRNAs (e.g., miR393) [187]. The first RSSs identified in a eukaryotic phytopathogen, PSR1 and PSR2, were isolated from the oomycete *P. sojae* and affect different endogenous plant PTGS pathways [188]. PSR1 targets endogenous miRNAs, whereas PSR2 hampers the expression of endogenous siRNAs. At present, no other RSSs have been characterized from filamentous pathogens, despite indirect evidence that *F. oxysporum* encodes RSSs [189]. In general, filamentous phytopathogen RSSs may be virulence factors if they hinder the silencing of the pathogen genome or may be defense elicitors if they block the negative regulation of host plant defense by miRNAs [169]. The reported data indicate that filamentous pathogen-encoded RSSs could be used to obtain disease-resistant GM crops.

When the potyviral RSS *helper component-protease* (HC-Pro) was expressed in GM tobacco, it increased susceptibility to a broad range of related viral pathogens, despite decreasing susceptibility to unrelated heterologous pathogens [182]. *Tobacco mosaic virus* (TMV) infection of HC-Pro-GM tobacco carrying the *R* gene resulted in fewer and smaller lesions compared with controls without HC-Pro [182]. HC-Pro-GM tobacco was also less susceptible to *Tomato black ring nepovirus* (TBRV) and to the oomycete *Peronospora tabacina* [182]. Thus, phytoviral RSSs may reduce susceptibility to multiple non-related-pathogens in GM plant contexts. The reported data indicate that pathogen-encoded RSSs could be used to develop GM crops with broad and durable disease resistance.

In conclusion, since RNAi pathways regulate the plant immunity gene expression, knowledge about RNAi mechanisms can be used to design several biotechnological approaches and strategies to develop disease-resistant GM plants, for instance, by suppressing pathogen genes (e.g., essential viral genes and phytopathogen virulence effectors) or by promoting the expression of innate immunity resistance genes (e.g., R proteins and PRRs). In the case of suppression of pathogen genes, despite not conferring broad resistance, the strategy confers sequence-specific, high and durable disease resistance to the GM plant. Moreover, the promotion of the expression of innate immunity resistance genes may result in broad and durable resistance as well.

6. Final remarks

Durable disease resistance is the goal of crop engineering science. A genetic modification strategy that uses genes involved in the co-evolutionary battle of specific pathosystems may lead to resistance breaking due to mutations in pathogen genes or simply due artificial selection of individuals within a pre-existing population of the pathogen. This phenomenon is already naturally observed in modern agriculture due to an artificial selection performed by conventional plant breeding. Naturally, several plant genes were reported to be counter-attacked by genes from co-evolving pests, such as plant chitinases and pathogen chitinase inhibitors, and vice-versa. Nevertheless, genetic modification strategies involving genes from heterologous sources (e.g., non-host plants or pathogens) and involving stacked layers of defense are more durable due to hampered co-evolutionary molecular recognition. In such cases, selection pressure may promote mutations in the pathogen that are deleterious and thus unlikely to occur.

Even with the identification and characterization of new biotechnological assets that potentially confer resistance to pathogens, only a few commercial GM crops resistant to viruses, bacteria and fungi/oomycetes were described. These data were initially revised by Wally and Punja (2010) and, at the time, only GM crops with resistance to viruses were described for commercialization [190]. Currently, according to ISAAA (International Service for the Acquisition of Agro-Biotech Applications), 28 GM crops are approved worldwide for commercialization presenting resistance to virus, and only three of them are combined for resistance to virus-fungus [191]. Several aspects can be considered a challenge for the generation of commercial biotech crops resistant to pathogens; three of them deserve special attention. The first aspect can be the high genetic variability of viruses, bacteria and fungi/oomycetes in regions of the genome that affect the ability of these pathogens to infect host plants. This genetic variation can reduce the magnitude of plant resistance and select pathogen populations resistant to new biotech assets. Another important aspect can be the possible undesirable pleiotropic effects conferred by some biotech assets. A good example could be the manipulation of elements of metabolic pathways that participate in the regulation of several development-related cellular routes and stress responses, such as early Ca^{2+} signaling dependent processes. The possible adverse pleiotropic effects caused by these assets could confer both significant reduction of biomass (and consequently yield reduction), and susceptibility to new classes of biotic and/or abiotic stresses. Finally, the speculative public perception of transgenic technologies, associated with the long period required from the development of the new GM crop until the commercial release, can also

be limiting factors by research groups in developing new biotechnological products.

The new technologies, such as “omics” and genome editing approaches, can overcome part of these different challenges by the characterization of more suitable biotech assets, since, up till now, no GM biotech product could be universally applied to increase viruses, bacteria, and fungi/oomycetes resistance. In this way, when the subject is plant breeding by genetic engineering, currently the tendency of research groups is to invest in the combination of technologies and traits more adequate for each situation, in order to engineer more specific and durable crop resistance, sometimes to more than one pathogen, without undesirable pleiotropic effects.

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Glossary

flg22: 22 amino acid epitope
AMPs: Antimicrobial proteins
AGO: Argonaute
amiRNAs: Artificial miRNAs
ACA: Autoinhibited Ca^{2+} ATPase
Avr factors: Avirulence
CDPK: Calcium-dependent protein kinase
CWDEs: Cell wall degrading enzymes
CERK1: Chitin elicitor receptor kinase
CIPs: Chitinase-inhibiting proteins
CEBiP: Chitin-elicitor binding protein
CC: Coiled-coil
CMV: Cucumber mosaic virus
cAMP: Cyclic AMP
CNGC: Cyclic nucleotide-gated channel
DAMPs: Damage-associated molecular patterns
dsRNA: Double-stranded RNA
ETI: Effector-triggered immunity
ETS: Effector-triggered susceptibility
FIGS: Filamentous pathogen-induced gene silencing
FLS2: Flagellin sensing 2
HIGS: Host-induced gene silencing
HR: Hypersensitive response
LRR: Leucine-rich repeat
LAR: Local acquired resistance
TrD: LRR trans-membrane *trans*-membrane domain
MAPKK: MAPK kinase
MAMPs: Microbe-associated molecular patterns
miRNAs: MicroRNAs
MLO: Mildew resistance locus O
MAPK: Mitogen activated protein kinase
RBOH: NAPDH oxidase
NO: Nitric oxide
NLS: Nuclear localization signal
NBS: Nucleotide-binding site
PTI: PAMP-triggered immunity
PAMPs: Pathogen-associated molecular patterns
PR proteins: Pathogenesis-related
PRRs: Pattern recognition receptors
PGIPs: Polygalacturonase-inhibiting proteins
PTGS: Post-transcriptional gene silencing
ROS: Reactive oxygen species
NbCSPR: Receptor-like protein required for csp22 responsiveness
R proteins: Resistance
RIPs: Ribosome-inhibiting proteins
DCL: RNA III-like endonuclease dicer-like
RNAi: RNA interference
RdDM: RNA-dependent DNA methylation
RdRPs: RNA-dependent RNA polymerases
SNO: S-nitrosylation
SPIPs: Serine proteinase-inhibiting proteins
ssRNA: Single-strand RNA
ssRNAs: Single-stranded RNAs
siRNAs: Small interfering RNAs
snciRNAs: Small-non-coding interfering RNA molecules
SGS3: Suppressor of gene silencing 3
SAR: Systemic acquired resistance
TIR: Toll interleukin-1 receptors
TGS: Transcriptional gene silencing
TCV: Turnip crinkle virus
VIGS: Virus-induced gene silencing
WMV: Watermelon mosaic potyvirus
TAXI-III: Xylanase inhibitor III
XIPs: Xylanase-inhibiting proteins
XEGIPs: Xyloglucan-specific endoglucanase-inhibiting proteins