Chapter

Pathogenesis-Related Proteins and Their Transgenic Expression for Developing Disease-Resistant Crops: Strategies Progress and Challenges

Anroop Kaur, Sukhpreet Kaur, Ajinder Kaur, Navraj Kaur Sarao and Devender Sharma

Abstract

Various pathogenic microorganisms (such as fungi, bacteria, viruses and nematodes) affect plant viability and productivity. However, plants combat these pathogens by inducing their defense mechanism to sustain their fitness. The aggregation of pathogenesis-related (PR) proteins in response to invading pathogens is a crucial component of a plant's self-defense mechanism. PR proteins induce innate resistance in plants through fungal cell wall disintegration, membrane permeabilization, transcriptional suppression, and ribosome inactivation. Earlier studies have demonstrated their crucial role in determining resistance against phytopathogens, making them a promising candidate for developing disease-resistant crop varieties. Plant genetic engineering is a potential approach for developing disease-resistant transgenic crops by employing several PR genes (thaumatin, osmotin-like proteins, chitinases, glucanases, defensins, thionins, oxalate oxidase, oxalate oxidases like proteins/germin-like proteins and LTPs). Furthermore, the overexpression of PR proteins enhances the resistance against phytopathogens. As a result, this chapter gives an overview of PR proteins, including their classification, functional characterization, signaling pathways, mode of action and role in defense against various phytopathogens. It also highlights genetic engineering advances in utilizing these genes singly or synergistically against various phytopathogens to impart disease resistance. Various challenges faced with the products of transgenic technology and synergistic expression of different groups of PR proteins were also discussed.

Keywords: biotic stress, pathogen-related proteins, plant genetic engineering, plant defense signaling

1. Introduction

With the rapid expansion in the world population, the area under cultivation has decreased [1]. Moreover, biotic stress has been a significant challenge for farmers since the dawn of agriculture. Global yield loss due to plant pathogens is estimated at 16% [2]. To overcome the economic loss in agricultural production, most research in this field focuses on protecting crops against pathogens, insect pests and nematodes. Crop production and productivity can be enhanced by significant breakthroughs in agricultural practices such as cultural controls, pesticide application, crop rotation, and plant breeding.

On the other hand, Pathogens frequently escape chemicals through strong selection and evolution, resulting in crop loss due to infection. Altering the genetic architecture of crops through breeding programmes is another option for crop protection, but it is a labor-intensive and time-consuming operation. In theory, genetic engineering, which refers to the use of biotechnology to alter an organism's genetic material directly [3], is a potential tool for improving disease resistance. Furthermore, genetic engineering can overcome the limitations of traditional breeding technology, including the introduction/alteration of specific genes with minimum undesirable changes to the rest of the genome; cross-species exchange of genetic material; and introduction of variations/genes into asexually propagated crops like bananas [4]. As a result, research studies have been directed toward the genes that impart long-term resistance to many pests or pathogens and are safe for consumption.

In plants, tolerance and susceptibility to a particular pathogen are determined by a complex interaction of signals and responses corresponding to specific environmental conditions. So, the major difference between resistant and susceptible varieties is the ability to recognize an invading pathogen and further activate host defense mechanisms. Plants have evolved various defense mechanisms, including activating both constitutive and inducible defense responses to combat the diseases. When pathogens are detected, immune receptors in plants recognize specific molecules that signal the activation of effective defense responses. Despite extensive research, details of host defense mechanisms that limit pathogenic infections have yet to be elucidated. The majority of defense responses are characterized by the transcriptional activation of a large number of genes (>1% of the genome), many of which have unknown functions [5, 6]. Pathogen identification activates signaling pathways that result in the formation of reactive oxygen species (ROS), protein kinases, phytohormones, phytoalexins, phenolic compounds and pathogenesis-related (PR) proteins, and eventually a hypersensitive response (HR). Production and accumulation of PR proteins, which are low molecular weight proteins, in plants during pathogen attack is vital [7]. In most plant species, nineteen families of PR proteins (PR-1 to PR-19) have been identified to date. The role of PR proteins in plant defense includes altering the integrity of pathogen and activating other defense pathways through the generation of elicitors.

Due to improvements in transformation techniques and isolation of numerous pathogenesis-related genes, plants can now be engineered to have effective and broad-spectrum resistance against pathogens. The transgenic approaches using PR genes have been proven to be efficient for obtaining pathogen resistance in plants [8, 9]. Several transgenic plants have been developed that offer varying degrees of protection against certain fungal and oomycete diseases.

This chapter overviews the PR proteins, including their classification, activation as defense signaling indicators, and mode of action against the pathogens. It also highlights the success and challenges of the transgenic approach using PR genes for disease resistance.

Families	Type member	Plant source	Gene accession no.	Classes/source	Size (kDa)	Properties	References
PR-1	Tobacco PR-1a	*Nicotiana tabacum*	YOO707		15–17	Antifungal	[54]
PR-2	Tobacco PR-2	*N. tabacum*	M59443.1	Classes III		β-1,3-Glucanase	[54]
				I plant vacuole	~33		[55]
				II, III extracellular proteins	~36		[55]
PR-3	Tobacco P, Q	*N. tabacum*	X77111.1	Classes V	25–30	Chitinase type I, II, IV, V, VI, VII	[56]
				I	~32		[55]
				II	27–28		[55]
				III	28–30		[55]
				IV	28–30		[55]
				V	41–43		[55]
PR-4	Tobacco "R"	*N. tabacum*	NW_015888419.1	Classes II	15–20	Chitinase type I, II	[56]
				I			[55]
				II			[55]
PR-5	Tobacco S	*N. tabacum*	NW_015793016		22–25	Thaumatin, antifungal, osmotin, zeamatin	[56, 57]
PR-6	Tomato inhibitor I	*Solanum lycopersicum*	NW_004196001.1		8	Proteinase inhibitor	[58]
PR-7	Tomato P69	*S. lycopersicum*	NC_015445.2		75	Endoproteinase	[59]
PR-8	Cucumber chitinase	*Cucumis sativus*	NC_026660.1		28	Chitinase type III	[60]
PR-9	Tobacco "lignin-forming peroxidase"	*Solanum* tuberosum	AJ401150		35	Peroxidase	[61]
PR-10	Parsley "PR1"	*Petroselinum crispum*	NC_026940.1	Classes III	17	Ribonuclease-like protein	[62]

Families	Type member	Plant source	Gene accession no.	Classes/source	Size (kDa)	Properties	References
				I	11 to 30		[55]
				II	~60		[55]
				III	~60		[55]
PR-11	Tobacco "class V" chitinase	*N. tabacum*	gi|899,342	—	40	Chitinase, type I	[63]
PR-12	Radish Rs-AFP3	*Raphanus raphanistrum*	NC_025209.1	Class IV	3–5	Defensin	[64, 55]
PR-13	Arabidopsis THI2.1	*Arabidopsis thaliana*	gi|1,181,531	—	5	Thionin	[65]
PR-14	Barley LTP4	*Hordeum vulgare*	gi|1,045,201	—	8.7–9	Lipid-transfer protein	[66, 55]
PR-15	Barley OxOa (germin)	*H. vulgare*	gi|2,266,668	—	20	Oxalate oxidase	[67]
PR-16	Barley OxOLP	*H. vulgare*	gi|1,070,358	—	20	Oxalate oxidase-like	[68]
PR-17	Tobacco PRp27	*N. tabacum*	—	—	27	Antifungal and antiviral	[69]
PR-18	Carbohydrate oxidases	*Helianthus annuns*	AF472608	—	60.9	Carbohydrate oxidases	[70]
PR-19	antimicrobial protein	*Pinus Sylvestris*	AF410954	—	—	antimicrobial protein	[15]

Table 1.
Classification and properties of PR proteins.

2. PR proteins and their classifications

PR proteins are defined as "Proteins encoded by the host plant but induced only in pathogenic or related conditions" [10]. Plant PR proteins were discovered and published for the first time in tobacco plants infected with the tobacco mosaic virus [11] and initially, only PR-1, PR-2, PR-3, PR-4, and PR-5 classes of PR proteins were reported from tobacco plants, but later different PR proteins were found from numerous plants [12]. These low molecular weight proteins (6–43 kDa) are heat stable, protease-resistant and soluble at acidic pH (<3) [13]. PR proteins are currently classified into 19 major families based on their enzymatic activity, biological roles, and amino acid sequences, as indicated in **Table 1** [14, 15]. These include antifungal (PR1), hydrolytic β-1,3- Glucanase (PR2), chitinases (PR 3, 4, 8,11), thaumatin (PR5), proteinase inhibitors (PR6), endo-proteinase (PR7), peroxidase (PR9), ribonuclease-like (PR10), plant defensins (PR12), plant thionins (PR13), lipid transfer proteins (PR14), oxalate oxidase protein family (PR15 and PR16) secretory protein (PR17) and carbohydrate oxidases (PR 18) [14, 7]. A novel antimicrobial protein from *Pinus sylvestris* was isolated and classified as PR19 [15].

3. PR proteins: functional characterization and mode of action

Plants are constantly being challenged by disease-causing organisms that have co-evolved with the evolution of plant hosts' defense mechanisms. Many PR proteins have been shown to possess antifungal, antibacterial, antiviral and antinematode properties [13]. Different PR proteins have a distinct mode of action against the pathogen depending upon the type of pathogen and the activities of the majority of these protein families are known or can be inferred. PR-1 protein, one of the dominant groups of PRs induced by the pathogen, inhibits pathogen growth by binding and sequestration of sterols from the pathogen. Moreover, the programmed cell death is also inhibited by PR1 upon pathogen infection by releasing a defense signal peptide CAPE1 (CAP-derived peptide 1) [16]. Some PR proteins function as hydrolytic enzymes, *viz.* the PR-2 (endo-β-1,3-glucanases) and PR-3, −4, −8 and − 11 (endo-chitinases) [17, 18]. They function as antifungal proteins by catalyzing hydrolytic cleavage of major components of fungal and oomycete cell wall, i.e. β-1,3-glucan (by the breakdown of β-1,3-glucosidic linkages) or chitin (by the breakdown of internal β-1,4-glycoside bonds) respectively, resulting in the breakdown of the fungal cell wall [19, 20]. Different isoforms of glucanases and chitinases are produced depending upon the plant-pathogen interaction.

Thaumatin-like proteins or Osmotin-like proteins such as PR5 inhibit hyphal growth and spore germination by producing transmembrane pores leading to fungal cell leakiness and blocking the function of plasma membrane receptors molecules involved in cAMP/RAS2 signaling pathways. Also, antifungal action has been demonstrated in some family members, predominantly against oomycetes. PR-5 was also demonstrated to exhibit potato cell's defense against *Phytophthora infestans* by forming a cytoplasmic aggregation through an actin-binding complex [21]. Proteinase inhibitors (PIs) such as trypsin inhibitors and serine inhibitors) belonging to PR6 family proteins, implicated in broad-spectrum defense activity, including suppressing pathogenic nematodes, insects and other herbivores, fungi and bacteria [22]. PIs can provide defense against pathogens, decreasing the lyase activity essential for fungal pathogenicity [23], inhibiting the viral replication cycle [24] and restricting

the digestive enzyme activity of nematodes and insects, limiting amino acid release [25]. In addition, HyPep (proteinase inhibitor peptide) also causes cell aggregation and pseudo-mycelia development by inhibiting amylase and serine proteinases [26]. Also, PIs can block chitin synthesis in fungal cell walls by inhibiting endogenous trypsin that is essential for chitin synthase, thus inhibiting fungal growth and development [27].

PR-7 is a major protein that has only been examined in tomatoes as an endoproteinase. It is an antifungal auxiliary protein that aids in destroying fungal cell wall proteins, chitinases, and glucanases [28]. The PR-9 family of peroxidases is believed to have a role in plant cell wall strengthening by facilitating lignin deposition in response to microbial invasion [29]. In susceptible wheat varieties, the transcription level of PR9 is considerably reduced after infestation with the aphid-transmitted fusarium virus and hessian flies [30]. This showed that PR9 catalyzes lignin deposition to protect susceptible cultivars from BPH.

The members of PR10 protein families exhibit ribonuclease activity required to inhibit the growth of pathogenic fungi. The antifungal activity of ribonucleases develops due to penetration of the pathogen and the destruction of cellular RNAs due to phosphorylation of PR10. It further leads to plant cell death at the inoculation site, causing apoptosis and the hypersensitivity reaction [31]. These intracellular PRs may be active against viruses due to their ribonuclease activity, although their ability to cleave viral RNA has yet to be shown.

The PR-12 type defensins, PR-13 type thionins, and PR-14 type lipid transfer proteins show antifungal and antibacterial activity, interacting with the target microorganism's biological membrane, leading to altered membrane permeability [32, 33]. Plant defensins are divided into two groups based on the structure of their precursor proteins: class I and class II. Class I defensins have endoplasmic reticulum (ER) signaling sequences along with defensin domains. In contrast, class II defensins contain an additional domain of 27–33 amino acid residues called C-terminal prepropeptide (CTPP) [34]. Due to a lack of signal sequences, class I defensins do not undergo post-translational modification or subcellular targeting. They accumulate in the cell wall and extracellular space directly upon synthesis through the secretory pathway [35]. However, class II defensins undergo proteolysis in the vesicles due to CTPP signal peptides targeting vesicles and releasing mature short peptides. Mature defensins consist of five segments of non-conserved loops, linking α-helices and β-strands to form high-level structures. Differences in the loop sequences confer different functions, including inhibition of protein synthesis, antimicrobial activity, heavy metal tolerance, plant development, and blocking of ion channels [36].

Oxalate oxidases (PR-15 family) and oxalate-oxidase-like proteins (PR-16 family) play an important role in plant defense [37]. These are essential enzymes to produce reactive oxygen species (ROS) during apoplastic oxidative burst [38]. ROS are produced in the apoplast by an enzyme that produces H_2O_2 and CO_2 when it reacts with oxalic acid. Proteolytic enzymes of the PR17 family play an important role in defense against fungi and viruses. PR19 protein binds to fungal cell wall glucans altering cell wall structure, leading to morphological distortion of hyphae [15].

4. PR protein activation as a defense response

Plant cells have evolved to activate and recruit the cellular machinery in response to various stresses to optimally utilize resources and sustain life. Accordingly, plants modulate genes' expression, activating a wide range of plant protectants and defense

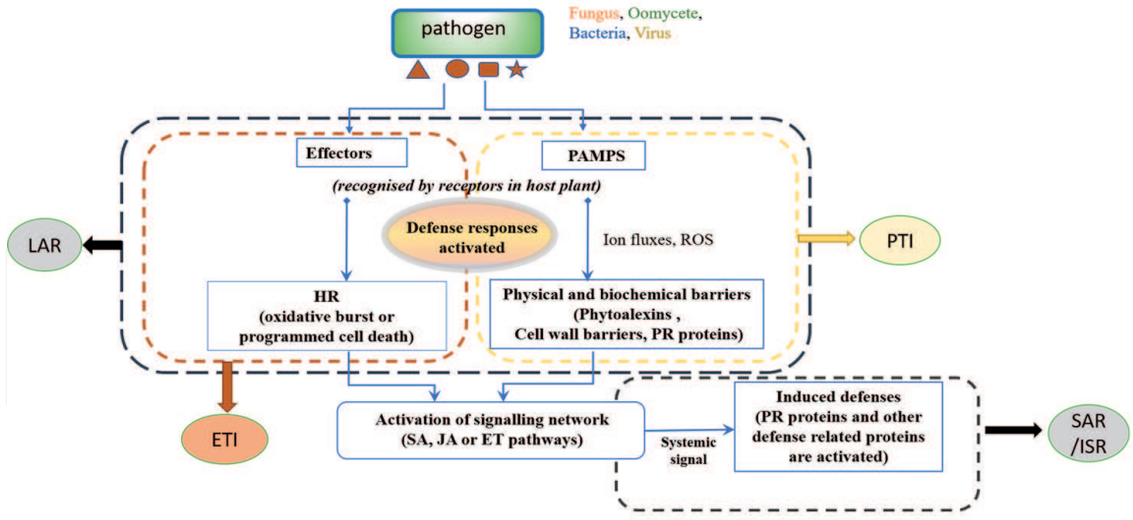

Figure 1.
Overview of the activation of defense response against the pathogen including induction of PR proteins locally as well as systematically.

genes [39]. The pathogenesis-related (PR) protein activation and production are crucial in response to an invading pathogen [40]. While healthy plants may produce a trace amount of PR proteins, they are produced in higher concentrations in response to pathogen attacks, elicitor treatment, wounding, or other stress.

Plants defend themselves against pathogen attacks by employing a variety of defense mechanisms for their survival and fitness [41]. After the pathogen challenge, plants trigger basal defense mechanism, i.e., pattern triggered immunity (PTI), by recognizing the pathogen-associated molecular patterns (PAMPs) and induced defense mechanism, i.e., effector-triggered immunity (ETI) [42]. PTI and ETI are accompanied by a set of preformed defenses (structural and biochemical barriers) and/or induced defense responses (hypersensitive reactions) that usually combat pathogen attacks [43]. Depending upon the plant-pathogen interaction, these defense responses are associated with a coordinated and integrated set of metabolic alterations that lead to induction of systemic acquired resistance (SAR) or induced systemic resistance (ISR) through activation of defense signaling pathways viz., salicylic acid (SA) and jasmonic acid (JA)/ethylene (ET) respectively. The activation of SA or JA signaling pathway leads to downstream activation and accumulation of PR gene products locally as well as systematically (**Figure 1**). As a result, PR proteins are related to the development of systemic acquired resistance (SAR) or a hypersensitive response (HR) to pathogenic fungi, bacteria, and viruses. Many plant species from many families have been shown to be induced by PRs, implying that PRs have a broad protective effect against biotic stress [40].

5. Role of signaling pathways in PR protein induction

Depending upon the host-pathogen interaction, different signaling systems are activated, producing different sets of PR proteins that provide disease resistance in plants. Basically, pathogens can be categorized into two types depending on the mode of infection: biotrophic and necrotrophic. Based on the type of pathogen, the pathogenic elicitors induce the production of different secondary signals such as ROS, jasmonates, salicylic acid or ethylene, which further induce the expression of different PR genes. Within the plant species, these secondary signals' spatial and temporal

production vary depending on pathogen type [44]. Classically, the resistance against biotrophic pathogens is conferred through the salicylic acid (SA) pathway, whereas against necrotrophic pathogens is conferred through activation of jasmonic acid/ethylene (JA/ET) pathways [45].

In plant-biotrophic pathogen interaction, the SA signaling system induces the expression of signature PR genes related to this pathway *viz* PR1, PR2, PR5, PR8, PR9 and PR10 [46]. The transcription studies (overexpression of PR genes), as well as mutational studies (SA mutants such as *nim1, npr1, sai1, nahG*), have provided evidence of the dependence of these PR genes on SA signaling pathway [47]. SA-mediated defense signaling regulates the expression of the PR genes through binding with and activating the NPR1 (due to conformational changes). Activated NPR1 interacts with transcription factors such as TGACG-binding factor (TGA), thus inducing defense gene expression [48]. However, in plant-necrotrophic pathogen interaction, it has been found through transgenic expression of PR genes as well as JA mutant analysis that the JA/ET signaling pathway induces the expression of PR3, PR4, PR10, PR11, PR12 and PR13 genes [47]. ET signaling pathway induces the expression of PR genes by activating the ETHYLENE RESPONSE FACTOR (ERF) transcription factor through activation of EIN2 and EIN3 proteins. However, in the JA signaling pathway, JAZ (jasmonate ZIM domain) protein is degraded by COL1 (coronatine insensitive 1) mediated 26S proteasome leading to activation of MYC2 transcription factor and hence transcription of JA responsive genes [49]. Furthermore, applying JA or SA hormones (defense hormones) increases the PR genes' transcription level, providing a broad spectrum of resistance [50]. During plant-pathogen interaction, hormonal crosstalks also occur, which can provide novel insights for disease resistance. PR-6 in tomato leaves generated by systemic and jasmonic acid was suppressed by exogenous application of SA. When a pathogen infects tobacco, ethylene may operate downstream of jasmonic acid to activate PR-2 and 3.

The ERF branch's ET/JA-regulated transcription factors are inhibited by the negative regulators of the SA signaling pathway. Also, SA biosynthesis is inhibited upon activation of the ET/JA signaling pathway, depicting these pathways' antagonistic role in defense response [51].

6. Pathogenesis-related proteins (PR-proteins) with their transgenic expression

With the development of modern DNA technology, it is possible to engineer transgenic plants transformed with genes to provide resistance against specific diseases. Recently the transgenic expression of various groups of PR proteins has enhanced the resistance of the transformed plant against several plant pathogens (**Table 2**). PR proteins are found in all organisms and are part of their innate immune systems. They have a wide range of activities, including disrupting fungal cell walls, permeabilizing membranes, inhibiting transcription, and inactivating ribosomes [52]. Genes coding for various PR proteins have been identified, cloned, and expressed in plants, preventing the development of specific diseases and conferring resistance to affected plants. Using modern biotechnology tools, various crops have been engineered to express, or over-express the PR proteins from different sources, such as (i) that are produced during the plant's defense response, (ii) derived from microorganisms or animal cells, (iii) synthetic peptides designed based on sequences of existing antimicrobial compounds [14, 53].

Enzyme	Genes	Source	Target pathogen	Transgenic plant	Transgenic system	Reference
Glucanase	*β-1,3-glucanase*	*Linum usitatissimum*	*Fusarium culmorum*	Potato	*Agrobacterium* -mediated transformation	[97]
	HbGLU	*Hevea brasiliensis*	*Rhizoctonia solani*	Potato	*Agrobacterium*-mediated transformation	[98]
	β-1,3-glucanase II cDNA	*Hordeum vulgare*	*Fusarium graminearum*	Wheat	Particle gun bombardment	[99]
	chi-2, ltp	*Hordeum vulgare, Triticum aestivum*	*Alternaria radicicola* and *Botrytis cinerea*	Carrot	*Agrobacterium*-mediated transformation	[100]
	McCHIT1	*Momordica charantia*	*Magnaporthe grisea* and *Rhizoctonia solani*	Rice	Electroporation	[101]
	OsPR4a–e	*Oryza sativa*	*Magnaporthe grisea*	Rice	*Agrobacterium*-mediated transformation	[102]
	RC7	*Oryza sativa*	*Rhizoctonia solani*	Rice	Biolistic and PEG-mediated transformation system	[103]
	BjCHI1	*Brassica juncea*	*Rhizoctonia solani*	Potato	*Agrobacterium*-mediated transformation	[98]
	chit cDNA	*Hordeum vulgare*	*Fusarium graminearum*	Wheat	biolistic bombardment	[104]
	Chitinase-I	*Oryza sativa*	*Verticillium dahliae and Fusarium oxysporum*	Eggplant	*Agrobacterium*-mediated transformation	[105]
	RC24	*Oryza sativa*	*Puccinia striiformis f.sp. tritici*	Wheat	Particle bombardment	[106]
	rcc2 and *rcg3*	*Oryza sativa*	*Puccinia striiformis f.sp. tritici*	Wheat	*Agrobacterium*-mediated transformation	[107]
	LcCHI2	*Leymus chinensis*	*Pseudomonas tabaci, A. alternata, Exserohilum turcicum, Curvularia lunata*	Maize	*Agrobacterium*-mediated transformation	[77]

Enzyme	Genes	Source	Target pathogen	Transgenic plant	Transgenic system	Reference
Thaumatin	*Thaumatin-likeTaLr19TLP1*	*Triticum aestivum*	*Puccinia triticina*	Wheat	virus-induced gene silencing	[108]
	Tlp	*Triticum aestivum*	*Fusarium graminearum*	Wheat	biolistic transformation	[109]
	Tlp	*Oryza sativa*	*Alternaria solani*	Tomato	*Agrobacterium*-mediated transformation	[110]
	Tlp	*Oryza sativa*	*Rhizoctonia solani*	Rice	Particle bombardment	[111]
	tlp-1	*Hordeum vulgare*	*Fusarium graminearum*	Wheat	*Agrobacterium*-mediated transformation	[99]
	CsTLP	*Camellia sinensis*	*Phytopthora infestans and Macrophomina phaseolina*	Potato	*Agrobacterium* transformation	[112]
	AdTLP	*Arachis diogoi*	*Rhizoctonia solani*	Tobacco	*Agrobacterium*-mediated transformation	[113]
Osmotin-like proteins	*OsOSM1*	*Oryza sativa*	*Rhizoctonia solani*	Rice	*Agrobacterium*-mediated transformation	[114]
	OsmWS	Withania somnifera	*A. solani*	Potato	*Agrobacterium*-mediated transformation	[78]
	JIOsPR10	*Oryza sativa*	*Magnaporthe oryzae*	Rice	*Agrobacterium*-mediated transformation	[115]
Ribonuclease like protein	*GmPRP*	*Glycine max*	*Phytophthora sojae*	Soybean	*Agrobacterium*-mediated transformation	[92]
Ribonuclease inactivating protein	*PAP*	*Phytolacca ameriacana*	Cucumber mosaic virus, Potato virus X, Potato virus Y	Tobacco and Potato	*Agrobacterium*-mediated transformation	[116]
	PAP	*Phytolacca ameriacana*	*Sclerotinia homoeocarpa*	Beet grass	Particle bombardment	[117]
Proteinase inhibitor	*mpi*	*Zea mays*	*Chilo suppressalis*	Rice	Particle bombardment or *Agrobacterium*-mediated transformation	[96]
	cry1B	*Zea mays*	*Chilo suppressalis*	Rice	particle bombardment	[95]

Enzyme	Genes	Source	Target pathogen	Transgenic plant	Transgenic system	Reference
Defensins	*Wasabi*	*Wasabia japonica* L.	*Magnaporthe grisea*	Rice	*Agrobacterium*-mediated transformation	[118]
	Wasabi	*Wasabia japonica* L.	*Botrytis cinerea*	Potato	*Agrobacterium*-mediated transformation	[119]
	MsDef1	*Medicago sativa*	*Fusarium oxysporum f. sp. lycopersici*	Tomato	-	[120]
	MtDef4.2	*Medicago truncatula*	*Puccinia triticina*	Wheat	*Agrobacterium*-mediated transformation	[50]
	RsAFP2	*Raphanus sativus*	Rhizoctonia solani and *Magnaporthe grisea*	Rice	*Agrobacterium*-mediated transformation	[121]
	RsAFP2	*Raphanus sativus*	*Rhizoctonia cerealis, Fusarium graminearum*	Wheat	Biolistic bombardment	[122]
	Wasabi	*Wasabia japonica* L.	*Alternaria solani* and *Fusarium oxysporum*	Melon	*Agrobacterium*-mediated transformation	[123]
	BoDFN	*Brassica oleracea*	Downy Mildew	Wild cabbage	*Agrobacterium*-mediated transformation	[124]
	VrPDF1	*Vigna radiata*	Weevils	mungbean	*Agrobacterium*-mediated transformation	[125]
	TAD1	*Triticum aestivum*	*Typhula ishikariensis, Fusarium graminearum*	Wheat	particle bombardment	[126]
Thionins	*AT1G12660 and AT1G12663*	*A. thaliana*	*R. solani* and *F. oxysporum*	Potato	*Agrobacterium*-mediated transformation	[83]
	Thionin	*Brassica oleracea* var. acephala, *Nasturtium officinale* and *Barbarea vulgaris*	*B. cinerea*	Potato	*Agrobacterium*-mediated transformation	[127]
	α-hordothionin (αHT)	*Hordeum vulgare*	*Ceratocystis fimbriata*	Sweet potato	*Agrobacterium*-mediated transformation	[128]
	Thi2.1	*A. thaliana*	*Fusarium oxysporum*	Tomato	*Agrobacterium*-mediated transformation	[129]

Enzyme	Genes	Source	Target pathogen	Transgenic plant	Transgenic system	Reference
Oxalate Oxidase	*OXO*	*Triticum aestivum*	*Sclerotinia sclerotiorum*	Soybean	*Agrobacterium*-mediated transformation	[86]
	Osoxo4	*Oryza sativa*	*Phytophthora infestans*	Potato	*Agrobacterium*-mediated transformation	[130]
	OXO	*Hordeum vulgare*	*Botrytis cinerea* and *Sclerotinia sclerotiorum*	Tomato	*Agrobacterium*-mediated transformation	[131]
	OXO	*Triticum aestivum*	*Phytophthora infestans*	Potato	*Agrobacterium*-mediated transformation	[132]
Lipid Transfer Proteins	*AtLTP4.4*	*A. thaliana*	*F. graminearum*	Wheat	particle bombardment	[90]
	Ace-AMP1	*Allium cepa*	*Sphaerotheca pannosa var. rosae*, *Blumeria graminis f. sp. tritici* and *Neovossia indica* , *Magnaporthe grisea* and *Rhizoctonia solani*	Wheat and rice	*Agrobacterium*-mediated transformation, microprojectile bombardment, In planta assays	[87, 133, 134, 88]
Carbohydrate oxidases	-	*Helianthus Annuus*	*Pectobacterium cartovorum* ssp. *cartovorum*	Tobacco	Electroporation	[70]
Antimicrobial protein	*Sp-AMP*	*Pinus Sylvestris*	*Heterobasidion annosum*	Tobacco	*Agrobacterium*-mediated transformation	[15]

Table 2.
Example of transgenic plants over-expressing PR proteins against plant pathogens.

7. Transgenic plants expressing antifungal activities

Fungi are one of the most harmful phytopathogens, resulting in considerable production losses in most agricultural crops [71]. PR proteins have proven effective in preventing fungal diseases in plants as many of these targets or hydrolyze fungal cell walls, resulting in cell death. PR1, PR2, PR3, PR4, PR5, PR8, PR11, PR12, and PR13 have been identified as plants' most effective antifungal proteins. Transgenic approaches using PR proteins are suitable for developing long-lasting fungal pathogen-resistant crops [47]. Of the various antifungal PR proteins, glucanases and chitinases are most widely used in transgenic technology to provide resistance against fungus.

The transgenic over-expression of glucanase and chitinase genes from different sources has been shown to be effective against pathogens, specifically fungus. It has been reported that overexpression of the tobacco glucanase gene imparted groundnut resistance to *Cercospora arachidicola* and *Aspergillus flavus*, demonstrating that fungal resistance is conferred via *in planta* transformation [72]. Transgenic Arabidopsis plants expressing grapevine *b-1,3-glucanase* (*VvGHF17*) confers resistance to *Colletotrichum higginsianum* and *Botrytis cinerea* [73]. Furthermore, tea with transgenic overexpression of the endo-*1,3-D-glucanase* gene, which expresses a potato glucanase, significantly improved tolerance to the blister blight fungus *Exobasidium vexans* [74]. Recently, oil palm resistance to *G. boninense* was improved by transgenic overexpression of *M. sativa* glucanase (*AGLU1*) [75]. Likewise, transgenic expression of chitinase genes have been reported to be antifungal generated transgenic zoysia grass was generated which overexpressed *Zjchi2* via *Agrobacterium-mediated* transformation and hence showed disease resistance against *Rhizoctonia solani* [76]. Currently, the overexpression of *LcCHI2* gene was identified that increasing the chitinase activity in transgenic tobacco and maize, resulting in improved resistance *to Pseudomonas tabaci*, *Alternaria alternata*, *Exserohilum turcicum*, *Curvularia lunata* [77].

Some other antifungal PR proteins that have been reported to be used in transgenics are thaumatin-like/osmotin-like proteins, defensin-like proteins, thionin, oxalate oxidase and lipid transfer protein. In fungal cells, thaumatin-like proteins are known to form transmembrane pores, whereas osmotin proteins are known to maintain the osmolarity of suitable solutes in cellular compartments [78]. In Arabidopsis thaliana, overexpression of the *TLP29* gene from grape *VqTLP29* improved resistance to powdery mildew and the bacteria *Pseudomonas syringae* [79]. Under *in vitro* conditions, transgenic poplars overexpressing *PeTLP* thaumatin genes showed enhanced resistance to *Marssonina brunnea* [80]. Similarly, in potatoes, overexpression of the osmotin gene (*OsmWS*) conferred resistance to the early blight fungus *A. solani* [78]. Many more transgenic plants have been generated that show increased resistance to phytopathogenic fungi by expressing the TLPs and OLPs as listed in **Table 2**.

The successful developed and characterized transgenic peanut and tobacco plants which overexpress the mustard *defenisn* gene and *Raphanus sativa*, *RsAFP2* gene for fungal resistance respectively [81]. The late leaf spot diseases *Cercospora arachidicola* and *Pheaoisariopsis personata* were more resistant to transgenic peanut plants whereas, *Phytophthora parasitica* pv. nicotianae and *Fusarium moniliforme* resistance was higher in transgenic tobacco plants. Similarly, the *rDrr230a* defensin protein gene suppressed spore germination and growth of both *Fusarium tucumaniae* and *Colletotrichum gossypii* var. cephalosporioides in transgenic *Pichia pastoris* [82]. The antifungal thionin genes (*AT1G12660* and *AT1G12663*) from *A. thaliana* had been used to produce transgenic potato conferring resistance against pathogenic fungi such as *Fusarium solani* and *Fusarium oxysporum* [83]. Furthermore, the overexpression of thionin

increased canker resistance and decreased canker bacterial development when transgenic Carrizo plants expressing the modified plant thionin were produced by *Agrobacterium-mediated* transformation [84]. Peanuts with transgenic expression of the oxalate oxidase expressing gene were more resistant to *Sclerotinia* blight [85]. Also, overexpression of oxalate oxidase genes has been developed to increase resistance against *Sclerotinia sclerotiorum* in transgenic Glycine max [86].

Transgenic expression of LTPs has been shown to improve resistance to phytopathogenic fungi in some studies. As an example, antimicrobial protein gene (*Ace-AMP1*) isolated from *Allium cepa* has been overexpressed in both *Triticum aestivum* and *Oryza sativa* through *Agrobacterium-mediated* transformation, microprojectile bombardment, in *planta* assays, conferring resistance against *Sphaerotheca pannosa* var. rosae [87], *Magnaporthe grisea*, *Rhizoctonia solani* and *Xanthomonas oryzae* [88] respectively. Recently, *A. thaliana* LTP overexpressing transgenics has been shown to increase resistance toward pathogens *Plasmodiophora brassicae* and *F. graminearum* [89, 90]. Some other examples of successfully generated transgenic plants with enhanced production of hydrolytic enzymes and resistance against phytopathogenic fungi are given in **Table 2**.

8. Transgenic plant expressing bacterial resistance

Numerous bacterial pathogens causing massive yield losses have been isolated and identified from different agriculturally important crops. Pathogenesis-related proteins are well-known weapons to combat resistance against these bacterial pathogens. Many in-vitro studies have shown the antibacterial properties of many PR proteins *viz* PR10 (Ribonuclease-like proteins), PR12 (defensins), PR13 (thionins) and PR14 (Lipid-transfer protein) [88, 91, 92]. Among these, PR10 shows broad spectrum of antibacterial activity against *P. syringae*, *Agrobacterium tumefaciens*, *A. radiobacter*, *Pseudomonas aureofaciens* and *Serratia marcescens* [92, 93]. Overexpression of lipid transfer protein (PR14) in rice plants showed increased resistance to bacterial as well as fungal pathogens (**Table 2**) [88]. The antibacterial efficacy of additional PR proteins and AMPs against a variety of bacterial diseases in economically significant crops has to be further investigated.

9. Transgenic plant expressing insect resistance

Plants expressing PR genes have been engineered in several experiments, resulting in enhanced pest resistance. The expression of both low and high levels of *MTI-2* was reported by using *Agrobacterium* transformation technique in tobacco and *Arabidopsis* plants leading to resistance against *Spodoptera littoralis* [94]. The wound-inducible expression of a *Bacillus thuringiensis* endotoxin gene which directed significant insecticidal gene expression to protect transgenic rice from *Chilo suppressalis* Walker [95]. Transgenic rice plants were developed by particle bombardment or *Agrobacterium-mediated* transformation of *mpi* gene leading to resistance against *C. suppressalis* (**Table 2**) [96].

10. Transgenic plant expressing viral resistance

Apart from their antifungal or antibacterial effects, PR proteins appear to be a promising candidate gene for producing virus-resistant transgenic crops based on

different studies of PR proteins, as given in **Table 2**. Antiviral activities of PR proteins such as defensins, thionins, peroxidase and lipid transfer proteins have been observed *in vitro* [134]. Antiviral activity has also been observed in ribosome-inactivating proteins (RIPs), which suppress translation by enzymatically damaging ribosomes [134]. Plant resistance to plant viruses was improved by a transformation study involving RIPs. In addition, CaPR10 from *Capsicum annuum* has been found to have increased ribonucleolytic activity against the Tobacco mosaic virus (TMV) RNA, allowing it to break viral RNAs [88].

11. Synergistic effect of transgenic PR proteins

In transgenic plants, the synergistic action of two or more PR genes reduces susceptibility to various pathogens. Researchers have reported that *β-1,3-glucanases* and *chitinases* synergistically inhibited the growth of *Fusarium oxysporum* by using *in planta* transformation [135]. Transgenic potato plants co-expressing chitinase (*BjCHI1*) and β-1,3-glucanase (*HbGLU*) suppressed *Rhizoctonia solani* and showed healthier root growth [98]. In another study, transgenes carrying the chitinase gene (*chi11*) and the thaumatin-like protein gene (*tlp*) from rice were introduced by co-bombardment, and overexpression of these antifungal *chi* and *tlp* proteins provided resistance to fungal infections in barley [136]. Likewise, in transgenic carrots, the synergistic action of three different PR-protein genes such as chitinase, β-1,3-glucanase and peroxidase, conferred disease resistance to necrotrophic pathogens namely, *Botrytis cinerea* and *Sclerotinia sclerotiorum* [137]. Amian *et al* [138] reported the development of transgenic pea plants with stable integration of two genes *viz* β-1,3-glucanase (*Hordeum vulgare*) and chitinase gene (*Streptomyces olivaceoviridis*) via *Agrobacterium*-mediated gene transformation and hence produced suppression of fungal spore germination. Chhikara *et al* [139] used *Agrobacterium*-mediated transformation to co-express the barley antifungal genes chitinase and ribosome-inactivating protein in Indian mustard, protecting against Alternaria leaf spot disease. Furthermore, transgenic potato plants expressing *rip30* and *chiA* genes transformed by *A. tumefaciens* strain *GV3101* showed improved resistance to *Rhophitulus solani* [140]. In the case of Oriental melon (*Cucumis melo* Makuwa Group), the fusion of chitinase (*CHI*) and antifungal protein (*AFP*) genes confers enhanced protection against *Rhizoctonia solani* and *Fusarium oxysporum* [141]. Rice plants co-transformed with chitinase (*OsCHI11*) and oxalate oxidase (*OsOXO4*), which are defense-related genes, showed improved resistance to the pathogen that causes sheath blight [142]. Boccardo *et al* [143] suggested co-expression of PR proteins *AP24* and *β-1,3 glucanase* enhanced resistance against *Rhizoctonia solani* in greenhouse conditions and *Peronospora hyoscyami* f.sp. *tabacina* and *phytophthora nicotianae* pathogens in field conditions.

12. Challenges faced by transgenic expression with PR proteins

Since the advent of plant genetic engineering, PR proteins have consistently been the top choice among scientists when creating transgenic plants to increase disease resistance against a variety of diseases. PR proteins expressed either singly or synergistically in transgenic plants can provide broader and more effective disease resistance against different pathogens as described above.

Aside from these successful outcomes, many studies have described the challenges of using PR proteins in transgenic technology. In contrast to the above findings, numerous studies have suggested that the transgenic expression of PR proteins did not lead to increased tolerance to pathogens. Szwacka *et al* [144] reported no relationship between transgenic protein expression level and increased tolerance against the pathogen. Transgenic cucumber plants with stably integrated thaumatin II cDNA under the control of the CAM35S promotor via *Agrobacterium* did not exhibit tolerance to *Pseudoperonospora cubensis*. Moravckova *et al* [145] co-introduced chitinase and glucanase into *Solanum tuberosum* to increase resistance to *R. solani* infection, but hyphal extension assay revealed that transformants did not affect *R. solani* growth in vitro.

Various transgenic plant modifications have been described, with varying degrees of protection against certain fungal and oomycete infections. However, the resulting resistance levels were frequently insufficient for breeding [146]. Furthermore, constitutive expression of PR proteins can lead to the spontaneous production of lesions that look like HR lesions in the absence of a pathogen), which can be an unfavorable outcome [147]. Disease resistance techniques must control specific diseases without affecting crop yield and quality.

Moreover, most researchers have used constitutive promoters to control the expression of PR genes in agricultural plants to enhance resistance, resulting in homology-dependent gene silencing. As a result, unregulated and untimely activation of PR genes or AMPs harms plant growth and development. Human allergenicity is one of the main issues hindering the success of transgenic technology with PR genes. According to the current classification, there are 19 different classes of PR-Proteins, and 8 of them have been confirmed to cause allergic reactions in humans by using *in-silco* approaches. These proteins have been known to trigger allergenic symptoms such as food allergens depending upon their mode of entry into the human body [148], dermatitis, airborne, asthma, airway allergy etc. and if all these allergens have been consumed in greater amount, the gastrointestinal symptoms are also triggered.

13. Conclusion

The goal of this chapter was to review the role of PR-proteins in plant defense and how transgenic expression of PR-proteins in agricultural plants resulted in increased resistance to stresses. Biotic stress has become a significant concern in modern agriculture and many research institutions are actively researching to generate resistant cultivars using PR proteins. PR proteins have become a highlighted topic between scientists because of their effectiveness against biotic agents. Genetic engineering is considered the best way to develop transgenic resistant plants using PR proteins. To increase agronomic characteristics worldwide, new inventions or novel approaches in PR protein transgenic technology are necessary and will continue to improve plant health in the future. Another future concern is that the formation of virulent phytopathogen strains increases as the global climatic change rate increases. So, to cope with such significant obstacles, it is necessary to define and identify novel PR genes functionally. Advances in genomics, transcriptomics, phenomics, proteomics, metabolomics, and ionomics, will substantially aid our understanding of the complex network of PR genes and the interaction of PR proteins with other proteins from plants and pathogens. Therefore, PR proteins could be utilized to develop crop plants

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more resistant to various stresses. They could also be employed as candidate genes for genetically engineering crop multi-trait factors. Future research is needed to assess the PR transgenic plants' responses to various traits, including biotics, plant development and yield.

Author details

Anroop Kaur[1], Sukhpreet Kaur[1], Ajinder Kaur[1], Navraj Kaur Sarao[1] and Devender Sharma[2*]

1 School of Agricultural Biotechnology, Punjab Agricultural University, Ludhiana, India

2 ICAR-Vivekananda Parvatiya Krishi Anusandhan Sansthan, Almora, India

*Address all correspondence to: devender.kumar1@icar.gov.in

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