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CURRENT TOPICS IN PLANT RESEARCH

Metabolic pathway genes for editing to enhance multiple disease resistance in plants

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Abstract

Diseases are one of the major constraints in commercial crop production. Genetic diversity in varieties is the best option to manage diseases. Molecular marker-assisted breeding has produced hundreds of varieties with good yields, but the resistance level is not satisfactory. With the advent of whole genome sequencing, genome editing is emerging as an excellent option to improve the inadequate traits in these varieties. Plants produce thousands of antimicrobial secondary metabolites, which as polymers and conjugates are deposited to reinforce the secondary cell walls to contain the pathogen to an initial infection area. The resistance metabolites or the structures produced from them by plants are either constitutive (CR) or induced (IR), following pathogen invasion. The production of each resistance metabolite is controlled by a network of biosynthetic *R* genes, which are regulated by a hierarchy of *R* genes. A commercial variety also has most of these *R* genes, as in resistant, but a few may be mutated (SNPs/InDels). A few mutated genes, in one or more metabolic pathways, depending on the host–pathogen interaction, can be edited, and stacked to increase resistance metabolites or structures produced by them, to achieve required levels of multiple pathogen resistance under field conditions.

Keywords Biotic stress resistance · Cell wall reinforcement · Innate immunity in plants · Metabolite biosynthetic genes · Multiple disease resistance · Transgene-free genome editing

Introduction

Atoms, since their origin about 13.7 billion years ago, have combined to form nucleic acids and genes, and evolved into life on earth, including prokaryotes and eukaryotes. Several microorganisms evolved as saprophytes or as plant pathogens. Domestication of plants for the last 10,000 years has led to the selection of the best phenotypes. Conventional breeding produced varieties that are dwarf in stature facilitating mechanical harvest, and plant response to high doses of fertilizers to develop high-yielding varieties, leading to the green revolution (Evenson and Gollin 2003). Lately, molecular breeding has produced hundreds of varieties, in different crops. To nourish the world's constantly increasing population, the high-yielding varieties with genetic uniformity are sought, whereas to meet the constantly evolving abiotic and biotic environmental stress agents with changing climate, the planting of varieties with high spatial and temporal genetic diversities is imperative, for sustainable crop production (Bailey-Serres et al. 2019; van Frank et al. 2020). Pathogens, being biotic agents, constantly evolve into more virulent and aggressive races, depending on pathogen types and crop production systems (McDonald and Linde 2002). The genetic variability and evolution of both host and pathogen in a production system determine the durability of a cultivar (Mundt 2014). To develop a variety with high yield and at the same time with minimum crop failure is very challenging but made possible based on molecular breeding. These methods of breeding also lead to genetic erosion, especially the genes involved in traits that are not targeted in breeding. In genetically uniform high-yielding varieties, the genetic diversity for environmental stress resistance can be improved based on genome editing, to develop high-yielding varieties with high levels of multiple pathogen resistance. Inadequate information on which genes to edit and how it can reduce pathogen progress in a plant is mainly limiting the

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use of genome editing tools to improve resistance to multiple pathogens in plants.

This review covers the basic concept of resistance, and different steps involved in editing metabolic pathway genes to enhance multiple pathogen resistance in plants to encourage researchers to undertake genome editing to improve plants (Fig. 1): (1) concept of resistance in plants to pathogen attack; (2) selections of *R* genes for genome editing; (3) CRISPR-Cas9 based genome editing; (4) evaluation of enhanced resistance to multiple pathogens in plants.

Concept of resistance in plants to pathogen attack

Resistance is the ability of a plant variety to restrict the invisible and visible responses of cells and tissues to a pathogenic organism that results in adverse changes in the form, function, and integrity of the plant, which may lead to partial impairment or death of plant part or the entire plant (Agrios 2005). The genetic bedrock of resistance is very complex, but still, certain general principles of plant disease resistance have been conceptualized. Three types of resistance have been recognized: (i) non-host resistance, defined as resistance in plants belonging to a taxonomic group outside the host range; (ii) apparent resistance or disease escape, which is generally controlled by the environment; (iii) true resistance, which is based on immune responses of plants to pathogen attack.

Plants have innate immunity, unlike animals which have both innate and adoptive immunities, and each cell responds to invasion by pathogens (Fig. S1). Following deposition, the plant pathogens produce elicitors or pathogen/microbe/damage-associated molecular patterns (PAMPs/MAMPs/DAMPs), and effectors, which are perceived by the plant membrane localized immune receptor proteins or *R* genes. These immune receptor *R* genes in turn generally trigger hierarchies of downstream regulatory *R* genes, such as MAPKs, phytohormones, microRNAs and transcription factors, which in turn regulate other *R* genes, such as resistance protein-coding and metabolite biosynthetic genes (Kushalappa et al. 2016a). Following recognition of elicitors and effectors, the immune receptor *R* genes in plants trigger reactive oxygen species (ROS), hydrogen peroxide (H_2O_2), and callose (β-1-3-glucans) biosynthesis, the latter is a metabolite deposited to form papillae around the hypha to suppress the advancing pathogen, eventually inducing hypersensitive response type of programed cell death (HR-PCD), leading to the pattern triggered immunity (PTI) and effector triggered immunity (ETI), respectively (Andersen et al. 2018; Camagna and Takemoto 2018). However, the hierarchies of genes involved in inducing the HR-PCD are still elusive. Generally, these types of specific resistance have been classified as qualitative resistance. If the HR-PCD fails to contain the pathogen, then the plant is susceptible or to have quantitative resistance (Andersen et al. 2018; Cowger and Brown 2019; Kushalappa et al. 2016b). However, several transcriptomic studies have revealed high expressions of several downstream *R* genes during the manifestation of HR-PCD, confirming that the qualitative and quantitative resistances are not distinct, rather a continued effort by the host to reduce the advancement of a pathogen, for an eventual reduction in invisible and visible cell responses and disease severity (Pollard et al. 2008). The pathogen

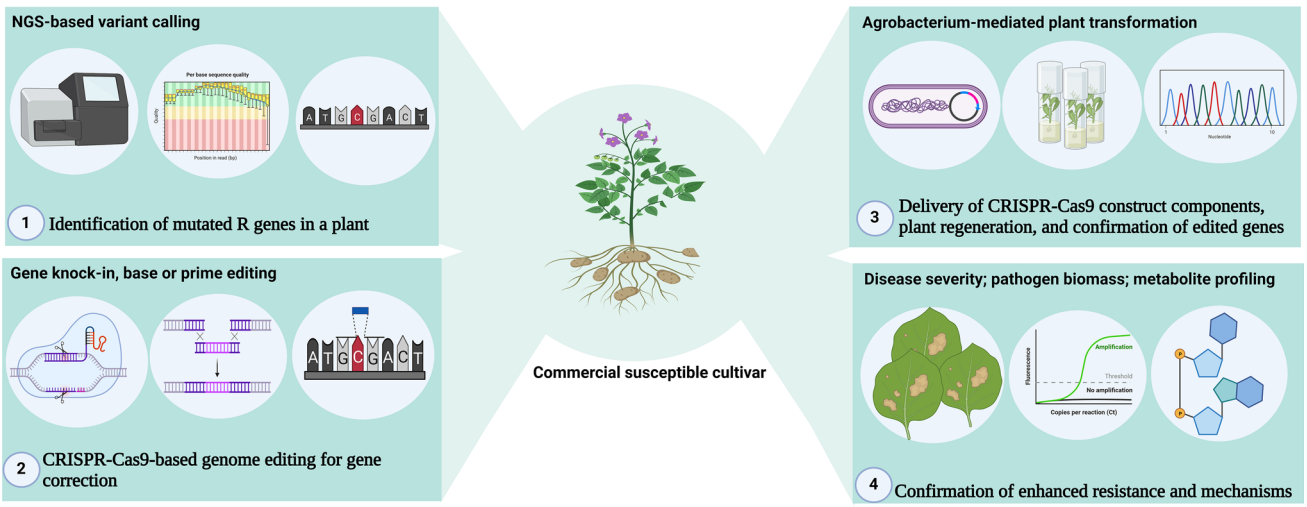

Fig. 1 Schematic diagram of steps involved in the discovery of mutated *R* genes in commercial crop cultivars and genome editing to enhance multiple disease resistance. The major steps are: (1) identification of candidate genes for editing; (2) CRISPR-Cas9-based genome editing for gene correction; (3) delivery of CRISPR-Cas9 construct components, plant tissue culture, and confirmation of edited genes; (4) confirmation of enhanced resistance and mechanisms

perception by plants, however, is quite complex and involves hundreds of genes (Couto and Zipfel 2016). The external stimuli (PAMP/MAMP) may involve membrane immune receptor *R* genes, but the internal stimuli may involve a diverse array of non-receptor-mediated stimuli, including radiation, toxins, viral infections, hypoxia, hyperthermia, free radicals, and involves intracellular sub-compartments such as mitochondria, nucleus, or others (Emanuele et al. 2018). Plants, following pathogen invasion, induce Ca^{2+} (Chen et al. 2015; Geng et al. 2013; Reape and McCabe 2008). In response to toxins produced by the pathogens, with hemibiotrophic and necrotrophic lifestyles, plants induce Ca^{2+} in the apoplast which is transported to the cytosol and cell organelles to induce apoptotic-like PCD (AL-PCD), and following this, the pathogen feeds on the dead cells to advance further causing sever diseases (Danon et al. 2000; Kushalappa et al. 2022; Reape and McCabe 2008). The increased colonization also increases the amount of toxins produced by these pathogens. Often, these toxins suppress specific gene functions and thus the metabolite biosynthesis (Chowdhury et al. 2017b). For example, in wheat, the pathogen *Fusarium graminearum* produces deoxynivalenol (DON), a protein biosynthesis inhibitor, which can inhibit resistance metabolite biosynthesis by *R* genes (Rocha et al. 2005). Natural mutation or silencing of the gene *HRC* that induces AL-PCD in plants can reduce pathogen progress and disease severity, thus enhancing the level of resistance in plants (Kushalappa et al. 2022). Transcriptome and metabolome profiling studies have revealed the expression of hierarchies of regulatory and biosynthetic *R* genes, that eventually code for resistance proteins and metabolites that reduce pathogen progress, leading to quantitative resistance (Karre et al. 2017; Kushalappa et al. 2016b; Neu et al. 2019). The resistance biochemicals, proteins and metabolites, may be constitutively present before pathogen invasion (CRP, CRM) or induced (IRP, IRM) following pathogen invasion (Karre et al. 2017). The constitutive biochemicals are also called phytoanticipins, which are either active or passive, the latter is often stored in vacuoles as glycoside conjugates, and the active compounds are released by simple hydrolysis, following pathogen perception. The biochemicals also form constitutive structures, such as cell membrane, wax, cuticle, epidermis, and secondary reinforced cell walls. The induced biochemicals are called phytoalexins, which are antimicrobial proteins and metabolites. The metabolites are biosynthesized in a network of different metabolic pathways (Fig. 2). Some of these biochemicals polymerize and/or conjugate with others to form complex molecules, which are deposited

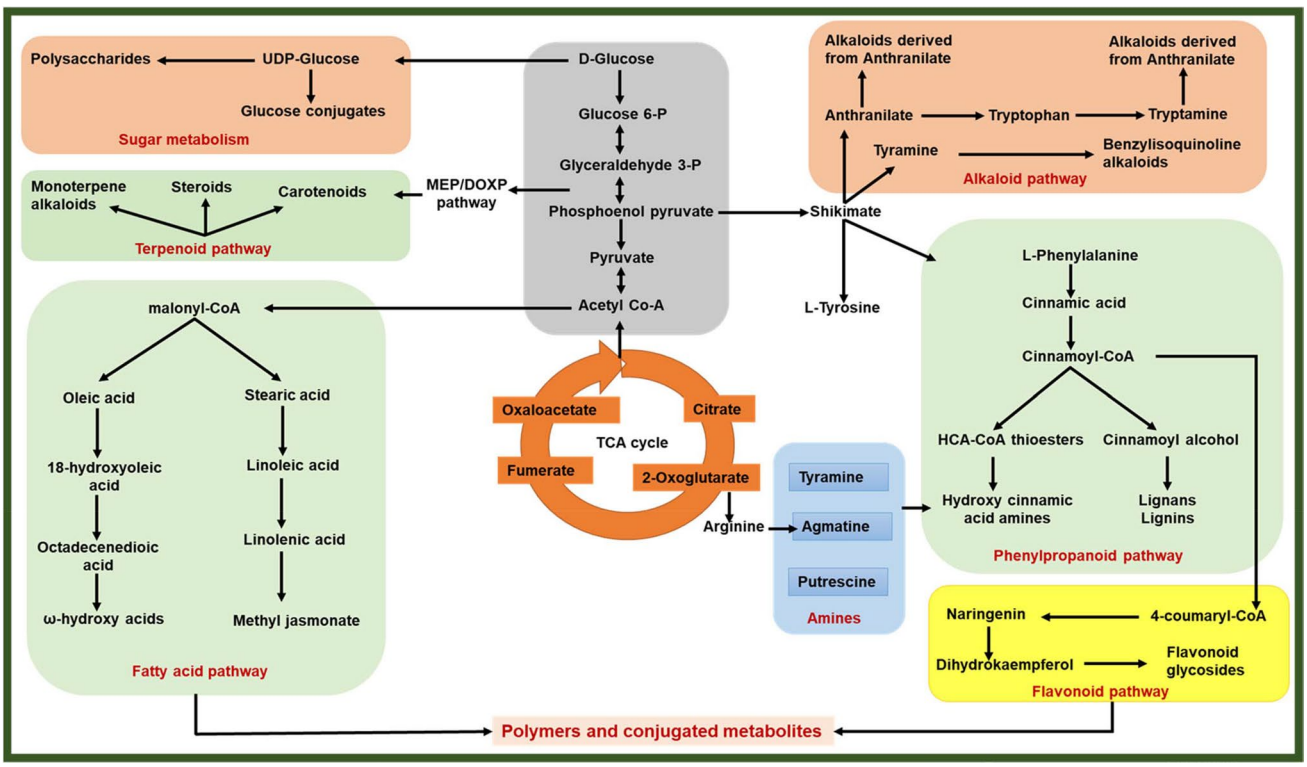

Fig. 2 Satellite metabolic pathways, involved in the biosynthesis of resistance metabolites by plants, in response to biotic stress. These resistance metabolites are biosynthesized by the catalytic proteins that are coded by the plant *R* genes. The biosynthesis of resistance metabolites in a plant is controlled by a hierarchy or several hierarchies of *R* genes, which may have regulatory or resistance metabolite biosynthetic roles

as structures reinforcing mainly the secondary cell walls, such as wax layers, cuticles, and epidermis to limit food supply to the pathogen in infected cells. The reinforced cell walls lead to the formation of abscission and cork layers limiting the food supply to the advancing pathogen, containing it to an initial infection area, forming only small necrotic lesions, instead of large necrosis involving tissues, organs or the entire plant (Cowger and Brown 2019; Kushalappa et al. 2016a). The reduced disease severity, due to reduction in the survival ratio and rates of infection, sporulation, and dissemination processes (the monocyclic process) of the pathogen, leading to reduced disease severity and rates of disease progress (polycyclic process) is considered quantitative disease resistance. Thus, silencing some of these *R* genes to enhance biofuel production should be discouraged to reduce future epidemics (Houston et al. 2016; Soni et al. 2020). To achieve sufficient levels of multiple disease resistance under commercial conditions, there is no need for a commercial cultivar to have all the resistance metabolites or functional *R* genes known in the metabolic pathway of that plant species. A few important resistance proteins and metabolites, the *R* genes involved in their biosynthesis, can offer high levels of resistance, depending on the plant-pathogen interaction. Even to biosynthesize a single metabolite a hierarchy of *R* genes are required, and these *R* genes are not localized in a QTL or in a chromosome, rather they are localized in several chromosomes (Karre et al. 2017). Thus, transferring a QTL identified to have high level of resistance in one variety to another variety may not always result in increased resistance, as the new variety may not have the other precursor metabolites, or hierarchy of genes to biosynthesize the resistance metabolites. Accordingly, the molecular breeding must be complemented with genome editing to enhance multiple pathogen resistance in commercial varieties (Hu et al. 2018).

The commercial varieties generally have several mutated r genes that occur due to (i) hybridization of land races and other genotypes used in breeding; (ii) mutations induced by environmental agents; (iii) gene transfer by microbes; (iv) horizontal gene transfer. The *R* genes that are mutated in these commercial varieties can be identified based on RNA sequencing, and edited to produce varieties with high multiple pathogen resistance (Hegde et al. 2020, 2021; Kushalappa et al. 2016b). Different combinations of *R* genes with different mechanisms of resistance can be stacked in subsets of cultivars or in different cultivars, which can be temporally and spatially rotated to make them more resilient to changing climates and durable in a locality (Miedaner and Juroszek 2021; Mundt 2014). However, stacking of only the functional immune receptor *R* genes may lead to a 'boom and bust cycle' as the resistance breaks down in the field within a few years (Xin et al. 2012). The immune receptor *R* genes are mainly the surveillance *R* genes that perceive the pathogen, and in turn, regulate the downstream *R* genes that produce reactive oxygen species or callose to induce HR-PCD (Camagna and Takemoto 2018), or they induce other resistance proteins and metabolites to reduce pathogen progress in plants (Kushalappa et al. 2016b).

The resistance due to constitutive and induced metabolites, as well their regulatory and biosynthetic *R* genes, will be focused here. Several *R* genes are also targeted by pathogens to suppress host resistance and invade further, which are generally referred to as the susceptibility genes (*S*-genes) (van Schie and Takken 2014; Zaidi et al. 2018) and these are not discussed.

Selection of *R* genes for genome editing

Genome editing is a technology to change the DNA of an organism, by adding, removing, or altering the genome at a specified location. There are several genome editing tools, but in this review, only the clustered regularly interspaced short palindromic repeats (CRISPR-Cas9) will be addressed, as this is the simplest, cost-effective and versatile (Chen et al. 2019; Wada et al. 2020). The major concern is which gene(s) to edit to improve a given trait, such as disease resistance.

The commercially grown cultivars, with unsatisfactory levels of resistance, also have resistance *R* genes, as in resistant genotypes, but some may be mutated, disabling the plant to code for resistance proteins or metabolites. Plants produce thousands of resistance metabolites, but only a few, depending on the plant-pathogen interaction, can offer high levels of resistance under commercial conditions. A cultivar may have functional *R* genes to biosynthesize a set of precursor monomer metabolites, but if the genes to biosynthesize a complex metabolite from these monomers is mutated, it would be unable to biosynthesize that complex metabolite, rendering the plant susceptible. Each metabolite biosynthesis involves a hierarchy of *R* genes, including both regulatory and biosynthetic *R* genes (Kushalappa et al. 2016a). The mutated (SNPs/InDels) *R* genes in a cultivar can be identified, based on whole genome sequencing (WGS), genotype by sequence (GBS), exome capture (EC) or RNA sequencing (Chung et al. 2017; He et al. 2019; Soni et al. 2020). The resistance metabolites that are produced in high amounts in a resistant genotype relative to a susceptible commercial cultivar can be identified based on metabolic profiling. The reduction in the amount of specific resistance metabolite in a cultivar may be due to mutation in the *R* gene in the respective metabolic pathway. The selection of a few mutated genes for editing to enhance resistance metabolites to enhance resistance, is very challenging, because the metabolic pathway regulation is very complex. Often, if a mutated gene in a metabolic pathway is edited, the immediate biosynthetic metabolite may not be accumulated in that cultivar, as it may be used to

biosynthesize other downstream metabolites, such as polymers and conjugated metabolites, depending on the *R* gene repertoire, the *R* genes induced following pathogen invasion and the current other needs of that cultivar (Hegde et al. 2021). Thus, certain resistance metabolites that enhance resistance to one pathogen may render the plant susceptible to another. Cultivars can be developed with a set of a few specific metabolites that are effective to manage the most devastating pathogens in a region. Cultivars with different combinations of resistance metabolites or *R* genes can be developed for use in crop rotations, to reduce the possible population buildup of specific races or chemotypes of the pathogen (Singh et al. 2011; Zhang et al. 2013). This review will focus mainly on the *R* genes that biosynthesize polymer and conjugate phytoalexins, which reinforce the secondary cell walls in plants to contain pathogens to initial infection areas (Fig. 2, Table 1). The *R* genes proved to be effective for a specific plant-pathogen interaction can also be effective against other plant-pathogen systems, and accordingly, the paralogs and orthologs of these *R* genes in other plants can be searched and used as candidate *R* genes for genome editing to enhance multiple disease resistance.

Polysaccharide metabolites and R genes

Polysaccharides are polymers of sugars or carbohydrates that are biosynthesized from D-glucose (Fig. 2, Table 1). *Callose:* The primary cell walls of the Poaceae are mainly composed of cellulose, arabinoxylans and (1,3;1,4)-β-glucans, whereas the other species, including dicotyledonous plants, contain mainly cellulose, xyloglucan and pectin. Callose is a polymer of β-1,3-glucan biosynthesized by callose synthase (*CalS*) or glucan synthase-like (*GSL*) using UDP glucose as a substrate. The *HvGSL6* enhances pre-penetration resistance to powdery mildew in barley (Chowdhury et al. 2016). In Arabidopsis, the *GSL5, GSL6,* and *GSL11* enhance resistance to powdery mildew (Jacobs et al. 2003). In citrus *CsCalS2* and *7* enhance resistance to Psyllid insect-transmitted bacteria (Granato et al. 2019). The role of fucosylated-xyloglucans and galactomannans was discussed recently (Molina et al. 2021). It was reported that these cell wall-derived xyloglucans are potential DMAPs which can trigger plant immunity (Molina et al. 2021) *Pectin*: This is a structural polysaccharide that contains 1,4-linked α-D-galactosyl uronic acid residues. In Arabidopsis, the powdery mildew resistant 5 (*PMR5*) acetylation protein transfers acetyl groups from acetyl-CoA to oligogalacturonides to resist powdery mildew (Chiniquy et al. 2019). *Glycoside conjugates*: Heteroxylans are (1,4)-β-xylan backbone and, depending upon the species and tissue type, the backbone is substituted to varying degrees with α-arabinofuranosyl (Araf) residues, α-glucuronosyl residues (GlcA), and with feruloylated arabinofuranosyl residues. Sugar glycosides of heteroxylans are biosynthesized by glucosyltransferases (*GT43* and *GT47*) which are deposited to form papillae to resist early penetration of powdery mildew in barley (Chowdhury et al. 2017a). *UDP-glycosides*: *HvUGT13248*, the UDP glucose, conjugates with the deoxynivalenol (DON) to detoxify this mycotoxin, the virulence factor, to resist fusarium head blight in wheat (Li et al. 2017b).

Phenylpropanoid metabolites and R genes

In the Shikimic acid pathway, the precursor metabolite phenylalanine is used to biosynthesize several complex phytoalexins by *R* genes (Fig. S2, Table 1) (Kashyap et al. 2021; Xin and Herburger 2021; Yadav et al. 2020). The enzyme phenylalanine ammonia-lyase (PAL) converts L-phenylalanine to trans-cinnamic acids and is the first dedicated step in the pathway. Genes encoding PAL were characterized and were confirmed to have cassava brown streak disease (CBSD) resistance in cassava (Kavil et al. 2021). *Monomers and polymers*: Of primary importance are the cinnamic acid thioesters and monolignols, biosynthesized by *4CL* and *CAD* genes, in this pathway, that can polymerize and conjugate with other metabolites to reinforce cell walls, thus containing the progress of the pathogen. *4CL* biosynthesizes cinnamic acid thioesters, which depending on the downstream functional genes lead to the biosynthesis of hydroxycinnamic acid amides (HCAAs), lignins and/or lignans in wheat to contain *Fusarium* (Dhokane et al. 2016); in beans to contain *Sclerotinia* (Oliveira et al. 2015); in potato to contain *Phytophthora* (Yogendra and Kushalappa 2016) and in rice to contain *Magnaporthe* (Liu et al. 2017). The *CAD* gene biosynthesizes monolignols, which depending on the downstream functional genes biosynthesizes lignins, lignans and/or glycoside conjugates in wheat to contain *Rhizoctonia* (Rong et al. 2016), in Arabidopsis to contain *Pseudomonas* (Tronchet et al. 2010), and in Populus to contain *Fusarium* and *Rhizoctonia* (Bagniewska-Zadworna et al. 2014). Coniferyl and sinapoyl aldehydes and alcohols are biosynthesized by *CCoAOMT* and *CCR* in maize, potato, tobacco and Arabidopsis to defend against several pathogens (Hegde et al. 2021; Lauvergeat et al. 2001; Maury et al. 1999; Yang et al. 2017). Scopoletin and scopolin are biosynthesized by *F6'H1* in potato and soybean to defend against *Phytophthora* and *Phakopsora*, respectively (Beyer et al. 2019; Hegde et al. 2021; Kai et al. 2008). *Phenol-glycosides*: The phenol monomers crosslink with polysaccharides and lignin to reinforce the cell walls (de O. Buanafina 2009; Reem et al. 2016). Scopoletin-glucoside biosynthesized by the UDP-Glc:phenylpropanoid glucosyltransferases (*UGT*s) enhanced resistance to tobacco mosaic virus and late blight of potato (Chong et al. 2002; Hegde et al. 2021). *Hydroxycinnamic acid amides (HCAAs)*: The HCAAs or phenylamides either directly or as conjugates with hemicellulose are deposited

Table 1 The resistance metabolites, mainly polymers and conjugated, produced in different pathways and their biosynthetic *R* genes in plants, which if mutated can be used as candidates, in genome editing to enhance plant disease resistance

Metabolites	Candidate genes	Host–pathogen interaction	References
Sugars or polysaccharide pathway			
Callose	Glucan synthase like (GSL6)	Barley—*Blumeria graminis*	Chowdhury et al. (2016)
Callose	(GSL5, GSL6, GSL11)	Arabidopsis—*Blumaria graminis*	Jacobs et al. (2003)
Callose	Callose synthase genes (CscalS2 and CscalS7)	Citrus sinencis—*Candidatus Liberibacter asiaticus*	Jacobs et al. (2003)
Pectin	Pectin acyltransferase (Mutant: PMR5)		Chiniquy et al. (2019)
Heteroxylan (Papillae)	Glycosyltransferases (GT43; GT47)	Arabidopsis—*Golovinomyces cichoracearum*	Chowdhury et al. (2017a)
Detoxification (deoxynivelenol—DON)	UDP-3-*O*-glucosyltransferase (HvUGT13248)	Barley—*Blumaria graminis* Wheat/Barley—*Fusarium graminearum*	Li et al. (2017b)
Phenylpropanoid pathway			Kashyap et al. (2021), Xin and Herburger (2021), Yadav et al. (2020)
Phenylalanine ammonia lyase	*PAL1*	Cassava—*Cassava brown streak virus*	Kavil et al. (2021)
p-coumaroyl CoA thioesters[a]	*4-Coumarate: coA ligase; Ta4CL3*	Wheat—*Fusarium graminearum*	Dhokane et al. (2016)
	Pv4CL	Beans—*Sclerotinia sclerotiorum*	Oliveira et al. (2015)
	St4Cl4	Potato—*Phytophthora infestans*	Yogendra et al. (2014)
	OsAAE3 (*4CL* like)	Rice—*Magnaporthe oryzae*	Liu et al. (2017)
Monolignols[a]	Cinnemoyl CoA dehydrogenase (CAD)	Wheat—*Rhizoctonia cerealis*	Rong et al. (2016)
	TaCAD12	Arabidopsis—*Pseudomonas syringae*	Tronchet et al. (2010)
	AtCAD-C and *AtCAD-D*	Populus—*Fusarium oxysporum* and *Rhizoctonia solani*	Bagniewska-Zadworna et al. (2014)
	PoptrCAD11 and *PoptrCAD15*		
Coniferaldehyde, Sinapaldehyde, Coniferyl alcohol, Sinapyl alcohol	Caffeoyl-CoA *O*-methyltransferase (CCoAOMT)	Maize—*Setosphaeria turcica*;	Yang et al. (2017)
	ZmCCoAOMT2	*Cochliobolus heterostrophus*;	Hegde et al. (2021)
	StCCoAOMT	*Cercospora zeae-maydis*	Maury et al. (1999)
	NtCCoAOMT	*Exserohilum turcicum* (NLB)	
		Potato—*Phytophthora infestans*	
		Tobacco—Tobacco mosaic virus	
Hydroxycinnamaldehydes Coniferaldehyde	Cinnamoyl-CoA reductase (CCR)	Arabidopsis -	Lauvergeat et al. (2001)
	AtCCR1 and *AtCCR2*	*Xanthomonas campestris pv. campestris*	
HCAAs	*TaACT; TaWRKY70*	Wheat—*Fusarium graminearum*	Kage et al. (2017b)
p-coumaroylagmatine	*TaMYB4*	Wheat—*Puccinia striformis*	Al-Attala et al. (2014)
Feruloylagmatine	*HvACT; HvWRKY23*	Barley—*Fusarium graminearum*	Karre et al. (2019)
p-coumaroylputrescine			
Feruloylputrescine			
Coumaroyltyramine	Tyramine	Tomato—*Pseudomonas syringae pv*	Campos et al. (2014)
Feruloyltyramine	hydroxycinnamoyl	*Tomato*	Pushpa et al. (2013), Yogendra et al. (2014, 2015, 2017b, a)
	transferase, *SlTHT*	Potato—*Phytophthora infestans*	
	StTHT; StTYDC; StNAC43; StWRKY1		
Hordatine A, B	*HvWRKY23*	Barley—*Fusarium graminearum*	Karre et al. (2019)
	(regulates peroxidase)		
Lignin	Laccase (LAC)	Wheat—*Fusarium graminearum*	Soni et al. (2020, 2021)
	TaLAC4; TaNAC032	Cotton—*Verticillium dahliae*	Hu et al. (2018)
	GhLAC1		

Table 1 (continued)

Metabolites	Candidate genes	Host–pathogen interaction	References
Lignans	Dirigent Gene	Soybean—*Phytophthora sojae*	Li et al. (2017a)
	GmDIR22	Wheat/Tobacco—*P. parasitica*	Ma and Liu (2015)
	TaDIR13	*Isatis indigotica*—Environmental	Xiao et al. (2020)
	IiWRKY		
p-coumaroyl shikimate caffeoyl CoA	Hydroxycinnamoyl transferase (HCT)	Alfalfa—*Colletotrichum trifolii*	Gallego-Giraldo et al. (2011)
Ferulate-polysaccharide-lignin complexes	Uredine diphosphate-dependent Glycosyl-transferases (UGT)	Plant—several pathogens	Reem et al. (2016)
Aromatic suberin	ShMYB78	*Saccharum* sp.—stress induced	Figueiredo et al. (2020)
Stilbenes (resveratrol; pinosylvin)[a]	*Stilbene synthase (VvSTS1)*	Grapes—*Plasmopara viticola*	Chong et al. (2009)
	VqWRKY53, VqMYB13, VqMYB14	*Vitis quinquangularis*—Powdery mildew	Wang et al. (2020)
Flavonoid pathway			Pushpa et al. (2013), Yogendra et al. (2017a)
Naringenin chalcone[a]	*Chalcone synthase (CHS) StCHS*	Potato—*Phytophthora infestans*	Yogendra et al. (2015)
	HvCHS	Barley—*Blumaria graminis*	Dao et al. (2011)
	HvCHS1	Barley—*Fusarium graminearum*	Dao et al. (2011)
Flavonols[a]	*Flavonol synthase (FLS)*	Potato—*Phytophthora infestans*	Yogendra et al. (2015)
Anthocyanidins and anthocyanins[a]	*Dihydroflavonol*	Apple- *Gymnosporangium yamadai*	Lu et al. (2017)
Anthocyanidins	*reductase (DFR); MYB10*	*Gossypium barbadense*—Veritcillium *dahliae*	Long et al. (2018)
	GbANS (anthocyanidin synthase)		
Flavonoid glycosides	HvWRKY23	Barley – *Fusarium graminearum*	
Flavonoid-glycosides:	StNAC43, StMYB8	Potato – *Phytophthora infestans*	Yogendra et al. (2017b)
Flavonoid-glycosides (Anthocyanidin, Kaempferol and Quercetin glycosides	*anthocyanin 7-O-glycosyltransferase (SsGT1)*	*Solanum sogarandinum* (over expressed in Flax)—*Fusarium* sp.	Lorenc-Kukuła et al. (2009)
Fatty acid pathway			Chong et al. (2002), Didi et al. (2015)
Wax (very long chain fatty acids)	β-ketoacyl-CoA synthase	Apple—*Botryosphaeria dothidea*	Zhang et al. (2019)
Cuticular wax and suberin	*MdKCS1;* MdMYB30	Arabidopsis—stress	Mahmood et al. (2019)
	ANAC046 regulates CYP86A1, CYP86B1		
Cutin in cuticle & Fatty acid glycosides	*KAS2, CYP86A2*	Barley—*Fusarium graminearum*	Kumar et al. (2016)
	CYP89A2, LACS2 (acyl-CoA synthase); HvWIN1		
Aliphatic suberin	*AtMYB107, AtMYB9*	Arabidopsis/tomato/potato—stress induced	Lashbrooke et al. (2016)
Suberin with wax	FHT (a fatty ω-hydroxyacid/fatty alcohol hydroxycinnamoyl	*Solanum tuberosum*—stress	Serra et al. (2010)
	Transferase)		
Terpenoid pathway			Li et al. (2007)
Sesquiterpene	*Sesquiterpene synthase*	Rice—*Magnaporthe oryzae*	Chen et al. (2018)
	OsTPS19	Alfalfa—*Aphanomyces euteiches*	Yadav et al. (2019)
	MtTPS10	*Poplar—Phytophthora cactorum*	Lackus et al. (2021)
	PtTPS5	Tobacco—*Potato Virus X*	Li et al. (2015)
	NbTPS1		

Table 1 (continued)

Metabolites	Candidate genes	Host–pathogen interaction	References
Sesquiterpene Gossypol	Sesquiterpene cyclase *(+)-δ-Cadinene Synthase*	Cotton—*Verticillium dahliae*	
Squalene withanolides (Phytosterols)	*Squalene synthase (SQS)* WsWRKY1	*Withania somnifera—Botrytis cinerea*	Singh et al. (2015)
Triterpene-glycoside (Avenacin A-1)	Arabinosyltransferase (AsAAT1 = UGT99D1)	*Avena strigosa – Gaeumannomyces graminis* (takeall disease)	Louveau et al. (2018)
Alkaloid pathway			
Benzylisoquinolines: morphinone, codeine-6-glucuronide and morphine-3-glucuronides	*Tyrosine decarboxylase (TyDC)* *(S)-norcoclaurine synthase (NCS) codeine reductase-2 (COR-2)* StWRKY8	Potato—*Phytophthora infestans*	Yogendra et al. (2017a)
Serotonin alkaloids	*Tryptophan decarboxylase (TDC)*	Capsicum—*Colletotrichum gloeosporioides*	Park et al. (2009)
Camalexin	*Cytochrome P450 monooxygenases CYP79B2 and CYP79B3* WRKY33	Arabidopsis—*Alternaria brassicicola* and *Botrytis cinerea*	Nafisi et al. (2007) Mao et al. (2011)
Alkylferulates in potato periderm, whereby fatty ω-hydroxyacids and fatty alcohols are esterified to feruloyl moieties	FHT Fatty ω-hydroxyacid/ fatty alcohol hydroxycinnamoyltransferase	*Solanum tuberosum*—stress	Serra et al. (2010), Jin et al. (2018)

[a]The monomers may not always accumulate as they may be further used to biosynthesize polymer and conjugated metabolites, if the downstream genes are functional

to reinforce the cell wall to contain progressing pathogens (Kage et al. 2017a, b; Macoy et al. 2015). The coumaroylagmatine biosynthesized by *TaACT* is regulated by TaWRKY70 to resist fusarium head blight in wheat (Kage et al. 2017a, b). Several HCAAs biosynthesized by *StTHT* and *StTyDC* were regulated by *StWRKY1*, *StNAC43* and *StMYB8* enhancing late blight resistance in potato (Pushpa et al. 2013; Yogendra et al. 2014, 2015, 2017a; b). In wheat, *TaMYB4 is* involved in the defense response against *Puccinia striiformis* (Al-Attala et al. 2014). Hydroxy-hordatine B is a dimer of feruloylagmatine that is biosynthesized by the gene peroxidase, which is regulated by *HvWRKY23* in barley to resist *F. graminearum* (Karre et al. 2019). Overexpressing *SlTHT* (tyramine N-hydroxycinnamoyltransferase) in tomato plants increased the hydroxycinnamic acid amide levels and enhanced resistance to *Pseudomonas syringae* (Campos et al. 2014). *Lignin:* This is the major metabolite for secondary cell wall reinforcement and is controlled by several biosynthetic and regulatory genes (Didi et al. 2015). The laccase (*LAC*) genes use oxygen, and the peroxidase (*POD*) genes use H_2O_2 to polymerize p-coumaroyl, coniferyl and sinapoyl alcohols, the monolignols, to hydroxy (H), guaiacyl (G) and syringyl (S) lignins, respectively. The lignin biosynthesized by *TaLAC4* in wheat gives high level of resistance to the spread of *F. graminearum* from the inoculated spikelet to other spikelets in the spike through rachis and this gene is regulated by *TaNAC032* (Didi et al. 2015; Soni et al. 2020, 2021). *GhLAC1* enhanced resistance to Verticillium wilt and is regulated by *GhWEKY1* TF (Hu et al. 2018). *OsNAC122*, 131 enhanced resistance in rice to *Magnaporthe grisea* (Sun et al. 2013). *Lignan:* Are phenylpropanoids with C_6C_3 coupling products, such as (+)-pinoresinol, podophyllotoxin, medioresinol, glucopyranoside and threocarolignan (Gunnaiah and Kushalappa 2014). *GmDIR22* and *TaDIR13* enhanced biotic stress resistance in soybean and wheat (Li et al. 2017a; Ma and Liu 2015). Lignan biosynthesis is regulated by *IiWRKY34* (Xiao et al. 2020). However, suppression of a polymer metabolite biosynthetic *R* gene can alter the metabolic fluxes, as the precursor metabolites can be used by other metabolic pathways, leading to an increase or decrease in resistance to a specific pathogen. The down-regulation of constitutive hydroxycinnamoyl CoA: shikimate hydroxycinnamoyl transferase (*HCT*) in *Medicago sativa* increased flavonoids enhancing resistance to *Colletotrichum* sp. (Gallego-Giraldo et al. 2011). *Aromatic suberins:* Are polyester fractions of phenylpropanoids (aromatic) and ω-hydroxy fatty acids (aliphatic), the former is mainly composed of hydroxycinnamic acids, monolignols and lignans (Pollard et al. 2008; Vishwanath et al. 2015). Suberins are deposited in epidermis, endodermis, and periderm layers (Gunnaiah and Kushalappa 2014; Yogendra et al. 2014). The sugarcane TF *ShMYB78* regulates caffeic acid methyltransferase (*ShCOMT*) to enhance aromatic suberin deposition (Figueiredo et al. 2020; Vishwanath et al. 2015). *Stilbenes*: Pinosylvin and resveratrol are synthesized by stilbene synthase (*STS*) from three malonyl-CoA and one CoA-ester of a cinnamic acid derivative p-coumaroyl-CoA or cinnamoyl-CoA (Chong et al. 2009). The *VvSTS1*, stilbene synthase biosynthesizes stilbenes, such as, resveratrol and pinosylvin to resist *Plasmopara viticola* (Chong et al. 2009). *VqWRKY53*, *VqMYB13*, and *VqMYB14* regulate *VqSTS32* and *VqSTS41* to synthesize resveratrol in grape vine to enhance resistance to powdery mildew (Wang et al. 2020).

Flavonoid metabolites and biosynthetic R genes

In the Shikimic acid pathway, the precursor metabolite p-coumaroyl CoA is used in the biosynthesis of chalcones, flavones, flavonols, anthocyanins, and anthocyanidins, which after glycosylation, methylation and acylation form different conjugated metabolites (Fig. S3, Table 1) (Saito et al. 2013; Tohge et al. 2017). *Monomers and polymers*: *CHS*, Chalcone synthase, in potato and barley, biosynthesize naringenin chalcone, which is further used in the biosynthesis of complex metabolites, to resist *Phytophthora*, *Blumaria* and *Fusarium* (Dao et al. 2011; Karre et al. 2019; Yogendra et al. 2015). *FLS*, Flavonol synthase, biosynthesizes flavonol in potato to resist *Phytophthora* (Yogendra et al. 2017a). *DFR*, dihydroflavonol reductase, regulated by *MYB10*, biosynthesizes anthocyanidins and anthocyanins in apple to resist *Gymnosporangium* (Lu et al. 2017). *GbANS* (anthocyanidin synthase) reduced wilt in cotton (Long et al. 2018). *Flavonoid-glycosides:* The *HvUDPGT* and *HvLAC15* are regulated by *HvCERK1* and *HvWRKY23* to biosynthesize flavonoid-glycosides to resist *F. graminearum* in barley (Karre et al. 2017, 2019). *SsGT1*, anthocyanin glucosyltransferase, over expression in flax significantly increased anthocyanidin, kaempferol and quercetin glycosides, enhancing resistance to *Fusarium* (Lorenc-Kukuła et al. 2009).

Fatty acid and lipid metabolites and biosynthetic R genes

The fatty acids are biosynthesized by acetyl-CoA carboxylase (*ACC*) and FA synthase (*FAS*) and lead to the formation of complex wax layers, cuticles, and aliphatic suberins (Fig. S4, Table 1) (Lim et al. 2017; Pollard et al. 2008). *Wax:* This is a polymer of fatty acids deposited on the cuticle and peridermal layers to prevent pathogen invasion. The C16 and C18 form very-long-chain fatty acids (VLCFA) and are biosynthesized by β-ketoacyl-CoA synthase, β-ketoacyl-CoA reductase, β-ketoacyl-CoA dehydratase, and enoyl-CoA reductase (Lim et al. 2017). The VLCFA biosynthesized by *MdKCS1* is regulated by *MdMYB30*, which is deposited on cuticle as wax layers enhancing resistance in apple to *Botryosphaeria dothidea* (Zhang et al. 2019). *TaKCS6* and *TaECR*

in wheat reduced the conidial germination of *Blumeria graminis*. Overexpression of *ANAC046* in transgenic Arabidopsis plants increased the suberin biosynthesis by regulating well-characterized suberin biosynthetic genes including *CYP86A1* and *CYP86B* (Mahmood et al. 2019). Feruloyl transferase (*StFHT*) enhanced suberin-associated wax biosynthesis in potato tuber periderm (Jin et al. 2018; Serra et al. 2010). *Cuticle*: Are made up of cutin monomers, C16 and C18 ω-hydroxy fatty acids, and polymers of ω-hydroxy fatty acids and glycerol (Pollard et al. 2008). Cutin is a major component of leaf, shoot and fruit epidermis. *LACS2* (Long-Chain Acyl-Coenzyme A Synthetase) in Arabidopsis biosynthesized cutin to resist *Botrytis cinerea* (Tang et al. 2007), and *GPAT4* and *GPAT8* (glycerol-3-phosphate acyltransferase) to resist *Alternaria brassicicola* (Li et al. 2007). Cutin biosynthesized by *LACS2, GPAT4, CYP86A4* and CYP86A7 are regulated by *WAX1* (wax inducer-1 gene) and transported by *WBC11* (ABC transporter) (Kannangara et al. 2007). The *CYP86A2*, *CYP89A2* and *LACS2* are regulated by the *HvWIN1* transcription factor to biosynthesize cutin in barley to enhance resistance to *F. graminearum* (Kumar et al. 2016). *Aliphatic suberins*: Are polyester fractions of ω-hydroxy fatty acids (aliphatic). Suberin is similar to cutin but it contains a wide range of more of α,ω-dicarboxylic acids with a wider range of chain lengths and varying oxygenation, and fatty alcohols and saturated aliphatic > C20 (Pollard et al. 2008; Vishwanath et al. 2015). *AtMYB107* and *AtMYB9* regulated the biosynthesis of suberins in Arabidopsis, potato and tomato (Lashbrooke et al. 2016).

Terpenoid metabolites and their biosynthetic R genes

The terpenoids have five-carbon building blocks of isopentenyl pyrophosphate (IPP) produced in the mevalonic acid pathway (Fig. S5, Table 1) (da Silva Magedans et al. 2021). *Monomers and polymers*: The acetyl-CoA (AcCoA) is the starting unit to biosynthesize farnesyl diphosphate (FDP) through the mevalonate (MVA) pathway. FDP is a central intermediate in the synthesis of triterpene saponins (da Silva Magedans et al. 2021). The *OsTPS19* (terpene synthase) biosynthesized monoterpene limonene to suppress *Magnaporthe oryzae* in rice (Chen et al. 2018). *Sesquiterpenes*: The *MtTPS10* biosynthesized sesquiterpenes in Medicago to resist *Aphanomyces euteiches* (Yadav et al. 2019). The *PtTPS5* biosynthesized two sesquiterpenes to resist *Phytophthora cactorum* in Populus (Lackus et al. 2021). The *NbTPS1* biosynthesized sesquiterpene to resist potato virus X in Nicotiana (Li et al. 2015). Farnesyl Diphosphate Synthase (FPS) and (+)-δ-cadinene synthase (CAD) were expressed in cotton suspension in response to *Verticillium dahliae* elicitors (Liu et al. 1999). *Triterpenes*: The *WsSQS* (sesquiterpene synthase) regulated by *WsWRKY1* biosynthesized squalene to suppress *Botrytis* in *Withania* (Singh et al. 2015). The *AsAAT1* (arabinosyltransferase) enhanced resistance to take-all disease in *Avena* (Louveau et al. 2018).

Alkaloid metabolites and their biosynthetic R genes

Alkaloids are nitrogen-containing compounds such as caffeine, nicotine, and cocaine (Fig. S6, Table 1) (Wink 2019). *Benzylisoquinoline alkaloids*: Are biosynthesized by *StTYDC* (tyrosine decarboxylase) which is regulated by *StWRKY8* in potato to defend against *Phytophthora* (Yogendra et al. 2017a). *Purine Alkaloids:* Caffeine (1,3,7-trimethylxanthine) and theobromine (3,7-dimethylxanthine) are biosynthesized by plants, including coffee, tea, and cacao. Tea caffeine synthase (TCS) biosynthesizes caffeine in tea to defend against *Colletotrichum* (Wang et al. 2016). *Indole-alkaloids*: Camalexin is an indole-alkaloid biosynthesized by *the PAD3* gene regulated by WRKY33 in Arabidopsis defends against *Botrytis* (Mao et al. 2011; Zhou et al. 2020). Cytochrome P450 monooxygenase (*CYP71A13*) catalyzed camalexin synthesis in Arabidopsis to resist *Alternaria brassicicola* (Nafisi et al. 2007). *Serotonin-alkaloids*: Serotonin is biosynthesized by *the T5H* gene and its conjugates feruloylserotinin by *SHT* in *Capsicum* to defend against *Colletotrichum* (Park et al. 2009).

CRISPR-Cas9-based genome editing to enhance multiple disease resistance

The CRISPR-Cas9 genome editing tool can be used either to knock out a gene, to make a functional gene to non-functional, or to knock in a gene, to make a non-functional gene to functional (Fig. S7a, b). The knock-out strategy is based on the CRISPR-induced double-stranded break (DSB) and error-prone non-homologous end joining (NHEJ) repair mechanism. However, with this approach, the development of a loss-of-function phenotype may increase the recessive resistance in plants but is known to have negative side effects on growth and yield (Brown and Rant 2013). CRISPR-Cas9 can also be utilized for gene targeting and generating gain-of-function mutations. There are several gene editing tools, but only three commonly used gene-editing tools will be discussed here (Fig. S7c): (i) CRISPR/Cas9 homology-directed repair (HDR) based gene knock-in; (ii) Base editing; (iii) Prime editing (PE). HDR-based gene targeting to introduce the sequence of choice (repair template or donor) has been considerably improved. The use of geminiviral replicons that provide an abundant supply of donor copies proved to increase the abundance of repair templates and overall HDR efficiency (Baltes et al. 2014; Čermák et al. 2015). Also, efficient gene targeting was achieved in maize by supplying

the donor repair templates from pre-integrated T-DNA (Barone et al. 2020). Gene targeting was achieved in Arabidopsis using the sequential transformation method (Miki et al. 2018). The method used egg cell- and early embryo-specific DD45 gene promoters to express Cas9 in parental lines followed by delivering single-guide RNA and donor template. These methods generally require stable transfer DNA (T-DNA) integration. T-DNA elimination is possible in the case of sexually propagated plant species and not in vegetatively propagated plants. HDR efficiency can also be improved by using different Cas9s. However, the availability of relatively new tools including CRISPR-Cas9-based base editing and prime editing tools has widened the prospects of precise gene modifications. These tools can be more versatile than the HDR-based CRISPR-Cas9 editing since they are simple, and no repair template is required. Adenine base editors (ABEs) were first used in Arabidopsis (Kang et al. 2018). Similarly, cytosine base editors (CBEs) were demonstrated first in wheat, rice, and potato (Zong et al. 2018), and the latter ones are being widely used in several other plant species. However, DNA base editors are restricted to only C to T and A to G substitutions and often result in off-target editing (Rees and Liu 2018; Mao et al. 2019). Prime editing (search and replace method) uses engineered Cas9 nickase (nCas9) fused to reverse transcriptase (RT) paired with a prime editing gRNA (pegRNA) for desired gene modification without DSBs and repair template (Anzalone et al. 2019). Prime editing was adopted in plants in both monocots and dicots with high efficiency (Lin et al. 2020; Lu et al. 2021; Wang et al. 2021). CRISPR-based prime editing has the potential to perform 12 possible base conversions or editing in plant cells (Hassan et al. 2020). Two Cas9 nickase variants, Cas9D10A nickase and a Cas9H840 nickase were used to increase the efficiency of base editors and prime editors, respectively (Anzalone et al. 2019; Mishra et al. 2020). In plants, prime editors were unable to develop homozygous and biallelic edits and were subjected to optimization further. The use of plant-derived promoters, codon optimization of Cas9H840 and different versions of plant editors (PE1, PE2 and PE3) are expected to enhance the efficiency in plants. In addition, the improved PE-PE3 system with engineered M-MLV-RT fused to the N terminal of the Cas9H840 nickase improved the editing efficiency in rice and maize (Sretenovic and Qi 2022). The use of paired pegRNAs and optimized melting temperature of the primer binding site (PBS) increased the editing efficiency in rice (Lin et al. 2020).

CRISPR-Cas9 construct components delivery for transgene-free genome editing

CRISPR-Cas9 can be delivered to plant cells using various methods including ribonucleoprotein (RNP) complexes, as virus particles, particle bombardment, and through *Agrobacterium*-mediated plant transformation. Delivery using *Agrobacterium* is the widely used method and is applicable in varieties of plant species, where different plant parts can be used as the explants. The *Agrobacterium*-mediated transformation method is a favoured method for delivering donor templates and gene targeting (Barone et al. 2020; Danilo et al. 2019).

In seed-producing plants, the segregating population obtained from back crossing of edited plants with non-edited control can be used to screen out any vector DNAs. PCR can be used to screen plants for the absence of marker genes, Cas9, and other transgenes. It can be further confirmed based on deep sequencing (Zong et al. 2018). Transgene sequence elimination through segregation and genotyping to screen transgene-free edited plants is a time-consuming and laborious strategy. TKC (transgene killer CRISPR) technology was developed to accelerate the screening of transgene-free genome-edited plants (Yubing et al. 2019). TKC plasmid vectors were reported to perform self-elimination of transgenes without compromising editing efficiency. The technology was reported to be a promising tool to conduct transgene-free gene editing experiments in cereal crops.

However, there is a challenge to have the elegant transgene or marker excision systems to develop transgene-free genome editing in clonally propagated crops. Only a few studies reported marker excision systems in plants using site-specific recombination methods; two important site-specific recombination systems including, Cre-loxP derived from bacteriophage P1 and FLP/FRT derived from *Saccharomyces cerevisiae*, have been used in plants to avoid marker gene or transgene integration (Chen et al. 2017; Woo et al. 2011). However, both systems are known to leave behind the recognition sequence of the recombinase on the genome. Despite these challenges, the animal-derived PiggyBac transposon system has been validated in plants, where it retained no DNA footprint at the excision site, proving the potential of piggyBac to carry out transgene-free genome editing in plants (Nishizawa-Yokoi and Toki 2021; Nishizawa-Yokoi et al. 2015). In one of the recent developments, PE was combined with piggyBac to produce transgene-free human cell lines, with very high efficiency (Eggenschwiler et al. 2021). In another study, the marker excision system using an I-SceI break and subsequent single-strand annealing (SSA)-mediated DNA repair system, was developed (Endo et al. 2021). Overall, the frequency of occurrence of vector DNA in the recipient plant can be reduced using prime editing alone or in combination with piggyBac (Wolff et al. 2021). This approach can be an alternative to the piggyBac system, to be used along with different types of CRISPR-Cas9 systems to generate transgene-free plants. However, prime editing alone can be used to generate transgene-free plants as was reported with base editing (Veillet et al. 2019).

Besides, transient expression of prime editors and base editors is achievable in plants and is useful in demonstrating transgene-free prime and base editing. DNA-independent delivery of prime editors is feasible since the particle bombardment CRISPR-Cas9 delivery system was optimized using CRISPR/Cas9 DNA or RNA (TECCDNA or TECCRNA)-based genome editing methods (Zhang et al. 2016). In addition, preassembled CRISPR-Cas9 Ribonucleoproteins (RNPs) are a well-established CRISPR delivery system in many plant cells for transgene-free genome editing (Zhang et al. 2021b). Recently, the prime editor was also delivered as RNPs in animal cells and a similar method can be tried in plant cells (Petri et al. 2022). Hence, prime, and base editors can be delivered either as a plasmid using biolistic and *Agrobacterium* or as an RNPs depending on the plant tissue types and regeneration. The antibiotic selection-free method developed by Bánfalvi et al. 2020, is also a suitable method in many plant species, to generate DNA-free plants using *Agrobacterium* transformation.

Plant regeneration and confirmation of edited genes

Irrespective of any available delivery methods, plant regeneration is always challenging in most plant species (Altpeter et al. 2016). The low plant regeneration restricts plant transformation and genome editing, especially in monocots like wheat and barley (Altpeter et al. 2016). However, attempts have been made to improve regeneration efficiency by expressing developmental regulators like BABY BOOM (*BBM*) and WUSCHEL (*WUS*) (Lowe et al. 2016; Maher et al. 2020). Concomitant expression of *WUS* and gene editing reagents in dicots resulted in de-novo meristem induction (Maher et al. 2020). Likewise, expression of *BBM* and *WUS* somatic cell embryogenesis (Lowe et al. 2016). Unfortunately, constitutive expression of *BBM* is shown to inhibit the other major developmental pathways in monocots. But, two recent reports demonstrated the use of regulators GRF-GIF, GROWTH-REGULATING FACTOR (GRF) and GRF-INTERACTING FACTOR (GIF), and their expression along with the gene-editing reagents (Debernardi et al. 2020; Kong et al. 2020). Overexpression of these growth-regulating transcription factors increases the regeneration efficiency in both monocots and dicots (Debernardi et al. 2020; Kong et al. 2020). So, the expression of GRF-GIF chimera along with the base and prime DNA editors can be employed in the rapid transgene-free gene targeting to improve the disease resistance in varieties of plant species.

Confirmation of enhanced resistance and mechanisms

Resistance can be quantified using ecological or epidemiological principles, the monocyclic (involves subprocesses: infection, sporulation and dissemination) and polycyclic processes (several monocyclic processes over time and space) (Kushalappa and Gunnaiah 2013): (i) infection efficiency: proportion of spores infected or proportion of host area infected, quantified as disease severity over time; (ii) lesion expansion: area of lesion or rate of lesion expansion; (iii) latent period: time in days since inoculation until sporulating lesion appearance; and (iv) sporulation: number of spores per unit plant area or rate of sporulation process. The polycyclic process quantification involves the quantification of epidemics over time and space, in the field.

The edited genes and alleles can be screened based on PCR and HRM-PCR (high-resolution melting analysis). Further, the putative clones or transformation events can be confirmed based on Sanger sequencing and chromatograms (Smedley et al. 2021). If any vector DNA is retained, the edited events can be screened to select the transgene-free plants. In the greenhouse, the edited and non-edited control plants can be inoculated with different pathogens, and the disease severity can be assessed, both under lab and field conditions. The disease severity is assessed, visually or using image analysis tools, over time, which then can be used to calculate the area under the disease progress curve (AUDPC) (Mukherjee et al. 2010). The disease progress, both cell damage and external manifestation of internal colonization of pathogen, can be precisely quantified using several digital image analysis tools (Bock et al. 2020; Fordyce et al. 2018; Landeovillanueva et al. 2021; Sarić et al. 2022; Tanner et al. 2022; Thomas et al. 2022). The disease symptoms can be due to internal colonization by pathogens or may also be due to toxins produced by pathogens. The pathogen biomass in the diseased area can be quantified, as mycelial or pathogen cell biomass, using several molecular tools (Ayliffe et al. 2013; Kulik et al. 2020; Lievens et al. 2006; Zhang et al. 2021a).

Resistance in plants is mainly due to resistance proteins and resistance metabolites, which as biochemicals can be antimicrobial or as structures are deposited around the infected cell(s) to contain the pathogen. Confirmation of the mechanisms of resistance due to *R* genes is important to have more confidence in its survival under field conditions. If the *R* gene is involved in metabolite biosynthesis, it can be proved based on metabolic profiling (Allwood and Goodacre 2010). The abundances of expected metabolite(s) and/or their further down conjugated metabolites can reveal the resistance functions (Kushalappa

et al. 2016a). The deposition of polymers and conjugated metabolites to reinforce cell wall can be proved based on histochemical analysis (Bhandari, et al. 2015; Tanner et al. 2022). All the same, the metabolic pathway regulation and eventual accumulation of the types of resistance metabolites at the site of infection is very complex. In Russet Burbank potato when the mutated *StCCoCAMT* gene was edited, instead of an increase in feruloyl-CoA metabolite accumulation, the plant accumulated several downstream conjugated metabolites to suppress the development of *Phytophthora infestans* (Hegde et al. 2021). A comprehensive study on gene functions, however, would require OMICs studies (Kushalappa et al. 2016a).

Conclusion and future perspectives

Plant diseases are one of the major constraints in commercial crop production. This is further exasperated by emerging new races and new pathogens in a region due to changing climate, thus further increasing greenhouse gases and air pollution t^{-1} of grain produced. To meet these challenges, we cannot always start from germplasms to develop new cultivars, as this takes several years to develop, and we should promote genome editing of hundreds of cultivars already developed for each crop to improve some of the traits they may be lacking. Commercial varieties also have several mutated genes, introduced during the hybridization of parents or landraces used in breeding (Wambugu et al. 2018). These mutated genes can be edited to make them functional to recover a given trait. Now that several models and crop plants are already genome sequenced, the number of genes with proven resistance functions is constantly increasing. CRISPR-Cas9 is a precise genome editing tool to improve plant traits but to meet all the regulatory requirements in different countries they need refinement. Patenting and royalty claimed is another major world concern. If the new knowledge originates from the public knowledge accumulated over years, then, the cost of novelty must be reassessed. To meet world hunger, the values of life in our society must encompass humanity, if not, the edited genes in a crop plant would be another destructive combination of atoms in the evolution.

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Declarations

Conflict of interest The authors report no conflict of interest.

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