Transgenic Technology for Disease Resistance in Crop Plants

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Keywords

Transgenic for disease resistance · Pathogen-derived resistance · RNAi · Genome editing tools

23.1 Introduction

The ever increasing demand for food production proportionate to exponential growth of global population evoked the need for applying innovative techniques for developing disease-resistant crop varieties, as the pest and pathogen attack causes considerable yield loss which in turn putting the agriculture sector and crop production in crisis. This is highly significant when the resistance conferred by conventional measures, like artificial hybridization, mutation breeding, marker-assisted selection, etc. appears to be inadequate in many cases, especially due to the evolution of new/more virulent strains/ pathovars/ isolates of pathogens and their unexpected host range expansion. Development of recombinant DNA technology, transgenic expression, and RNA silencing strategies lead to a new era of transgenically engineered resistance in several crop species, many of which succeeded in field trials and got commercialized. Elucidation of genetic and molecular mechanisms underlying host-pathogen interactions, resistance, susceptibility, and different levels of plant immune responses (PTI, ETI, HR, etc.) revealed the key genes in the host as well as pathogens that can be manipulated by transgenic approaches/ techniques for conferring effective resistance. Reprogramming of phytohormonal regulatory pathways determining defense response and remodeling of molecular receptors/

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K. P. Singh et al. (eds.), *Emerging Trends in Plant Pathology*,
https://doi.org/10.1007/978-981-15-6275-4_23

transcription factors involved in resistance or disease development can be brought about by transgenic methods to enhance the resistance in host plants. Targeting of conserved sequences or molecular components could provide broad-spectrum resistance in certain cases. Mining of R genes, transgenic expression of foreign R gene, etc. is another useful strategy. Potential of genome editing based on engineered nucleases like ZFNs, TALENs, and CRISPR/ Cas9 to precisely mutate the genomic sequence of interest can be exploited for specific targeting of the host/ pathogenic genes associated with the biotic stress response. Application of all these approaches for the management of plant pathogens were discussed in this chapter.

During the earlier times, resistant varieties were created by artificial hybridization with wild-type resistant varieties, which was purely depended on phenotypic selection. Diminishing genetic variability and lack of resistant germplasm limited the possibilities of resistant gene introgression via conventional breeding. Moreover, it is time-consuming and associated linkage drag may result in introgression of undesirable traits too. Mutation breeding was an alternative to this, where chemical mutagens are used for inducing mutation in target genes to create desirable phenotype. Resistance to powdery mildew has been successfully engineered in this way by mutating Mlo locus. As mutagenesis is random, the chances of off-target mutations is high. QTL mapping and marker-associated selection and breeding are other ways to create disease-resistant varieties but require large mapping population and are time-consuming and labour-intensive as in the case of hybridization. Even though these conventional strategies enabled crop protection to a great extent, over the centuries, rapid genetic variation and adaptive evolution of pathogens engendered for novel, robust and sustainable methods for controlling pathogens and pest management.

The development of genetic engineering (GE) technology for designing tailor-made plants by transforming plant with the desired gene from other organism was a major breakthrough in crop improvement programs. Initially, transgenic experiments were confined to model plants merely for demonstration and proof-of-concept purpose; later, species wide dispersal of technology was made possible by the development of optimum transformation and regeneration protocols, and this widened the applicability of GE for many economically important crops. Since disease resistance is an important yield determinant, transgenic technologies have given considerable focus on developing/reinforcing resistance in economically important crop species. Crop improvement program for disease resistance gained a significant progress by deploying various GE techniques. Rapid introgression of promising candidate genes, even into phylogenetically unrelated species, precise alteration or modification of host factors involved in the perception of pathogenic effectors and deployment of defence signals, metabolic pathways and biomolecules involved in direct defence responses as well as targeted by pathogen effectors have been successfully implemented using transgenic strategies. Similarly, multitude of pathogenic genes and effectors have also been manipulated transgenically, in order to reduce virulence and pathogenicity. Furthermore, transgenic strategies permit precise spatio-temporal regulation of trait of interest. The emergence of new breeding techniques (NBT), including targeted mutagenesis and precision breeding,

further revolutionized crop improvement strategies. Genome editing tools, like ZFNs, TALENs and CRISPR/Cas9, based on site-directed nucleases enabled precise editing of targeted genomic loci to confer/modify resistance traits in many of the crop plant species. Altogether transgenic strategies facilitated the fine-tuning of the plant immune system for ensuring sustainable production. In this chapter, we will describe about the strategies adopted for creating resistance in transgenic plants against virus, bacteria and fungi and at then the upcoming strategies based on the information obtain from plant–pathogen interactions.

23.2 Transgenic Technology for Virus Resistance

Viruses are notorious plant pathogens causing devastating damage to crop yield. These obligate parasites invade, replicate and proliferate in host cell and result in infection, which reduces fitness and productivity of crop plants and decreases market and aesthetic values of the products. Attributes like great evolvability, large population sizes, error-prone replication and efficient host-dependence render the control of plant viruses extremely difficult. Development of sustained resistance against pathogenic virus, broad-spectrum as well as durable, throughout the productive stage while infection pressure persists is always a challenge in crop improvement. Farmers rely on combining traditional cultural management practices, such as field sanitation, crop rotation, planting of trap plants, spraying for vector, rouging and manual removal of infected plants upon detection of disease symptoms, use of certified virus-free seeds or planting materials (Kamala and Makeshkumar 2015; Deepthi and Makeshkumar 2016), as there is no specific direct control strategy; even chemical pesticides are not available. All these measures are laborious and time-consuming. Chemical control of vectors, in addition, causes health and environmental hazards. The most efficient practical solution available is the use of resistant varieties. But elite cultivars might not always be endowed with resistance traits, which further make the problem complicated and uneconomical. Even though resistance genes can be introgressed from wild varieties, either by conventional breeding or marker-assisted selection, linkage drag and other inherent shortcomings of these techniques render it inadequate for successful crop improvement. Resistance genes in unrelated species or sometimes related species cannot be introgressed by hybridization due to barriers, like sexual incompatibility, male sterility, etc. Similarly, attaining broad-spectrum resistance requires stacking of resistance genes from different sources, which is again a laborious and less efficient strategy. Moreover, virus resistance conferred by R genes is less durable due to the suppression of R–Avr interaction by creating variant Avr protein, which might be unrecognizable by the host R protein, by quickly evolving viral genome, rendering the host susceptible for compatible host-pathogen interaction. However, transgenic approaches have succeeded to a great extend in engineering efficient virus resistance in crop species.

Recessive resistance, conferred by mutating host factors required for infection cycle completion and thereby making the host non-permissive to virus, is more efficient and durable strategy. Resistance thus conferred is passive as there is no

active involvement of plant immune system. Resistance mechanism, to be adopted, and respective targets vary with stage of infection (Johnson 1981).

Transgenic antiviral approaches used so far were based on the expression of various viral proteins; RNAs; nonviral genes like nucleases, antiviral inhibitors and plantibodies; plant defence response elicitors; host-derived resistance genes (dominant resistance genes and recessive resistance genes); and various factors involved in host defence responses. Viral proteins usually used for engineering resistance are capsid protein, replicase proteins and movement proteins. Several RNA molecules, viz. sense RNAs, antisense RNAs, satellite RNAs, defective interfering RNAs, hairpin RNAs and artificial microRNAs, noncoding RNAs, antisense RNAs, ribozymes, double-stranded RNAs (dsRNAs) and inverted repeat RNAs (irRNAs), have been employed for conferring virus resistance by post-transcriptional or transcriptional gene silencing. Transgenic virus resistance strategies are deployed principally by three mechanisms: pathogen-derived resistance, pathogen-targeted resistance and RNA interference.

23.2.1 Pathogen-Derived Resistance

Pathogen-derived resistance (PDR) is protein-mediated resistance conferred by viral protein expressed in host cells (Sanford and Johnston 1985). PDR is accomplished by different a mode of action, which varies with strains and stages of infection cycle, like whether it is in movement/transport/replication phase. It can be inhibition of replication and viral particle accumulation in the early stages of infection while limiting the spread via apoplastic/symplastic/phloem stream during the movement stage (Galvez et al. 2014). The first type of PDR is based on the silencing of pathogenic gene by expression of a part of that gene in host.

23.2.2 Coat Protein–Mediated Resistance (CPMR)

Since coat protein (CP) has implications in almost all stages of infection, like uncoating, systemic movement, long-distance transport, replication, symptom development, etc., it can be the ideal candidate for engineering PDR, even in non-host plants (Galvez et al. 2014). CPMR is manifested by expression of coat protein gene in the host cell and the subsequent interaction between transgenic CP and viral CP (Koo et al. 2004). The very first time application of CPMR was showed in tobacco (*Nicotiana tabacum*) plants, expressing the capsid protein-encoding sequences of *Tobacco mosaic virus* (TMV), and this resulted in partial resistance to TMV (Abel et al. 1986). Later it was expanded to tomato, using the same CP construct, with resistance to *Tomato mosaic virus* (ToMV) (Nelson et al. 1988). Similarly, capsid protein gene of a potyvirus was expressed in a non-host plant, tobacco, conferring resistance to other potyviruses. CPMR has been used to confer resistance to at least 35 viruses, representing more than 15 different taxonomic groups (Table 23.1). Several virus-resistant transgenic crop plants were developed by using a suitable

Table 23.1 Transgenic plant species showing coat protein-mediated resistance

Host plants	Virus	References
Citrus	*Citrus mosaic virus* (CiMV)	Iwanami et al. (2004)
	Citrus psorosis virus (CPsV)	Reyes et al. (2011)
	Citrus tristeza virus (CTV)	Domínguez et al. (2002)
		Febres et al. (2008)
		Loeza-Kuk et al. (2011)
Cucumber	*Zucchini yellow mosaic virus* (ZYMV)	Wako et al. (2001)
Squash	*Squash mosaic virus* (SqMV)	Pang et al. (2000)
Watermelon	*Cucumber green mottle mosaic virus* (CGMMV)	Park et al. (2005)
	Cucumber mosaic virus (CMV) and *Watermelon mosaic virus* (WMV)	Lin et al. (2012)
	WMV	Wang et al. (2003)
	Zucchini yellow mosaic virus (ZYMV) ZYMV	
	ZYMV and *Papaya ringspot virus* -W (PRSV-W) PRSV-W	Yu et al. (2011)
Brinjal	CMV	Pratap et al. (2011)
Lettuce	*Lettuce big-vein associated virus* (LBVaV)	Kawazu et al. (2006)
Melon	CMV	Xu et al. (2005)
	ZYMV	Wu et al. (2009)
	ZYMV and PRSV-W	Wu et al. (2010)
Soybean	*Bean pod mottle virus* (BPMV)	Reddyet al. (2001)
	Soybean dwarf virus (SDV)	Tougou et al. (2007)
		Wang et al. (2001)
	Soybean mosaic virus (SMV)	Furutani et al. (2006)
Rice	*Rice stripe virus* (RSV)	Park et al. (2012)
	Rice tungro bacilliform virus (RTBV)	Ganesan et al. (2009)
	Rice tungro spherical virus (RTSV)	Verma et al. (2012)
	Rice yellow mottle virus (RYMV)	Kouassi et al. (2006)
Maize	*Maize dwarf mosaic virus* (MDMV)	Liu et al. (2005)
	Sugarcane mosaic virus (SCMV)	Liu et al. (2009)
Wheat	*Wheat streak mosaic virus* (WSMV)	Sivamani et al. (2002)
		Li et al. (2005)
Orchid	*Cymbidium mosaic virus* (CymMV)	Chang et al. (2005)
	CymMV	Liao et al. (2004)
Papaya	*Papaya ringspot virus* (PRSV)	Lines et al. (2002)
		Ferreira et al. (2002)
		Fermin et al. (2004)
		Souza et al. (2005)
		Kertbundit et al. (2007)
	PRSV and *Papaya leaf-distortion mosaic virus* (PLDMV)	Kung et al. (2009)
		Kung et al. (2010)

(continued)

Table 23.1 (continued)

Host plants	Virus	References
Groundnut	*Peanut stripe virus* (PStV)	Higgins, et al. (2004)
	Tobacco streak virus (TSV)	Mehta et al. (2013)
Capsicum	CMV	Lee et al. (2009)
		Pack et al. (2012)
	CMV and *Pepper mild mottle virus* (PMMoV)	Shin et al. (2002)
	CMV and *Tomato mosaic virus* (ToMV)	Shin et al. (2002)
Sugarcane	*Sugarcane mosaic virus* (SCMV)	Guo et al. (2008)
	Sugarcane yellow leaf virus (SCYLV)	Zhu et al. (2011)
Sweet potato	*Sweet potato feathery mottle virus* (SPFMV)	Okada et al. (2001)
		Okada and Saito (2008)
		Okada and Yoshinaga (2010)
Potato	*Potato virus Y* (PVY)	Rachman et al. (2001)
Plum	*Plum pox virus (PPV)*	Scorza et al. (1997); Ilardi and Tavazza (2015)
Sweet pepper	*Cucumber mosaic virus*	Zhu et al. (1996)
Nicotiana tabacum cv. Petit Havana	*Cucumber mosaic virus* (CMV) subgroup IA	Dubey et al. (2015)
Tomato	CMV	Pratap et al. (2012)
	Physalis mottle virus (PhMV)	Vidya et al. (2000)
	Tomato leaf curl virus (ToLCV)	Raj et al. (2005)
	Tomato leaf curl Taiwan virus (ToLCTWV)	Sengoda et al. (2012)
	Tomato spotted wilt virus (TSWV)	Gubba et al. (2002)

coat protein gene (Mundembe et al. 2009; Nomura et al. 2004). CPMR has been successfully established in host plants, including potato, tomato, tobacco and papaya, exhibiting resistance to *Potato virus Y* (PVY) and *Potato leaf roll virus* (PLRV), *Cucumber mosaic virus* (CMV) and *Papaya ring spot virus* (PRSV) (Makeshkumar et al. 2002). The efficiency of CPMR varies for each virus with different stages of infection cycle (Bendahmane et al. 2007). The underlying molecular mechanism of resistance manifestation is either through recoating of invading viral particles or by blocking of receptors in transgenic plants (Saharan et al. 2016).

23.2.3 Replicase- or Rep-Associated Protein-Mediated Resistance

Replicase is another potential candidate to engineer resistance against viral genome. Expression of intact or truncated or mutant virus-encoded replicase in host can confer resistance to that virus. It was reported for the first time when a 54 kDa truncated protein of TMV replicase protein was expressed in *N. benthamiana*, where it conferred high level of resistance against TMV infection (Golemboski et al. 1990).

Table 23.2 List of various transgenic plants developed with replicase-mediated resistance

Plant species	Virus
Potato	*Potato leafroll virus* (PLRV)
	Potato virus Y (PVY-N) tobacco rattle virus (TRV)
Rice	*Maize dwarf mosaic virus* (MDMV)
	Rice tungro spherical virus (RTSV)
	Rice yellow mottle virus (RYMV)
Tomato	CMV
Watermelon	CMV, *Zucchini yellow mosaic virus* (ZYMV), and WMV
Wheat	*Wheat streak mosaic virus* (WSMV)
	Wheat yellow mosaic virus (WYMV)
Papaya	*Papaya ringspot virus* (PRSV)
Citrus	*Citrus tristeza virus* (CTV)
Cucumber	*Cucumber fruit mottle mosaic virus* (CFMoMV)
Barley	*Barley yellow dwarf virus* (BYDV-PAV)

This strategy has been extended to many food crops, including rice, bean, potato etc., and mostly resulted in narrow-spectrum resistance towards a particular race of the pathogen (Saharan et al. 2016) (Table 23.2). Rep-associated protein, which interacts with host DNA polymerase during replication of ssDNA virus, can also be manipulated in a similar manner. Resistance to two ssDNA viruses, *Tomato yellow leaf curl virus-Israel* (TYLCV-Is [Mild]) and *Bean golden mosaic virus* were conferred by transgenic expression of a truncated replication-associated protein gene and rep gene, respectively, in tomato and *P. vulgaris* (Brunetti et al. 1997; Faria et al. 2006). As in the case of CPMR, the active entity conferring resistance can either be protein or RNA or both.

23.2.4 Movement Protein–Mediated Resistance

Movement proteins (MP) facilitate the intracellular or cellular movement of viral particles through plasmodesmata, by modifying the gating channels of plasmodesmata. Resistance conferred by dysfunctional or mutated MP is mostly broad-spectrum in nature when compared to CP or replicase-mediated resistance (Prins et al. 2008). The first MP-mediated resistance was shown in transgenic tobacco plants expressing a 30 kDA mutant defective MP (dMP), which competed with the wild-type virus-encoded MP for the binding sites in the plasmodesmata and conferred resistance against eponymous virus infection. dMP conferred broad-spectrum resistance by preventing the systemic spread of distantly related and unrelated viruses (Lapidot et al. 1993; Cooper et al. 1995). Similar broad-spectrum resistance was observed in transgenic potato expressing wild-type *Potato leafroll virus* (PLRV) movement protein against PLRV, PVY and PVX (Tacke et al. 1996), while narrow-spectrum resistance was shown in transgenic *N. benthamiana* expressing wild-type movement proteins of *Cowpea mosaic virus* (CPMV) and transgenic tobacco expressing PVX movement protein (Sijen et al. 1995).

23.2.5 Other Viral Protein-Mediated Resistance

Transgenic expression of viral proteins other than those discussed previously, such as replication-associated protein, NIa protease, P1 protein and HC- Pro, has been tried out in order to achieve resistance against viruses (Cillo and Palukaitis 2014). Transgenic expression of partial or complete Rep gene was found to confer resistance against *Tomato golden mosaic virus* (TGMV), *Tomato yellow leaf curl virus* (TYLCV) (Yang et al. 2004; Antignus et al. 2004; Lucioli et al. 2003), *African cassava mosaic virus* (ACMV) (Chellappan et al. 2004), *Bean golden mosaic virus* (BGMV) (Faria et al. 2006), *Maize streak virus* (Shepherd et al. 2007), *Cotton leaf curl virus* (CLCuV) (Hashmi et al. 2011) and *Tomato leaf curl Taiwan virus* (ToLCTWV) (Lin et al. 2012) in transgenic plants.

Transgenic tobacco plants expressing the NIa protein of *Tobacco vein mottling virus* (TVMV) exhibited resistance against TVMV, whereas it failed to confer resistance to two other potyviruses, *Tobacco etch virus* (TEV) and PVY (Maiti et al. 1999). Transgenic tobacco lines expressing paired NIa protease coding sequences for two viruses, like TEV–PVY, TEV–TVMV and TVMV–PVY, were assessed for virus resistance and mostly resulted in the recovery-type resistance (Fellers et al. 1998). However, expression of multiple genes (NIa/NIb/capsid protein) from a single potyvirus using a single construct failed to confer any enhanced resistance in most cases (Maiti et al. 1999).

Transgenic expression of complete or partial sequence encoding P1 protein conferred resistance, of varying degree, against viruses like PVY, PPV, TVMV and PVA. A recovery-type resistance was obtained in most cases rather than complete resistance (Germundsson and Valkonen 2006). Expression P1 coding sequence of PVY-O and PPV, respectively, showed either complete resistance or a recovery-type resistance against PVY-O infection in potato and PPV infection in *N. benthamiana* (Maki-Valkama et al. 2001; Tavert-Roudet et al. 1998).

Expression of viral HC-Pro protein resulted in recovery phenotypes instead of conferring resistance to PVA and PPV in transgenic *N. benthamiana* and SMV in transgenic soybean (Savenkov and Valkonen 2002; Barajas et al. 2004; Lim et al. 2007). Expression level of transgene considerably influenced the resistance mediated by HC-Pro in most of the experiments, wherein low expression levels are mostly favoured for resistant or recovery phenotype rather than high expression levels. Deletion of central domain of HC-Pro protein has found to confer recover disease symptoms of *Cowpea aphid-borne mosaic virus* (CABMV) in transgenic *N. benthamiana* compared to intact protein (Mlotshwa et al. 2002).

Other viral genes, such as VPg-protease coding region of *Tomato ringspot virus* (ToRSV), capsid protein domain of BNYVV (Andika et al. 2005) and p23 silencing suppressor protein of *Citrus tristeza virus* (CTV) (Fagoaga et al. 2006), were also used as potential transgene candidates for providing resistance against ToRSV infection in *N. benthamiana*, *olymyxa betae* infection in transgenic *N. benthamiana* plants and CTV infection in transgenic Mexican lime plants, respectively (Table 23.3).

Table 23.3 List of transgenic crop plants with hairpin-mediated resistance against viruses

Transgenic host plant	Disease/virus	Hairpin construct used	References
Cassava	ACMV	Rep gene of ACMV	Vanderschuren et al. 2012
	Cassava brown streak virus	Capsid protein of CBSV	Ogwok et al. (2012)
	Cassava brown streak Uganda virus	Rep gene of CBSV	Chauhan et al. (2015), Wagaba et al. (2017), Vanderschuren et al. (2012), Yadav et al. (2011)
	SLCMV	AV1 and AV2	Ntui et al. (2015)
Citrus	*Citrus psorosis virus* (CPsV)	RNA3 of CPsV	Reyes et al. (2009)
		p24 gene on RNA1	Reyes et al. (2011)
	CTV	p23 gene and 3'NTR	Lopez et al. (2010)
		Capsid protein gene	Muniz et al. (2012)
		RNA silencing suppressor (CTV)	Soler et al. (2012)
Cucurbits	PRSV	Capsid protein coding sequences of PRSV	Krubphachaya et al. (2007)
	CGMMV	Capsid protein gene of CGMMV	Kamachi et al. (2007)
	Melon necrotic spot virus (NSV)	Cm-eIF4E translation initiation factors (eIF)	Rodríguez-Hernández et al. (2012)
	ZYMV	HC-pro encoding sequences of ZYMV	Leibman et al. (2011)
Common bean	BGMV	C1 gene of BGMV	Bonfim et al. (2007)
Soybean	*Soybean mosaic virus*	Coat protein, VSR Hc-pro, P3 cistron	Kim et al. (2016), Gao et al. (2015), Yang et al. (2018)
	Soybean dwarf virus (SbDV)	Coat protein	Tougou et al. (2006)
Cowpea	*Cowpea aphid-borne mosaic virus*	Coat protein	Cruz et al. (2014)
White clover	WCIMV	Replicase gene of WClMV	Ludlow et al. (2009)
Maize	SCMV	NIb coding sequences of SCMV	Zhang et al. (2010)
	Maize dwarf mosaic virus (MDMV)	P1 coding sequence or capsid protein coding sequence of MDMV	Zhang et al. (2011)
Potato	PVY-N, certain isolates of PVY-NTN and PVY-O	3'part of the PVY-N capsid protein coding sequences of PVY-N	Missiou et al. (2004)
	PVX, PVY-O, PVY-N, PVY-C	Fused PVX capsid protein and PVY Nib encoding region	Bai et al. (2009)

(continued)

Table 23.3 (continued)

Transgenic host plant	Disease/virus	Hairpin construct used	References
	PVX, PVY and PLRV	Fused sequences from PVX, PVY and PLRV	Arif et al. (2012)
	PVY-O and PVY-NTN	PVY capsid protein	McCue et al. (2012)
	PVA, PVY, PLRV	Fused hpRNA sequences of three viruses (PVA, PVY and PLRV)	Chung et al. (2013)
Sweet potato	SPFMV and SPCSV	Capsid protein of *Sweet potato feathery mottle virus* (SPFMV)	Nyaboga et al. (2008)
Tomato	TYLCV	C1 (rep) gene	Fuentes et al. (2006)
	TYLCV	TYLCV capsid protein	Zrachya et al. (2007)
	TYLCV (Sardinia variant), TYLCV	Rep gene of TYLCV (Sardinia variant)	Tamarzizt et al. (2009)
	PSTVd	hpRNAs derived from PSTVd	Schwind et al. (2009)
	CMV	Replicase	Ntui et al. (2015)
Wheat	WSMV	NIa coding sequence (WSMV)	Fahim et al. (2010)
	BYDV (PAV)	Polymerase gene of BYDV (PAV)	Yassaie et al. (2011)
Maize	*Maize dwarf mosaic virus* (MDMV)	Coat protein	Zhang et al. (2011)
Barley	*Barley yellow dwarf virus* (BYDV)	Polymerase gene	Wang et al. (2000)
Banana	*Banana bunchy top virus*	Master replication initiation protein of bunchy top virus	Elayabalan et al. (2013)
Rice	*RDV*	Pns4 or Pns12 genes of RDV	Shimizu et al. (2009)
	RSV	pC1 (replicase), pC3 (nucleocapsid) and pC4 (movement protein) of RSV	Shimizu et al. (2011)
	RSV	Nucleocapsid protein gene or disease-specific protein gene of RSV	Ma et al. (2011)
			Zhou et al. (2012)
			Park et al. (2012)
	Rice black streaked dwarf virus (RBSDV)	Non-structural gene (P9–1) of *Fijivirus* (RBSDV)	Shimizu et al. (2012)

(continued)

Table 23.3 (continued)

Transgenic host plant	Disease/virus	Hairpin construct used	References
	Rice gall dwarf virus	Non-structural gene (Pns9) of *Rice gall dwarf virus*	Shimizu et al. (2012)
	Rice ragged stunt virus	Nucleocapsid protein or movement protein gene of *Rice ragged stunt virus*	Shimizu et al. (2013)
Tobacco	CMV	Coat protein	Chen et al. (2004)
	PPV	VSR P1 and HC- pro	Di Nicola-Negri et al. (2005)
	CGMMV	Coat protein	Kamachi et al. (2007)

23.2.6 Viral RNA-Mediated Resistance

Although virus resistance has been successfully manifested in several crop species by transgenic expression of intact or dysfunctional or mutated structural proteins of virus, resistance in many plants has been found to be mediated by corresponding mRNAs rather than the encoded protein moieties (Saharan et al. 2016). The active role of RNA in conferring resistance was first revealed when an untranslatable coat protein gene in transgenic tobacco plants provided resistance against *Tobacco etch virus* (TEV) (Lindbo and Dougherty 1992a, b). Many cases of transgenic expression of untranslatable viral proteins further substantiated the direct participation of RNA in developing resistance. Later, it has been shown that the virus resistance can be RNA-mediated, and viral protein expression is not necessary for the same (Dougherty et al. 1994). Viral RNA-mediated resistance is carried out by transcriptional gene silencing (PTGS), RNA interference or RNAi or RNA silencing, where a transgenic viral RNA introduced into the host plant drives sequence-specific homology-dependent degradation of viral genomic RNA or viral mRNAs by employing the small interfering RNA (siRNA) pathway of host cell machinery (Voinnet 2008; Waterhouse et al. 2001; Lindbo and Dougherty 2005). This homology-dependent gene silencing mechanism is conserved among higher eukaryotes and operates for gene expression regulation and in host defence against transposable elements and viruses (Hannon 2002; Mawassi and Gera 2012).

23.2.7 RNA Interference: Mechanism

RNA interference is widely accepted as a potential gene silencing strategy that can be easily manipulated to obtain desirable trait in organism of interest. RNA silencing process requires a double-stranded RNA precursor to trigger the silencing process. This dsRNA precursor is derived from viral RNA intermediates or viral RNA secondary structures. After recognition of this dsRNA by the RNase III-like enzyme, namely, DICER, it is cleaved into 21–25 nucleotide duplexes, called as small

interfering RNAs (siRNAs). The siRNAs thus formed are incorporated and converted to ssRNAs by Argonaut (Ago) containing multisubunit ribonuclease named as RNA-induced silencing complex (RISC). Subsequently RISC targets the specific mRNAs that share sequence similarity with siRNA through degradation of transcript (Waterhouse et al. 1998; Hamilton and Baulcombe 1999; Voinnet 2008).

RNA-mediated resistance engineering approaches include expression of non-coding regions of viral genome, viral CP mRNA, satellite (sat) RNA, defective interfering (DI) RNA and viral sequences in sense or antisense orientation or in double-stranded forms in host plants (Cillo and Palukaitis 2014).

Non-coding ssRNA Viral non-coding RNAs like 5′ and 3′ NTRs, intergenomic regions and non-coding RNAs, either in sense or antisense orientation, have been used for engineering resistance in various crop species. Transgenic expression of 5′ and 3′ NTRs and intergenomic regions could successfully confer resistance in crop plants. Sometimes simultaneous expression of non-coding NTR regions with mRNAs also confer resistance. For instance, transgenic tobacco expressing 3′-NTR of *Andean potato mottle virus* (APMoV), which was expressed with a smaller portion of capsid protein-coding region, showed excellent resistance in several lines (Vaskin et al. 2001). Transgenic oilseed rape plant expressing 3′-NTR of *Turnip yellow mosaic virus* (Zaccomer et al. 1993) and intergenic region of PLRV expressing transgenic potato (Dong et al. 1999) showed resistance to respective pathogens.

Non-translatable Sense RNAs Non-translatable sense RNAs of coat protein created by frameshift mutation have been widely used to confer resistance to a large number of crops, including tobacco, rice, papaya, peanut, grape, sugar cane, etc. Expression of frameshifted, capsid protein coding sequences of PVY-NTN in transgenic potato was shown to be resistant to PVY^{NTN} infection (Rachman et al. 2001). There are some other viral proteins which can be suitable candidates to be manipulated in similar manner to engineer resistance. Potential of non-translatable coding sequences of movement protein, nucleoprotein, Vpg, NSm, etc. to mediate viral gene silencing has been proved through different experiments. Expression of non-translatable form of the capsid protein gene has been conferred resistance to *Papaya leaf-distortion mosaic virus* (PLDMV) (Kung et al. 2010), *Zucchini yellow mosaic virus* (ZYMV) and PRSV-W (Wu et al. 2010; Yu et al. 2011) and *Sugarcane yellow leaf virus* (Zhu et al. 2011). Transgenic tobacco expressing a non-translatable form of the NSM coding region of TSWV and a non-translatable form of the TEV 6-kDa/VPg encoding region exhibited resistance towards TSWV and TEV, respectively (Prins et al. 1996; 1997; Swaney et al. 1995). Moreover, hybrid constructs, designed by a fusion of non-translatable coding sequence of coat protein of different viruses, have found to be efficient for developing broad-spectrum resistance.

Antisense RNAs Transformation of host plant with antisense RNAs of certain viral genes is a powerful strategy to silence invading viral genes. Transgenic plants expressing sense RNAs for viral replicase, transcription activator protein (TrAP),

replication enhancer protein (Ren), AV1, etc. had been developed. Antisense RNA-mediated silencing mostly resulted in attenuated or delayed symptoms and complete resistance in some cases. Trials to engineer resistance to *Tomato leaf curl virus* (Praveen et al. 2005), *African cassava mosaic virus* (Zhang et al. 2005) and *Mung bean yellow mosaic India virus* (Singh et al. 2013) have been performed.

Satellite RNA Some viruses exhibit a supernumerary RNA component that does not show any apparent homology to the viral RNA genome but depends on its helper virus for replication, encapsidation and transmission and is defined as a satellite RNA (Simon et al. 2004). These sequences have found to have a highly variable range of effects on various components like viral replication, pathogenesis and symptom expression in certain host-pathogen interactions. Sat variants with attenuating effects are regarded as potential biocontrol agents in transgenic plants. Transgenic expression of an attenuating CMV-associated satRNA suppressed viral replication and symptom development and thereby conferred tolerance to CMV in tobacco and other solanaceous plants including tomato (Baulcombe et al. 1986; Harrison et al. 1987; Kim et al. 1997; Kim et al. 1995)). Transgenic *N. benthamiana* and *A. thaliana* plants expressing *Bamboo mosaic virus* (BaMV) satRNA showed high resistance to helper virus (Lin et al. 2013).

Defective Interfering DNAs and RNAs Defective DNAs and RNAs are produced during the replication of certain viral species, which can reduce the full-length genome accumulation and results in the denomination of DI nucleic acids, associated with the modulation of symptom expression, and thus serves as a source for transgenic resistance strategies. ssDNA geminiviruses, like ACMV, and ssRNA *Tombusvirus*, like *Cymbidium ringspot virus* (CymRSV), are known to form DIs. Interfered viral replication and milder symptoms were observed in transgenic *N. benthamiana* expressing naturally occurring subgenomic ACMV DNA B upon ACMV infection (Stanley et al. 1990). Tolerance to other viral species, including *Beet curly top virus* (BCTV) (Frischmuth and Stanley 1998; Stenger 1994), *Cucumber necrosis virus* (CNV), *Carnation Italian ringspot virus* and CymRSV (Kollàr et al. 1993; Rubio et al. 1999), has been obtained by employing DI DNA and DI RNA.

Silencing by Ds Inverted Repeat Sequence/hpRNA Combined expression of sense and antisense transcripts in the same transgenic plants is advantageous for obtaining more transgenic lines exhibiting resistance. It is more efficient than the expression of either strand alone (Waterhouse et al. 1998; Smith et al. 2000; Waterhouse and Helliwell 2003). This was made possible by designing a single transcript of both polarities, with sense and antisense sequences separated by introns or other spacer sequences, to generate dsRNAs with loops, known as hpRNAs, intron hpRNAs (ihpRNAs) or irRNAs, which stabilize the inverted repeat DNA sequences in *Escherichia coli* (Smith et al. 2000; Wesley et al. 2001). dsRNA thus produced is then acted upon by siRNA pathway, resulting in virus resistance (Castel and Martienssen 2013; Csorba et al. 2009; Ding 2010; Eames et al. 2008; Pumplin

and Voinnet 2013). Broad-spectrum resistance can be achieved by creating a chimeric, fused hpRNAs expressing sequences of several viruses from the same vector (Cillo and Palukaitis 2014). The application of hpRNAs as a transgenic resistance strategy was first demonstrated in transgenic tobacco and barley expressing a hpRNA specifically targeting NIa protease coding sequences of PVY and polymerase coding sequences of BYDV, respectively (Smith et al. 2000, Abbott et al. 2000). Development of viral resistance in crop plants was tremendously advantaged from the establishment of simple and efficient technique for stable integration of self-complementary hairpin construct, designed specifically for the cognate RNA target. dsRNA and siRNA are generated in host cell and silence the pathogen gene expression by cleaving target RNA. The major crop plants, like rice, maize, citrus, cassava, legumes, etc., have been conferred durable and efficient resistance using this method (Cillo and Palukaitis 2014). Although RNAi confers efficient and durable resistance, plant viruses have evolved smart measures, like suppressors of RNA-induced gene silencing, to escape RNAi which is a challenge before the advancements of transgenic resistance (Table 23.3).

Silencing by Artificial MicroRNAs MicroRNAs are small (20–250 nt), non-coding RNAs present in eukaryotic cells and facilitate gene regulation at post-transcriptional or translational level (Bartel 2004). The precursor miRNA transcripts are processed into mature miRNA and directed to target mRNA by the same mechanism involving DICER, Ago and RISC, as that of siRNAs (Jones-Rhoades et al. 2006). Customized miRNA precursors producing target-specific siRNAs, which are having no similarity with mature endogenous miRNAs, known as artificial microRNAs (amiRNAs), can be transgenically expressed to target invading viral sequences. amiRNA-mediated virus resistance was first reported in transgenic *Arabidopsis* showing resistance against *Turnip yellow mosaic virus* (TYMV) and *Turnip mosaic virus* (TuMV) by stable expression of amiRNAs targeting the RNA sequences encoding silencing suppressors P69 and HC-Pro of the virus (Niu et al. 2006). amiRNAs targeting RNA silencing suppressors of virus have found to be more efficient in conferring resistance either complete resistance or delayed infection/susceptibility, when compared to those targeting CP or other structural proteins (van Vu et al. 2013). Silencing suppressors Hc-Pro and TGB1/p25 of PVY and PVX, respectively, were targeted efficiently by amiRNAs (Ai et al. 2011). amiRNAs have been demonstrated to confer resistance against a vast range of viruses, including positive-sense ssRNA genome such as CMV, PVY and PVX; negative-sense ssRNA viruses like WSMoV (genus *Tospovirus*); and ssDNA viruses of the genus *Begomovirus* like *ToLCV New Delhi* variant (van Vu et al. 2013) and *Cotton leaf curl Burewala virus* (Ali et al. 2013). Efficiency of amiRNA-induced viral gene silencing is determined by several factors, like expression levels of pre-miRNA backbone and sequence complementarity of amiRNA with target and structural features of target RNA and accessibility of amiRNA to target RNA (Ali et al. 2013; Cillo and Palukaitis 2014; Simón-Mateo and García 2011; Duan et al. 2008). Viruses may quickly evolve new strains with mutations in amiRNA target site and escape silencing, which can be circumvented by simultaneous targeting of

multiple regions of highly conserved RNA motifs of viral genome with multiple amiRNAs. A polycistronic amiRNA designed from a modified rice miRNA395, targeting different conserved regions of the WSMV, conferred resistance to WSMV in wheat (Fahim et al. 2012).

Silencing by Co-suppression Resistance to virus can be achieved by activating co-suppression by introducing surplus amount of viral transcripts in the host, where overabundance of sense strand induces downregulation or suppression of transgene and invaded viral gene. Such overabundance of sense strand leads to the removal of all homologous transcripts, beyond a critical threshold, by respective cellular machineries (Stam et al. 1997). Overexpressed transcripts, when recognized by plant RNA-dependent RNA polymerase, act as primers for dsRNA synthesis, which then subjected to cleavage and degradation by DICER and RISC complex (Dougherty and Parks 1995).

23.2.8 Transgenic Technology to Engineer Pathogen-Targeted Virus Resistance

PDR and R gene often do not confer complete resistance to invading viruses, and mostly resistance is not stable over several generations. Pathogen-targeted resistance is manifested by introducing silencing constructs into host plants, where it targets viral genome. Synthetic constructs designed to target viral sequences are neither of plant origin nor pathogen-derived. Such non-viral-mediated resistance is manifested by transgenic expression of synthetic nucleases like zinc finger nucleases, transcription activator like effector nucleases, CRISPR/Cas9, antiviral inhibitor proteins like ribosome-inhibiting proteins, peptide aptamers and plantibodies (Bastet et al. 2018).

Nucleases Resistance against *Fijivirus* RBSDV and TMV was conferred by transgenic expression of an *E. coli* dsRNase gene (Cao et al. 2013), bovine pancreatic RNase (Trifonova et al. 2007) and an inducible extracellular RNase from *Zinnia elegans* (Trifonova et al. 2012). *E. coli* dsRNase (RNase III) conferred high-level resistance to a tomato isolate of TSWV also (Langenberg et al. 1997). Feasibility of transgenic expression of antiviral pathways or invading nucleic acid targeting pathways from heterologous systems in host plant for targeting infectious viruses has been demonstrated using OAS system 2,5A-oligoadenylate synthetase (OAS)/RNase L system, which is also called as 2,5A oligoadenylate pathway, an animal antiviral pathway induced by interferons in mammalian cells. The OAS catalyses the polymerization of ATP producing 2-5-linked oligoadenylates, pppA (2_p5_A) nor 2,5A, when it recognizes a dsRNA, which can be replicative intermediates of single-stranded RNA viruses or viral dsRNA genomes. The subsequent activation of the latent ribonuclease, RNAse L, by the 2,5A oligonucleotides produced by OAS, carries out the degradation of both viral and cellular RNAs, thereby hampering the viral replication and infection (Floyd-Smith et al. 1981). The efficacy of this RNA targeting mechanism to confer broad-spectrum resistance has been demonstrated in

tobacco plants (Mitra et al. 1996). Resistance to several DNA and RNA viruses has been engineered employing ZFNs, TALENs and CRISPR/Cas9. Artificial TALE proteins and FokI nuclease expressing *Nicotiana benthamiana* plants showed resistance to different *Begomoviruses*. Transgenic expression of yeast-derived dsRNase, Pac1, confer broad-spectrum resistance to phytopathogenic viruses. Pac1 has been successfully used to confer resistance to CMV, *Tomato mosaic virus* (ToMV) and PVY in tobacco (Watanabe et al. 1995) and PSTVd in potato (Sano et al. 1997).

Ribosome-Inactivating Proteins Ribosome-inhibiting proteins or ribosome-inactivating proteins found in certain plant species possess anti-viral activities. Transgenic expression of RIP from the pokeweed (*Phytolacca americana*) conferred broad-spectrum resistance to virus infection in several plant species, including tobacco, potato and *N. benthamiana* and *Brassica napus* against PVX, PVY and TuMV (Lodge et al. 1993; Zhang et al. 1999), respectively. Transgenic expression of RIP from *Dianthus caryophyllus*, *Trichosanthes kirilowii* and *Iris hollandica* also was observed to provide varying degrees of resistance to ACMV, TMV and CMV and TEV and TMV, respectively. However, any of the RIPs evaluated so far has not found to have an efficient broad-spectrum anti-viral activity.

Peptide Aptamers There are some short-peptide molecules, which can confer low-level broad-spectrum resistance and are advantageous in that the need for producing high level of transgene to induce PTGS can be avoided. Such short-peptide-mediated resistance sometimes provides better resistance than RNAi- and protein-mediated PDR as in the case of transgenic *N. benthamiana* with an introduced target-specific peptide aptamer showing broad-spectrum resistance to *Tomato spotted wilt virus* (TSWV), *Groundnut ringspot virus* (GRSV) and *Chrysanthemum stem necrosis virus* (CSNV) (Rudolph et al. 2003). Similarly, a conformationally constrained peptide aptamer was found to interfere the replication of *Tomato golden mosaic virus* (TGMV) and *Cabbage leaf curl virus* (CaLCuV) (Lopez-Ochoa et al. 2006). Further investigation of such peptides and understanding of their mode of action enable their application for engineering broad-spectrum resistance.

Plantibodies Antibody-mediated resistance to a plant virus was first demonstrated in transgenic *N. benthamiana* plants expressing an scFv against *Artichoke mottled crinkle virus* (Tavladoraki et al. 1993). Later, transgenic expression of scFv in different crop plants, like Chinese cabbage, citrus, gladiolus, potato and tomato, conferred them with virus resistance. Initial experiments resulted in delayed or reduced disease symptoms only, and complete resistance was not observed. Improvements in plantibody targeting and stabilization and development of non-structural viral proteins targeting plantibodies enhanced the level of resistance provided by them (Gargouri-Bouzid et al. 2006; Nickel et al. 2008, Boonrod et al. 2004; Gil et al. 2011). Transgenic *N. benthamiana* expressing scFvs targeted against the TBSV replicase showed broad-spectrum resistance to TBSV, two members of

family *Tombusviridae* (CNV and TCV) and *Dianthovirus Red clover necrotic mosaic virus* (Boonrod et al. 2004).

23.3 Engineering Resistance to Vectors

Many pathogens, particularly viruses, spread by different insect vectors, mostly hemipterans, aphids, dipterans, etc. Efficient vector control strategies significantly reduce the infection and crop damage. In certain cases, a single vector may harbour multiple pathogens, which are infecting same or different species. Transgenic strategies for vector control can be implemented by taking cues from vector-pathogen–host interactions and underlying genetic, molecular and biochemical processes. Persistent, semi-persistent and non-persistent vectors differ highly in their feeding nature, subcellular localization of virus in them and the status of virus in vector, whether replicating or non-replicative, circulating (Jones 2014; Brault et al. 2010). Viruses themselves influences host interaction with vectors and environment and modulate host immune response accordingly to regulate the feeding pattern and preference in order to manage the acquisition as well as injection of pathogens from viruliferous and non-viruliferous plants (Ingwell et al. 2012; Stafford et al. 2011). All these are accomplished by regulating host defence signalling hubs, manipulation of plant biochemistry for enhanced vector performance and virus-mediated suppression of plant immunity. Suppression of JA- and SA-mediated immune response to reduce insect repellents signals, i.e. different volatile compounds is a typical example for how pests reprogramme plant immunity for enhancing infection (Westwood et al. 2013). Interestingly, such negative regulation of defence-related signalling can be manipulated transgenically to reverse the effects so that resistance to both pests and pathogens can be achieved.

Understanding of involvement of semiochemicals and subsequent and signal generation and transduction can give an insight into potential semiochemical targets that can be modified transgenically to control vectors. Transgenic plants constitutively emitting the semiochemical (E)-b-farnesene, an aphid alarm pheromone and predator attractant, showed repulsion to aphid species and attraction to aphid-parasitizing wasps under controlled conditions (Bruce et al. 2015). However, the metabolically engineered plants showed no differences in aphid infestation or presence of natural enemies in field. Stimulated expression or mixed expression of different semiochemicals can be an alternative strategy to effectively control vectors.

Introgression of R genes conferring vector resistance, like the melon Vat ('virus aphid transmission') gene and the tomato Mi-1.2 gene, is useful for controlling vectors. Vat confers effective resistance to multiple viruses but only when transmitted by specific aphid species (Goggin et al. 2006), whereas Mi-1.2 provide resistance to the nematode *Meloidogyne incognita* and many phloem-feeding insects, including *B. tabaci* (Guo et al. 2016; Peng et al. 2016). As the vectors that are identified by NB-LRR protein domains of plants are limited, modifying these receptors in order to expand the range of defendable vectors using transgenic methods can be a promising strategy (Harris et al. 2013; Kim et al. 2016). The vector genes/transcripts encoding

effectors can be targeted by transgenic expression of cognate RNA molecules in host, which is taken up by feeding invertebrates and silences the respective target. Fusion protein consisting of luteoviral coat protein–spider toxin was expressed in host, where viral coat protein moiety facilitates the uptake of toxin by vector (Whitfield and Rotenberg 2015; Bonning et al. 2014). Disruption of transmission of three groups of vectors – whiteflies, aphids and mealy bugs – was obtained by phloem-specific expression of a spider-derived toxin and a lectin (Javaid et al. 2016). Engineering plant as vectors of viruses, which are pathogenic on aphids and leafhoppers, including *Densoviruses* (DNA) and *Dicistroviruses* (positive-sense RNA), is a potential strategy, where host plants act as biocontrol agents. It was tested using cricket paralysing virus, and it disrupted normal aphid responses to olfactory cues from other aphids, causing them to scatter and predisposing them to attack by predators and parasitoids or promote transition to winged morphs, enhancing virus dissemination (Kerr et al. 2015; Ban et al. 2008; Ryabov et al. 2009).

23.4 Transgenic Approach for Resistance Against Viroids

Viroids are small (~250 to 400 nt) non-protein-coding, circular, single-stranded RNAs, which autonomously replicate through an RNA–RNA rolling-circle mechanism that is catalysed by host enzymes (RNA polymerases, RNases and RNA ligases) in the nucleus (*Pospiviroidae*), or in plastids, primarily chloroplasts (*Avsunviroidae*). These small infectious nucleic acids can infect economically important higher plants potato, tomato, citrus, hop and temperate fruits, like peach, apple and pear. Systemic infection of viroids involves entry into specific subcellular organelles, replication, exit of the organelles and cell-to-cell trafficking, access into and long-distance trafficking within the vasculature and exit and invasion of non-vascular tissue to restart the cycle. Interaction of the viroid RNA with cellular proteins involved in its replication/trafficking or from defensive responses is triggered by the host results in infection and disease development (Navarro et al. 2012; Flores et al. 2005). As conventional control measures sound insufficient to offer complete protection against these pathogens, transgenic approaches, like targeting the viroid RNA for degradation or manipulating the host defensive response in order to disrupt host–viroid interactions, may efficiently alleviate the disease symptoms. A peculiar resistance mechanism, namely, cross-protection, wherein a previous infection from mild strain induces development of resistance against further infection from a severely virulent strain, operates against viroids. The underlying molecular mechanism of cross-protection can be sequence specific, which may be related to RNA silencing (Niblett et al. 1978; Khoury et al. 1988; Flores et al. 2005).

As viroid replicates via dsRNA intermediates, yeast-derived dsRNA-specific RNase pac1 has been engineered to target this dsRNA of PSTVd in potato, which resulted in reduced infection and symptoms. Such RNAi approach can confer additional resistance to some RNA viruses as well. These pac1 expressing transgenic potato lines showed resistance to *Tomato spotted wilt virus* also (Flores et al. 2017). Expression of a catalytic single-chain variable antibody (3D8 scFv) with intrinsic

RNase (and DNase) activities, in chrysanthemum plants, resulted in resistance to CSVd infection. This strategy is advantageous in that antibody accumulates to very low levels and antibody expression does not induce any phenotypic alteration, even though it acts in sequence-independent manner, and protects plants not only against CSVd but also confers resistance to DNA and RNA viruses (Lee et al. 2013; Tran et al. 2016). Different trials for antisense RNA-mediated silencing of viroid genomic RNA couldn't establish a stable resistance strategy based on RNAi. Even so, CSVd resistance was conferred by *Agrobacterium*-mediated transformation of a commercial chrysanthemum cultivar with four different constructs carrying sense or antisense CSVd-specific RNAs of 75–82 nt (Matoušek et al. 1994; Jo et al. 2015). Although initial attempts to engineer hammerhead ribozymes to target the plus and minus strands of CEVd did not confer viroid resistance in vivo, hammerhead ribozyme with shorter recognition sequences (9–11 bases) engineered to target PSTVd minus RNA suppressed viroid accumulation in transgenic potato lines (Yang et al. 1997). Tertiary stabilizing motifs (TSMs) of hammerhead ribozymes is suspected to play a critical role in their potential to cleave specific RNA in trans, and this was substantiated when hammerhead ribozymes, with their tertiary stabilizing motifs (TSMs) preserved, engineered to target PSTVd minus RNA successfully interfered with systemic PSTVd infection in in transgenic *N. benthamiana*. Target accessibility of the substrate and the subcellular co-localization of ribozyme and substrate are major hurdles to extract the complete potential of these small RNAs for crop protection (Flores et al. 2017). RNA interference strategy for targeting viral genome can be applied to viroids also. Transgenic tomato and *N. benthamiana* expressing hpRNA construct with near full-length (340-nt) and specific truncated sequences of PSTVD, respectively, showed resistance to this viroid. As RISC is active in cytoplasm, it may not interfere with viroid replication and accumulation host in nucleus but can be targeted while they move to cytoplasm for invasion into neighbouring cells (Schwind et al. 2009). Overexpression of AGO also has found to attenuate viroid accumulation (Minoia et al. 2014). Artificial small RNAs, artificial miRNAs (amiR-NAs) and synthetic trans-acting siRNAs (syn-tasiRNAs) are another efficient transgenic strategy for controlling viroids, when stably expressed in transgenic plants (Carbonell et al. 2014). Concurrent application of more than one strategy might have synergistic effects, resulting in enhanced resistance to viroids.

23.5 Transgenic Technology for Resistance Against Fungi

Fungal pathogens cause several diseases in plants resulting in catastrophic effects on crop yield (10% of global crop loss). Conventional control strategies include the application of chemical fungicides and biocontrol agents, phytosanitation, crop rotation, destruction of intermediate hosts, etc., which are inadequate to provide complete protection from infection. Transgenic strategies can be adopted to confer/enhance the resistance to fungal pathogens so that yield loss and disease management expenses can be considerably reduced. Several plants naturally produce

antifungal compounds, and genes encoding these compounds or regulating their biosynthesis can be manipulated to confer fungal resistance. Prime plant-derived antifungal compounds are chitinase and glucanases, enzymes that degrade major components of fungal cell wall, chitin and glucan, thereby imparting resistance (Silva et al. 2018, 2019). Stacking of these two genes can yield enhanced resistance against fungal pathogens. Transgenic carrot expressing chitinase and β1,3-glucanase, together with AP24 gene, exhibited broad-spectrum resistance against fungal pathogens (Ram and Mohandas 2003). Transgenic grape and cotton plants constitutively expressing chitinase from *Trichoderma* species conferred resistance to several fungal diseases (Rubio et al. 2015; Emani et al. 2003). Plant-derived inhibitors of microbial cell wall degrading enzymes are other potential candidates for engineering resistance. Transgenic expression of inhibitors of cell wall degrading enzymes has conferred resistance against many dreaded fungal pathogens in crop plants. Reduced susceptibility to necrotrophic fungus *Botrytis cinerea* has been observed in PGIP overexpressing transgenic tomato, grapevine, tobacco and *Arabidopsis thaliana* (Ferrari et al. 2012; Liu et al. 2017; Manfredini et al. 2005). PGIP degrades microbial polygalacturonases and thereby delays plant cell pectin hydrolysis, which in turn restricts fungal infection (Ferrari et al. 2013). Xylanase inhibitor protein controls the xylanases, which degrades the main component of cell wall, xylan, as in the case of effective inhibition and counteraction of the *F. graminearum* necrotic xylanase activity by constitutive expression of *Triticum aestivum* xylanase inhibitor III (TAXI-III) in GM wheat (Tundo et al. 2016). Expression of pectin methyl esterase (PME) inhibitors in *Arabidopsis* could prevent damage to the plant cell wall during *Botrytis cinerea* infection (Lionetti et al. 2017). Transgenic strategies to control insect pests, including cry genes from *Bacillus thuringiensis*, provide dual protection by reducing the chances of fungal infection and mycotoxin (fumonisin, *Fusarium* spp.; aflatoxin ergotoxine, *Claviceps* spp.; aflatoxins, *Aspergillus* spp.) contamination by reducing the rate of insect wounding. This would reduce management costs and yield loss considerably. Overexpression of PnAMP-h2 gene from *Pharbitis nil* and barley chitinase (chi-2) genes were found to provide enhanced resistance to fungal pathogens. Antimicrobial peptides derived from non-plant, non-phytopathogenic microbes can also be used for imparting disease resistance as in the case of enhanced resistance to *R. solani* and soil-borne pathogen *A. alternata* observed in transgenic cotton and tobacco plants expressing endochitinase gene from a mycoparasitic fungus *Trichoderma virens*. Phytoalexin capsidiol provided resistance to the potato blight pathogen, *Phytophthora infestans* (*P. infestans*) in pepper (*Capsicum* spp.) (Lee et al. 2017) (Table 23.4).

Defensins are cysteine-rich antimicrobial peptide of plant origin, which is a potential candidate to protect crop plants with phytopathogenic fungal infection by their transgenic expression in plants. Constitutive expression of an alfalfa seed defensin MsDef1 and NmDef02 defensin in potato provided strong resistance to *Verticillium dahliae* and *Phytophthora infestans*, respectively (Gao et al. 2000). Constitutive expression of defensins mostly causes undesirable side effects, including reduced growth and yield performance (Chen and Chen 2002). Regulation of defensin expression by using (native or heterologous) pathogen-induced promoter

Table 23.4 List of transgenic plants developed for resistance to fungal pathogens

Plant species	Antifungal genes transferred	Resistance against	References
Rice	Chitinase (chi11); glucanase (gluc)	*Rhizoctonia solani*	Sridevi et al. (2008)
	stress-inducible b-glucanase (Gns1)	*Magnaporthe grisea*	Nishizawa et al. (2003)
	Ribosome-inactivating protein (MOD1) Chitinase (RCH10)	*Rhizoctonia solani*	Kim et al. (2003)
	Chitinases (RCH10 and RAC22); glucanase (b-Glu); ribosome-inactivating protein (B-RIP)	*Magnaporthe grisea*	Zhu et al. (2007)
	ER-CecA; Ap-CecA (cecropin A)	*Magnaporthe grisea*	Coca et al. (2006)
	Dahlia merckii defensin DM-AMP1*)*	*Magnaporthe oryzae* and *Rhizoctonia solani*	Jha et al. (2009)
Potato	Chitinase (ChiC)	*Alternaria solani*	Khan et al. (2008)
	Chitinase (CHIT); glucanase (GLUC)	*Rhizoctonia solani*	Moravcikova et al. (2007) Moravcikova et al. (2004)
	Chitinase (BjCHI1); glucanase (HbGLU)	*Rhizoctonia solani*	Chye et al. (2005)
	Cationic peptide (msrA3)	*Phytophthora infestans* *Phytophthora erythroseptica* *Erwinia carotovora*	Osusky et al. (2004)
	RPI-BLB2	*Phytophthora infestans*	van der Vossen et al. (2005)
Tomato	Glucanase (GLU); antifungal protein (alfAFP); glucanase (GLU-AFP)	*Ralstonia solanacearum*	Chen et al. (2006)
Carrot	Chitinase383; glucanase638; cationic peroxidise (POC1)	*Botrytis cinerea* and *Sclerotinia sclerotiorum*	Wally et al. (2009)
Taro	Chitinase (ricchi11)	*Sclerotium rolfsii*	He et al. (2008)
Cucumber	Chitinase (RCC2)	*Botryti cinerea*	Kishimoto et al. (2002)
Nicotiana benthamiana	Polygalacturonase-inhibiting protein from grapevine	*Botrytis cinerea, Bipolaris sorokiniana*	Joubert et al. (2007)
Tobacco	Polygalacturonase-inhibiting protein (PGIPs) (pepper)	*Phytophthora capsici*	Wang et al. (2013)

(continued)

Table 23.4 (continued)

Plant species	Antifungal genes transferred	Resistance against	References
Alfalfa	*RCT1* from (*Medicago truncatula)*	*Colletotrichum trifolii*	Yang et al. (2008)
Potato	RPI-BLB2 (*Solanum bulbocastanum)*	*Phytophthora infestans*	Van der Vossen et al. (2005)
Wheat	*Fusarium Tri101*	*F. graminearum*	Okubara et al. (2002)
Peanut	Chitinase	*Cercospora arachidicola*, *Aspergillus flavus*	Prasad et al. (2013)

like barley GER4c promoter or tissue-specific promoter, which mainly depends on the infection biology and tissues affected, would be advantageous to avoid the negative fitness effects associated with their constitutive expression (Himmelbach et al. 2010; Rushton et al. 2002). Expression of antifungal defensins under root-specific promoter confers resistance to sudden death syndrome in soybean, because it is caused by a root-colonizing pathogen *Fusarium virguliforme*. Similarly, lifestyle-specific expression of defensins by fine-tuning their subcellular localization, which in turn depends upon whether pathogen is biotrophic or hemibiotrophic, is also effective to confer durable resistance without compromising yield. Extracellularly targeted defensins enable protection from biotrophic fungi, whereas extra- and intracellularly targeted defensins should be coexpressed in order to confer resistance to hemibiotroph. However, the effect of transgene encoded antimicrobial compound on animal/mammalian system should be assessed before employing this strategy for transforming edible crops. A detailed understanding of the underlying molecular mechanisms of their mode of action would permit the development of much sophisticated strategies for the regulated expression of antifungal defensins to confer broad-spectrum durable resistance against many notorious fungal pathogens (Kaur et al. 2011). Synergistic effects by coexpression of different plant defensins with different mode of action or plant defensin with antifungal pathogenesis-related protein (PR) or HIGS with plant defensins enhance resistance to a wide range of fungal infection (Chen et al. 2009; Ntui et al. 2011).

Silencing of pathogen genes by RNAi-based host-induced gene silencing (HIGS) is another efficient transgenic strategy to develop resistance. Resistance to *Blumeria graminis* and *F. verticillioides* was achieved by HIGs of their endogenous gene and fungal transgene, respectively (Nowara et al. 2010; Tinoco et al. 2010).

Plant resistance genes have extensively used for engineering resistance against phytopathogenic fungi. Introgression of R genes from related or unrelated species is facilitated by transgenic technologies. Overexpression of NPR1, a key regulator of SAR, conferred resistance to blight and blast disease causing fungal and bacterial pathogens, respectively (Chern et al. 2005; Yuan et al. 2007). Transgenic rice plants overexpressing AtNPR1 gene and translational suppressor uORFs from the TBF1 gene displayed resistance towards bacterial and fungal pathogens and reduced negative fitness costs associated with constitutive expression of NPR (Xu et al.

2017). In another case, introgression of the gene involved in non-host resistance, *Phytophthora soaje* susceptible 1 (AtPSS1), from *Arabidopsis* into soybean by transformation enhanced resistance to the soybean host fungal pathogen *Fusarium virguliforme*, by a suspected autophagy mechanism without yield penalty (Wang et al. 2018). Transgenic expression of R gene RPI-BLB2 from *Solanum bulbocastanum* in cultivated potato showed resistance to *Phytophthora infestans* (van der Vossen et al. 2005). Resistance conferred by single R gene is mostly not/less durable as pathogen is smart enough to circumvent the resistance. Stacking of multiple R genes, either targeting single or different pathogens, in single plant is an efficient alternative to impart durable and/or broad-spectrum resistance against destructive fungal pathogens. Robust transgenic strategies for gene pyramiding are required for executing this. Host genes manipulated by fungus for apressoria formation and haustoria development can be a potential target to be modified in order to confer pre-haustorial resistance. *Arabidopsis* PENETRATION genes involved in haustoria establishment is a suitable candidate in that regard (Fonseca and Mysore 2019). Integrated transgenic approach combining resistance genes, defensins and antifungal proteins is expected to confer effective durable resistance.

23.6 Transgenic Technology for Bacterial Resistance in Crop Plants

Bacteria are causing many diseases in economically important plants, where it severely impairs the growth, development and yield potential of plants. Plants defend bacterial pathogens with common components of immunity, viz. R genes, ETI-associated HR, antimicrobial compounds, inhabitants of bacterial enzymes, etc. Transgenic approaches for engineering resistance to bacteria in plants can be executed in the following ways: introduction of host R genes and bacterial avirulence genes, incorporation of pathogen-derived genes for resistance to bacterial phytotoxins and expression of antibacterial proteins from plants, insects or bacteriophages as bactericidal or bacteriolytic agents (Panopoulos et al. 1996). Transfer of maize R gene RESISTANCE TO XANTHOMONAS ORYZAE 1 (RXO1) to rice conferred enhanced resistance to the rice host pathogen *Xanthomonas oryzae* pv. *oryzicola* causing bacterial streak disease. Similarly, single R gene obtained from pepper was introgressed to tomato in order to control highly destructive bacterial leaf spot (Zhao et al. 2004, 2005; Tai et al. 1999). Unravelling novel candidate receptors or enzymes or structural proteins from bacteria that can be targeted by transgenic methods will have promising results in controlling bacterial diseases (Table 23.5).

Antimicrobial peptides of plant origin as well as those from heterologous non-plant systems are efficient candidates for engineering disease resistance. Plant-derived antimicrobial peptides contain cysteine-rich short amino acid sequence and target the outer membrane structures. Overexpression of pepper antimicrobial peptide-encoding gene CaAMP1 in *Arabidopsis* conferred broad-spectrum resistance to bacterial, fungal and oomycete pathogens (Lee et al. 2008).

Table 23.5 List of transgenic plants developed for resistance to bacterial pathogens

Plant species	Transgene	Target species	Reference
Nicotiana tabacum var. Petit Havana	Magainin analogue, Myp30, MSI-99	*P. syringae* pv. *tabaci*	Li et al. (2001)
			De Gray et al. (2001)
Tobacco Potato Apple, Poplar	Cecropin B, mutant (SB37, MB39) and synthetic (Shiva-1, D4E1)	*P. syringae* pv. *tabaci* *Erwinia carotovora* subsp. *atroseptica* *E. amylovora* *Agrobacterium tumefaciens* and *Xanthomonas populi*	Huang et al. (1997)
			Arce et al. (1999)
			Norelli et al. (1998)
			Mentag et al. (2003)
Tobacco	LTPs (lipid transfer protein)	*Pseudomonas syringae* pv. *tabaci.*	Sarowar et al. (2009)
Arabidopsis	Plant PRRs (RLPs) *N. benthamiana* (NbCSPR)	*Pseudomonas syringae*	Saur et al. (2016)
Tomato	R proteins (NB-LRR) pepper Bs2	*Xanthomonas perforans*	Horvath et al. (2012)
Tomato	*Pto*	X. campestris pv. vesicatoria	Tang et al. (1999)
	Bs2	X. campestris pv. vesicatoria	Tai et al. (1999)
Tomato (tomato snakin-2)	Plant antimicrobial defence proteins (plant encoded defensins (PR-12), thionins (PR- 13), lipid transfer proteins (PR-14), snakins, cyclotides, knottins and hevein-like proteins	*Clavibacter michiganensis*	Balaji and Smart (2012)
Banana	*Hrap* and *Pflp* (sweet pepper)	*X. campestris* pv. *musacearum*	Tripathi et al. (2010, 2014), Namukwaya et al. (2012)
Rice	*Rxo1*	X. oryzae pv. oryzicola	Zhao et al. (2005)
	Npr1	*X. oryzae pv. oryzae*	Chern et al. (2005)
	NH1	*X. oryzae pv. oryzae*	Yuan et al. (2007)

A significant reduction of fire blight symptoms in pear and partial resistance to bacterial blight in tomato was achieved by expression of lactoferrin, a mammalian glycoprotein (Malnoy et al. 2003). Attacin is another heterologous protein that has been used for conferring resistance against *Erwinia amylovora*, causing fire blight in

transgenic apple (Aldwinckle et al. 2003). Transgenic expression of another protein, pectate lyase 3 (PL3), in potato resulted in increased resistance to *Erwinia* soft rot (Wegener 2002).

Cecropins are antimicrobial amphipathic peptides of 31–39 aa long and interact with bacterial membranes or induce pore formation in membrane. Transgenic tomato expressing high level of cecropin displayed enhanced resistance against bacterial speck disease. An increased resistance to bacterial blight in rice was engineered by targeting cecropin towards intercellular species (Alan et al. 2004; Jan et al. 2010).

Increased expression and accumulation of ROS, hydrolytic enzymes, antimicrobial peptides and proteins in transgenic host plant can enhance resistance, including HR, to bacterial pathogens and avoid the energy and resource loss in inducing immune responses, which in turn enable plant to channelize the saved energy and nutrients/metabolites into growth and development process so that yield is not compromised. Resistance to several bacterial and fungal pathogens has been achieved by transgenic expression of genes encoding cell wall degrading hydrolytic enzymes, defence-related proteins with protease inhibitor activity, etc. (Shin et al. 2008; Senthilkumar et al. 2010).

23.7 Genome Editing Techniques for Engineering Disease Resistance

Genome editing technology, which emerged in the 1990s, enabled targeted mutagenesis of genomic loci of interest by exploiting cell's intrinsic DNA repair pathways. Site-directed nuclease fused with sequence-specific DNA-binding protein domains or RNAs creates double-stranded breaks in target genomic site. DSBs thus formed are subsequently repaired either by error-prone DNA repair pathway non-homologous end joining (NHEJ) or high-fidelity homology-directed repair pathway (HDR). NHEJ usually results in point mutations at the site of DSB, and HDR enables replacement or introduction of a DNA fragment, while a repair template is provided. Mutations thus induced are stably inherited over generations. Genome editing techniques are useful for functional genomic studies as well as for creating or modifying desired phenotype or trait in organism of interest. Meganucleases, zinc finger nucleases (ZFN), TALENs and CRISPR/Cas are the commonly used genome editing tools. ZFNs and TALENS are fusion proteins in which specific DNA-binding proteins domains are fused with endonuclease domain of FokI nuclease, so that protein guides FokI to respective DNA targets. CRISPR/Cas9, in contrast, is an RNA-guided engineered nuclease, where a single guide RNA with 5′ target-specific 20 nt spacer directs Cas nuclease to target DNA. CRISPR/Cas is a part of bacterial adaptive immune system, providing resistance to invading viruses or nucleic acids. There are different classes of CRISPR/Cas systems, of which dsDNA targeting class 2, type II system seen in *Streptococcus pyogenes*, popular as CRISPR/Cas9 is the most common CRISPR system exploited for genome editing. Several variants of CRISPR systems and Cas protein have been discovered, each of which varies in their target recognition and cleavage properties.

Genome editing techniques have been successfully employed to develop resistance against bacterial, fungal and viral pathogens. ZFNs conferred broad-spectrum resistance to various *Begomovirus*es, including *Tomato yellow leaf curl China virus* (TYLCCNV) and *Tobacco curly shoot virus* (TbCSV) by targeting a single site in viral genome (Chen et al. 2014). Modified ZFN called as artificial zinc finger protein (AZP), which lacks the cleavage domain compared to ZFN, has been used to confer resistance to *Beet severe curly top virus* (BSCTV, family *Geminiviridae*) and *Rice tungro bacilliform virus* (RTBV) in *Arabidopsis* by targeting the intergenic region (IR) of BSCTV and blocking viral promoter sequences of RTBV (Ordiz et al. 2010). TALEs have been engineered to confer resistance to TbCSV and TYLCCNV in tobacco (Cheng et al. 2015). Complexity of protein engineering and off-target effects limited the use of these modular protein toolboxes. CRISPR/Cas9, whose genome editing potential was revealed recently only, quickly superseded and emerged as a promising tool for crop improvement with high efficiency, precision, robustness and simple designing strategy.

CRISPR/Cas9 has been widely used for engineering disease resistance in many crop plants by targeting pathogenic as well as host factors. Pathogenic genes that are inevitable for survival and infection cycle are appropriate candidate targets for CRISPR/Cas9-mediated knockout. Adaptive potentials enable pathogens to overcome the dominant R gene-mediated resistance. Thus, susceptibility genes are considered as potential candidates to be targeted in order to develop durable resistance. Disruption of susceptibility genes or S proteins by CRISPR/Cas9-mediated targeted mutagenesis has been successfully demonstrated. The first report of the application of CRISPR/Cas9 for virus resistance came when double-stranded replicative from geminiviral DNA was targeted to confer resistance. Later, this strategy was used to provide resistance to several DNA viruses. Apart from the demonstration for proof of concept of the CRISPR/Cas9 in model plants, highly efficient resistance against different viral, bacterial and fungal pathogens has been obtained in various crops, including major food crops like wheat and rice (Table 23.6, 23.7 and 23.8). For instance, mutagenesis of ERF transcription factor (OsERF922) gene using CRISPR/Cas9 imparted resistance in rice against fungal pathogen *Magnaporthe oryzae* (Wang et al. 2016). Similarly, CRISPR/Cas9-induced mutation in homeoalleles of mildew resistance locus (MLO), encoding a transmembrane protein, and enhanced disease resistance 1 (EDR1), encoding a Raf-like mitogen-activated protein, conferred resistance to powdery mildew in hexaploid wheat (Wang et al. 2014). CRISPR/Cas9-mediated loss-of-function mutation in eukaryotic translation initiation factor eIF4e, which is essential for infection of *Potyviridae* family viruses, conferred resistance against potyvirus (*Zucchini yellow mosaic virus* and *Papaya ringspot mosaic virus-W*) and *Ipomovirus* in *Arabidopsis* and cucumber (*Cucumber vein yellowing virus*) (Chandrasekaran et al. 2016). CRISPR/Cas9-mediated targeted mutagenesis of promoter sequence of susceptibility gene CsLOB1 conferred resistance to bacterial pathogen *Xanthomonas citri* subsp. *citri* in citrus (Peng et al. 2017). This tool has also been shown to be effective to control dsDNA viruses, as in the case of resistance to *Cauliflower mosaic virus* (CaMV) in *Arabidopsis* (Liu et al. 2018).

Table 23.6 Crop plants harnessing CRISPR/Cas-mediated resistance to viruses

Virus/viruses	Plant	Target (viral/host)	Function	Strategy	GM/ transgene-free	References
BSCTV	*N. benthamiana* and *A. thaliana*	IR, CP and rep	RCA mechanism	*Agrobacterium*-mediated transformation of leaves with Cas9/gRNA expression plasmid vectors	GM	Ji et al. (2015)
BeYDV	*N. benthamiana*	LIR and rep/RepA	RCA mechanism	*Agrobacterium*-mediated transformation of leaves with Cas9/gRNA expression plasmid vectors	GM	Baltes et al. (2015)
TYLCV, BCTV and MeMV	*N. benthamiana*	IR, CP, and rep	RCA mechanism	*Agrobacterium*-mediated transformation of leaves with a TRV vector in Cas9 overexpressing plants	GM	Ali et al. (2015)
CLCuKoV, TYLCV 2.3, TYLCSV-Logan, BCTV-Worland, MeMV	*N. benthamiana*	IR, CP, and rep	RCA mechanism	Agrobacterium-mediated transformation of leaves with a TRV vector in Cas9 overexpressing plants	GM	Ali et al. (2016)
TuMV	*A. thaliana*	Host factor eIF(iso)4E	Host factor for RNA virus translation	*Agrobacterium*-mediated transformation with Cas9/ gRNA recombinant plasmid binary vectors (floral dipping)	Transgene-free	Pyott et al. (2016)
CVYV, ZYMV and PRSMV	*Cucumis sativus*	Host factor eIF4E	Host factor for RNA virus translation	*Agrobacterium*-mediated transformation of cut cotyledons (without embryo) with Cas9/gRNA binary vectors	Transgene-free	Chandrasekaran et al. (2016)

(continued)

Table 23.6 (continued)

Virus/viruses	Plant	Target (viral/host)	Function	Strategy	GM/ transgene-free	References
CMV, TMV	*Nicotiana benthamiana* and *Arabidopsis thaliana*	ORF1, 2, 3, CP and 3′UTR	Replication mechanism	*Agrobacterium*-mediated transformation of leaves with FnCas9/gRNA expression binary vectors floral dipping for *Arabidopsis*	GM	Zhang et al. (2018)
RTSV	*Oryza sativa* L. *japonica*	eIF4G	Host factor for RNA virus translation	*Agrobacterium*-mediated transformation of immature embryos with Cas9/gRNA expression plasmid vectors	GM	Macovei et al. (2018)
TYLCV	*Solanum lycopersicum*	CP, rep	RCA mechanism	*Agrobacterium*-mediated transformation of cotyledons with Cas9/gRNA expression vectors	GM	Tashkandi et al. (2018)
TuMV	*Nicotiana benthamiana*	GFP1, GFP2, HC-Pro, CP	Replication mechanism	Agrobacterium-mediated transformation of leaves with a TRV vector in Cas13a overexpressing plants	GM	Aman et al. (2018)
Endogenous *Banana streak virus* (eBSV)	*Musa balbisiana*	ORF 1, 2 and 3 (aspartic protease gene)	Transcription or/and translation/post-translational modification of vital viral proteins	*Agrobacterium* mediated transformation of cell suspension culture with CRISPR/Cas9 construct	GM	Tripathi et al. (2019)

Table 23.7 Crop plants harnessing CRISPR/Cas-mediated resistance to bacterial pathogens

Plant species	Disease and causative bacteria	Target gene	Gene function	Strategy	Reference
Oryza sativa	Bacterial blight (*Xanthomonas oryzae* pv. *oryzae*)	SWEET13	Sucrose transporter gene	*Agrobacterium*-mediated transformation of embryogenic callus with Cas9/gRNA expression plasmid vectors and TALEN	Li et al. (2012), Chen et al. (2012), Zhou et al. (2015)
Citrus paradisi	Citrus canker (*Xanthomonas citri* subspecies *citri*)	LOB1	Susceptibility (*S*) gene promoting pathogen growth and pustule formation	*Agrobacterium*-mediated transformation of epicotyl with Cas9/gRNA expression plasmid vectors	Jia et al. (2016)
Citrus sinensis Osbeck	Citrus canker (*Xanthomonas citri* subspecies *citri*)	LOB1	Susceptibility (*S*) gene promoting pathogen growth and pustule formation	*Agrobacterium*-mediated transformation of epicotyl with Cas9/gRNA expression plasmid vectors	Peng et al. (2017)
Malus domestica	Fire blight (*Erwinia amylovora*)	DIPM-1 DIPM-2 DIPM-4	Susceptibility factor involved in fire blight disease	PEG-mediated protoplast transformation with CRISPR ribonucleoproteins	Malnoy et al. (2016)
Solanum lycopersicum	*Pseudomonas syringae* and *Xanthomonas* spp.	DMR6	Susceptibility (*S*) gene involved in downy mildew disease	*Agrobacterium*-mediated transformation of cotyledons with Cas9/gRNA expression vectors	Thomazella et al. (2016)
	Tomato bacterial speck disease (*Pseudomonas syringae* pv. *tomato* (*Pto*))	JAZ2	Regulation of stomatal opening by coronatine	*Agrobacterium*-mediated transformation of cotyledons with Cas9/gRNA expression vectors	Ortigosa et al. (2018)

Table 23.8 Crop plants harnessing CRISPR/Cas-mediated fungal resistance

Plant species	Disease and target species	Target gene	Gene function	Strategy	Reference
Vitis vinifera	Powdery mildew (*Erysiphe necator*)	MLO-7	Susceptibility (*S*) gene involved in powdery mildew disease	PEG-mediated protoplast transformation with CRISPR ribonucleoproteins	Malnoy et al. (2016)
Oryza sativa L. *japonica*	Rice blast disease (*Magnaporthe oryzae*)	ERF922	Transcription factor implicated in multiple stress responses	*Agrobacterium*-mediated transformation of embryogenic calli with Cas9/gRNA expression binary vectors	Wang et al. (2016)
Oryza sativa L. *japonica*	Rice blast disease (*Magnaporthe oryzae*)	SEC3A	Subunit of the exocyst complex	Protoplast transformation with Cas9/gRNA expression binary vectors	Ma et al. (2018)
Solanum lycopersicum	Powdery mildew (*Oidium neolycopersici*)	MLO1, PMR4	Major responsible for powdery mildew vulnerability	*Agrobacterium*-mediated transformation of cotyledons with Cas9/gRNA expression plasmid vectors	Nekrasov et al. (2017), Koseoglou (2017)
Triticum aestivum	Powdery mildew (*Blumeria graminis* f. sp. *tritici*)	MLO-A1	Susceptibility (*S*) gene involved in powdery mildew disease	Particle bombardment of immature wheat embryos with Cas9/gRNA expression plasmid vectors	Wang et al. (2014)
Theobroma cacao	Black pod disease (*Phytophthora tropicalis*)	NPR3	Regulator of the immune system	*Agrobacterium*-mediated transient transformation of stage C leaves with Cas9/gRNA expression binary vectors	Fister et al. (2018)

Loss-of-function mutations created by CRISPR/Cas9 is useful for identifying resistance and susceptibility-determining genes and corresponding regulatory elements like promoters or enhancers. As the NHEJ-induced mutations are stably inherited and the CRISPR/Cas expression cassettes can be eliminated in successive generations, the resulting plant remains transgene-free and thus overcomes the regulatory issues associated with *Agrobacterium* transformed conventional transgenic plants. Delivery of CRISPR/Cas9 constructs as ribonucleoprotein (RNP) complex composed of sgRNA and Cas9 protein into host plant also yields non-transgenic edited plant with disease resistance. Non-transgenic tomato, resistant to powdery mildew, has been created in this manner (Nekrasov et al. 2017). Multiple DNA sequences can be targeted simultaneously by designing gRNA cassettes using golden gate cloning/tRNA: gRNA/gRNA: ribozyme assembly strategy. The ease of multiplexing is highly beneficial for conferring host with sound resistance against single pathogen as well as with broad-spectrum resistance. Multiple regions of pathogen genome can be targeted to alleviate/reduce the possibilities for the evolution of cleavage or recognition resistant variants by overcoming mutations at single site. Although incorporation of resistance gene from foreign species can also be performed by inducing HDR repair pathway of DSB created, efficiency of such events is very less and still needs to be optimized for obtaining high rate of HDR. Cisgenic applications of genome editing can be useful for enhancing disease resistance in species, which are difficult to hybridize by introducing a desirable gene fragment from its natural gene pool using CRISPR/Cas9-induced HDR-based pathway. Genetic engineering approaches manipulating endogenous genetic variations related to specific traits are preferred to exogenous genomic targeting recombinant DNA technology.

Apart from dsDNA targeting Cas9, there are certain CRISPR systems with RNA targeting properties, which can be engineered to confer resistance to RNA viruses or silencing of viral transcripts in host. Class II, type VI Cas effector proteins, Cas13a (C2c2), Cas13b (C2c6), Cas13c (C2c7), Cas13d and Cas9 from *Francisella novicida* (FnCas9), RNA targeting SpCas9 (RCas9), are the RNA-guided RNA targeting Cas effector variants identified in different microbial genomes so far (Abudayyeh et al. 2017). Expression of Fncas9 and sgRNA targeting conserved 3′ UTR of *Cucumber mosaic virus* (CMV) and *Tobacco mosaic virus* (TMV) developed stable immunity in *N. benthamiana* against CMV and/or TMV (Zhang et al. 2018). CRISPR-Cas13a was used to confer resistance to *Turnip mosaic virus* (TuMV) in *N. benthamiana* by targeting Hc-Pro and GFR regions of genome (Aman et al. 2018).

A class II, type V CRISPR/Cpf1 system that efficiently target DNA was discovered from *Prevotella* and *Francisella* species and named as Cas12a (Zetsche et al. 2015; Makarova et al. 2015). Cpf1 from *Acidaminococcus sp. BV3L6* Cpf1 (AsCpf1) and *Lachnospiraceae bacterium* Cpf1 (LbCpf1) are other two promising nucleases that can be used for genome editing purposes (Zetsche et al. 2015).

Gene expression regulation and epigenetic modification is also facilitated by mutant Cas9, which lacks cleavage activity. Transcriptional activators or repressors can be recruited to a particular gene of interest by fusing them with dCas9, which then is directed by specific sgRNA to the target site. Similar strategy can be adopted

for editing epigenetic marks using appropriate epigenetic modifiers (Dominguez et al. 2016). This enable fine-tuning of defence regulatory pathways and metabolic pathways involved in immune response. Transcriptional activation of positive regulators of immunity and biomolecules like pathogenesis-related proteins or hydrolytic enzymes, which are directly involved in defence manifestation, repression of negative regulators of immunity, etc., can be achieved using CRISPR/dCas9 system. Resistance traits that are unexpressed due to epigenetic suppression can be activated by reversing the methylation and similarly epigenetically controlled susceptibility also modified. Tissue and developmental stage or stress status specific activity of CRISPR components and target editing events can be achieved by employing tissue-specific/inducible promoters for the expression of Cas effector and guide RNA. This is particularly beneficial for reducing unwanted or off-target editing.

Genome-wide mining of S genes may unravel novel candidate S genes that can confer resistance, durable as well as broad-spectrum, without having any fitness costs. Several trace elements, like Fe, Ca, Cu, K, Mn and Zn, are required by pathogens to meet their nutritional requirements, whereas the same are required in host plants for nutrition as well as to activate/drive certain critical defence responses like Cu-dependent binding of SA binding to its NPR1 receptor (Yuan et al. 2010). Coordinated activities of plant immune signalling pathways to inhibit/suppress bacterial iron acquisition mechanisms substantiated the role of Fe in establishing infection (Nobori et al. 2018). Pathogens compete with host to acquire these mineral nutrients, which is counteracted by plant, by limiting their availability to invading pathogens by a mechanism called as nutritional immunity. Pathogenic mineral transporters strive to acquire Fe, Mn, Zn, K and Cu, which are essential for their virulence/survival, from the host, leaving host deprived of these trace elements and thereby inhibiting immune responses that require these minerals (Ren et al. 2016; Hood and Skaar 2012). Thus, such trace element transporters or their acquisition mechanisms can be potential nutritional immunity-related S gene targets for genome editing (Zaidi et al. 2018).

Complete knockout of S genes mostly has negative fitness effects, like reduced growth, yield and fertility; early senescence; and reduced tolerance to abiotic stress, as most of them being primary in function. Even though fitness cost is not lethal, it can cause phenotypic abnormalities. Alternative strategies, like introduction of S gene variant, creation of intermediate alleles by promoter targeting, transient knockout of S genes by using pathogen-inducible promoter, etc., using CRISPR/Cas9 would efficiently confer resistance, avoiding any cost of fitness. Identification of S gene allelic variants, which can confer resistance, leads to create resistant S gene variant by specifically editing the respective SNP using CRISPR/Cas9 base editors (Rodríguez-Leal et al. 2017; Yan et al. 2018).

Even though the characteristics, like specificity, efficiency, simplicity, flexibility, etc., of this genome editing tool are highlighted, several shortcomings and complications, including off-target effects, low rate of successful transformation events and recalcitrance for regeneration and establishment of edited plants, are associated with CRISPR/Cas. Each stage of experiment, from the selection of target

sequence to stable integration of mutations, needs to be optimized for each and every species for utilizing maximum potential of this genome editing tool for disease resistance.

23.8 Strategies Based on the Mechanism of Plant–Pathogen Interaction

Plants are constantly subjected to stress from various biotic agents/pathogens, like virus, bacteria, fungi, oomycetes, nematodes, insects and parasitic plants, that are causing serious crop loss. Plant–pathogen interaction studies have revealed various components involved in immunity and their mechanism of action during defence response. Interaction is specific characteristic of each group of pathogens. A vast array of well-programmed defensive mechanisms driven by multiple biomolecules which are involved in each stage, viz. invasion of pathogen into host, recognition and initiation of immune response, active deployment of defensive strategies and establishment of resistance, together constitute plant immunity. Pathogen-derived signals during infection process is perceived by specific receptors, mostly located in the cell membrane, which then trigger a cascade of defence activities/pathways making use of a large spectrum of biomolecules and molecular mechanisms (Silva et al. 2018).

Plant innate immunity has organized as a three-layered system with pathogen-triggered immunity (PTI), effector-triggered susceptibility (ETS) and effector-triggered immunity (ETI). The first active line of plant immunity is PTI, which is triggered when pathogen-associated molecular patterns (PAMP), together with this damage-associated molecular patterns (DAMP) released during pathogen invasion and damage, are recognized by transmembrane pattern recognition receptors (PRRs) (Boutrot and Zipfel 2017; Uma et al. 2011). PTI is suppressed by effector-triggered susceptibility (ETS) induced by pathogen-derived susceptibility proteins/effector and results in infection (Jones and Dangl 2006; Chisholm et al. 2006). This activates the second line of defence, effector-triggered immunity induced by recognition of specific effectors/cognate factors, pathogen avirulence (Avr) proteins by another group of receptors encoded by resistance genes (R). A co-evolutionary gene-to-gene molecular arm race occurs between pathogen effectors and host R genes, while pathogen evolves a new effector to restore the compatible interaction to facilitate infection, parallel to which host evolves a new R protein, to strengthen immunity. PTI is conserved over a range of organisms, whereas ETI specific to Avr protein is produced by each organism. ETI mostly/usually activates localized cell death pathway, otherwise known as hypersensitive response (HR), to restrain infection by transmitting defence signals to neighbouring non-infected cells via plasmodesmata and to other systemic organs via phloem, resulting in distal resistance responses, namely, local acquired resistance (LAR) and systemic acquired resistance (SAR), respectively (Coll et al. 2011; Dangl and Jones 2001). Like HR, ETI responses also involve production of salicylic acid (SA), reactive oxygen species (ROS), necrosis and also structural changes, such as lignification and callose deposition. Various

biomolecules, like pathogenesis-related proteins (PR), antimicrobial peptides (AMP), ribosome-inhibiting proteins (RIPs), defensive secondary metabolites, etc., are produced by plants as a part of defence response (Kachroo and Robin 2013; Dempsey and Klessig 2012). Increasing the cytosolic calcium levels in response to exogeneous signals, like H_2O_2/PAMP/DAMPs, etc., produced during infection is sensed by cellular and membrane calcium receptors, which in turn transduce signals to elicit defence responses, like HR, ROs production, transcriptional regulation of stress responsive genes, etc. (Seybold et al. 2014; Silva et al. 2018).

Incompatible interaction between non-host species and pathogen results in immunity against non-host pathogen in non-host plant. Preinvasive NHR is mostly passive mainly based on different physical and chemical barriers to interfere proliferation and accumulation of pathogen. Sometimes, NHR involves active defence response mediated by incompatible R–Avr gene-to-gene interaction result in ETI, and this shows that common mechanisms converge at some points in NHR and HR (Flor 1971; Glazebrook 2005; Gill et al. 2015). Prolonged interaction between host and pathogen occurs, wherein pathogens are under constant dual selection pressures, for increased resistance from host and on pathogen for increased performance of pathogen, which results in co-evolution of pathogen and host with virulence specificities on specific hosts (Allen et al. 2004). Such co-evolved pathogens become unable to infect phylogenetically unrelated hosts, resulting in non-host resistance, which is highly beneficial to control pathogens. NHR is a quantitative trait with multiple genes and pathways, whereas HR has specific R gene (Senthil-Kumar and Mysore 2013). Based on plant response, NHR is considered to be of two types. Type 1 NHR involves no visible symptom as pathogen fails to penetrate tissues. It is manifested by different physical, chemical and metabolical barriers that block pathogen penetration without activating ETI and PTI. Type II NHR involves some degree of pathogen penetration or entry in plant tissue, not as strong as a susceptible host pathogen infection but in a dose sufficient to trigger HR (cell death), and involves ETI. During post-invasive NHR, some pathogens penetrate host by overcoming PTI responses and involve activation of defence responses, like HR, ROS, cell death or the formation of cell wall appositions in infected cells (Collins et al. 2003; Rojas et al. 2012).

23.9 Enhancing Immunity: Transgenic Approaches to Manipulate Plant Innate Immunity

23.9.1 Upregulation of Defence Pathways

Upregulation of molecules involved in defence regulation, signalling and other allied cellular processes can boost hosts' general immune responses, including ROs production, callose deposition, PR proteins and activation of SAR, etc. Resistance to several microbial (bacterial and fungal) pathogens has been achieved by employing this strategy without introducing new metabolic pathway or new gene but exploiting plant's own immune system. Resistance to *Rhizoctonia solani* and

Magnaporthe oryzae (rice blast causative fungi) has been achieved by expressing a native rice gene under the control of a constitutive promotor from maize (Bundó and Coca 2016; Chen et al. 2016; Vincelli 2016). Genome editing techniques, like CRISPR/cas9-mediated transcriptional regulation, can be a potential strategy to serve this purpose.

23.9.2 Production of Antimicrobial Compounds

Microbial pathogens secrete different cell wall degrading enzymes (CWDE) like cellulases, polygalacturonases, xylanases, xyloglucan endoglucanase, chitinases and protease inhibitors to damage cell wall for making their way into host cells. In order to combat CWDEs, plants initiate cell wall strengthening/damage preventing mechanisms, like production of polygalacturonase-inhibiting proteins (PGIPs), xylanase-inhibiting proteins (XIPs) and xyloglucan-specific endoglucanase-inhibiting proteins (XEGIPs) (Schüttelkopf et al. 2010; Xu et al. 2011). As these mechanisms are mostly evolutionarily conserved, their manipulation could confer durable and broad-spectrum resistance in many crop species. Reduced susceptibility to *Phytophthora capsici* by expressing a pepper PGIP in GM tobacco; resistance to *Verticillium* and *Fusarium* wilts by expressing protein GhPGIP1 in *Arabidopsis* and cotton; resistance to *Phytophthora sojae* in soybean by constitutive expression of elevated levels of a *Glycine max XEGIP* (GmGIP1), an inhibitor of a *Phytophthora sojae,* xyloglucan-specific endoglucanase (PsXEG1); etc. have proved the efficacy of enhancing plant's own immune mechanisms by transgenic strategies to confer resistance (Silva et al. 2013; Liu et al. 2017; Wang et al. 2013; Ma et al. 2015).

23.9.3 Enhancing Plant Recognition of Infection

PTI results from perception of self-derived DAMPs and non-self PAMPs signals by PRR comprising receptor like kinases and receptor like proteases, whose active domains include leucine-rich repeats (LRR), lysine M domain (LysM), epidermal growth factor (EGF) like domain and lectin motif (Wu and Zhou 2013; Silva et al. 2018). Transfer and expression of PRRs in heterologous species by transgenic methods broaden the range of pathogens that can be recognized by the host while plant's own defence system or metabolic pathways are left unmodified. Expansion of pathogen recognition window is of prime importance in field conditions, where multiple infections prevail. Even though certain microbes are highly adapted with species-/race-/strain-specific PAMPs and have specific residues/motifs to block perception by PRRs, most of the PAMPs are conserved across microbial species, and thus individual PRR genes can confer broad-spectrum resistance (Zipfel and Felix 2005). Manipulation of PRRs and other receptors or downstream components involved, from a non-host species or unrelated family, is useful for boosting plant immunity as it will be difficult for pathogen to overcome the resulting new PRR/PAMP recognition system and the downstream signalling pathways.

Resistance to *Xanthomonas citri* in transgenic Hamlin sweet orange expressing NbFLS2 and resistance to *Pseudomonas syringae* in *Arabidopsis* expressing *Nicotiana benthamiana* receptor-like protein are required for Csp22 responsiveness (NbCSPR); NbCSPR exemplifies the effectiveness of transgenic expression of PRRs, even in heterologous system (Hao et al. 2016). Similarly, the transgenic expression of AtEFR in tomato (against *P. syringae* pv. *syringae*, *Ralstonia solanacearum* and *Xanthomonas perforans*), tomato PRR Ve1 in *Arabidopsis* and the *Arabidopsis* PRR AtEFR-Tu in wheat and potato against bacterial wilt (BW) causing soil-borne pathogen *Ralstonia solanacearum* is also expected to confer durable and effective resistance to microbial pathogens (Fradin et al. 2011; Lacombe et al. 2010; Schoonbeek et al. 2015). As most of the PAMPs are conserved and metabolically vital molecules of pathogen, it may not evolve quickly along with transferred PRRs in order to widen the host range.

23.9.4 Targeting Susceptible Gene/Protein

PTI is modulated or suppressed by the binding of pathogen effector proteins to host S proteins and thereby triggering ETS, which facilitate pathogen's invasion and establishment. Plant S genes are responsible for susceptibility to pathogen, ETS establishment and supporting compatibility. Molecular mechanisms associated with S genes are exercised in three levels: basic compatibility or early establishment in preinvasive stage when recognition and invasion occurs, sustained compatibility during which proliferation and spread of pathogen occurs and, finally, negative regulation of immune signals via respective signalling during post-penetration stage (van Schie and Takken 2014; Pavan et al. 2010). S proteins can either be positive regulators of immunity by promoting infection and disease development or negative regulators of immunity, and S gene products either facilitate pathogen's growth in host system or negatively regulate the plant immunity (Silva et al. 2018). Disarming/dysfunctioning, such susceptibility genes, by knockout or loss-of-function mutation is an efficient strategy to develop immunity, and the resulting resistance is mostly recessive and may be associated with pleiotropic effects and negative fitness costs, which may be due to constitutive activation of defence response. Durable and broad-spectrum resistance to powdery mildew in different plant species has been conferred in the same manner, by mutating mildew resistance locus O (MLO). Effector targets in host are activated by pathogenic effectors, which then negatively regulate plant immunity. S gene-mediated resistance can be pathogen specific, if the impaired pathway is inevitable for pathogen in any of the pre-penetration/penetration/post-penetration events, and it can be broad-spectrum, if the target S gene has implications in constitutive defence response. Another point to be noted while S proteins are encoded by multiple S genes is that all of them need to be targeted simultaneously; otherwise, the feasibility of same also should be checked. S gene LOF confers plants with broad-spectrum durable resistance than dominant R gene-mediated resistance as this is race/pathovar/strain non-specific. In order to overcome a modified or silenced susceptibility gene conferring host-

pathogen compatibility, pathogen should acquire a new function to replace the host factor it was exploiting, which is a tough task.

Eukaryotic translation initiation factors eIF4e and eIF4g usually interact with the cap structure of transcripts. Upon *Potyviridae* family virus attack, this interaction is disrupted and initiates interaction with Vpg proteins from virus and facilitates translation of viral proteins in host cell, thereby eIF4e and eIF4g act as negative regulators of plant immunity (Zhang et al. 2006; Callot and Gallois 2014). Similar factor rwm1 has been identified in watermelon under *Cucumber mosaic virus* infection (Ouibrahim et al. 2014). Loss-of-function mutation of such factors confers recessive resistance towards respective pathogens and is advantageous as far as it does not cause any serious pleiotropic effect. Sugar efflux from the host cell is activated by TAL activator from *Xanthomonas oryzae*, by binding to promoter region of SWEET14 gene in order to nourish bacterial pathogen (Yuan and Wang 2013; Boch et al. 2009; Chen et al. 2012; Li et al. 2012). Since this may have an adverse impact on plant health and agronomic performance due to altered sugar flux, modification of binding sites or specific residues of SWEET14 gene render is non-accessible or useless for pathogen. This can be accomplished by CRISPR/Cas9 genome editing tool.

23.9.5 Mining of R Genes

Overexpression or transfer of resistance genes from other related/unrelated species can confer durable broad-spectrum resistance. This is particularly advantageous in some crop species, where natural R gene is lacking or endogenous R genes fail to confer effective broad-spectrum resistance to most dreaded pathogens. In such cases, resistance genes can be introgressed from distantly related or even from unrelated species by genetic engineering, which in any way is impossible with conventional breeding. Conventional breeding itself is a hurdle in the case of polyploid crops, like potato, grape, banana, apple, etc. In such cases, the useful Cis genes also can be introgressed by genetic engineering, by avoiding major drawbacks of breeding techniques, viz, time consumption and linkage drag (Jones et al. 2014). Resistance to potato late blight has been achieved by this strategy (Jo et al. 2014). Though resistance genes efficiently confer resistance, their durability in heterologous hosts, over the course of time in field conditions, is limited. Since R gene action is strain-/race-specific, it confers species/subspecies level durable, broad-spectrum resistance, particularly in phylogenetically related hosts by heterologous expression of R genes (Eg: Bs2 gene). GM tomato plants expressing Bs2, an R gene from pepper that recognizes AvrBs2 present in some *Xanthomonas campestris* pathovars, were conferred with resistance against *Xanthomonas perforans* (Horvath et al. 2012). The efficacy of R genes in heterologous systems in conferring disease resistance has been demonstrated by similar transgenic plants. Bs2 gene functions as a solid source of resistance to different *Xanthomonas* species and *X. campestris* pathovars (e.g. pv. *vesicatoria* (*Xcv*)) in multiple solanaceous species. Co-transformation of R genes with cognate Avr gene can be followed by stacking of heterologous R genes, once

the R gene becomes able to overcome phytopathogen. Such interspecific R gene transfer confers host with broad-spectrum resistance in unrelated host as well (Gururani et al. 2012; Horvath et al. 2012). Overexpression of certain post-invasion-induced non-host resistance genes (PINGs) and bright trichomes (BRT1) genes that encode UDP glycosyltransferase 84A2, both of which have been found to be involved in NHR, conferred enhanced resistance against *P. pachyrhizi* in soybean (Langenbach et al. 2016). It has been found that expression of translational repressor domain from the TBF1 gene (uORFs), along with the defence-related gene, can circumvent the negative fitness costs associated with expression of defence-related genes in plants. For example, transgenic rice plants overexpressing the AtNPR1 gene and translational suppressor uORFs from the TBF1 gene displayed the expected resistance phenotype towards bacterial and fungal pathogens and reduced negative fitness costs associated with constitutive expression of NPR (Xu et al. 2017).

The principal functional domains of R genes are Toll/interleukin-1 receptor (TIR), nucleotide-binding site (NBS), leucine-rich repeat (LRR), LRR transmembrane domain (TrD), coiled coil (CC), nuclear localization signal (NLS), mitogen-activated protein kinase (MAPK) and MAPK kinase (MAPKK) domains. R proteins are classified into eight groups based on the arrangement of functional elements, and the major subset among them is LRR-NBS protein (LRR interact to Avr proteins with their highly variable, specificity-determining C- terminus. Mostly, R–Avr interactions occur via intermediate molecules regarded as effector targets. Mutation of host receptors mostly by altering or modifying functional domains by transgenic methods would enhance resistance and can be used in agriculture even though it is quite difficult to achieve broad-spectrum resistance by this strategy due to high R–Avr specificities. Mutation of functional domains will alter binding specificities of R proteins, which in turn strengthen the defence response either by blocking perception of specific pathogen by native R gene or by broadening the range of pathogens that can be recognized by R protein. This strategy handles pathogen recognition mechanisms only, while the rest of the defence response is part of the innate immunity only. *Solanum tuberosum* R3a gene (from the CNL group) confers resistance to the *P. infestans* Avr3aKI isolate but not to the Avr3aEM isolate. *N. benthamiana* expressing R3a* mutant variants showed significantly improved recognition of AVR3aEM (Chapman et al. 2014). Such mutated plant R genes enhance possibilities of recognizing pathogens/races that usually escape original R gene perception and thus confer durable resistance in field.

23.9.6 Modifying Pathogen Effector Targets in Host and Targeting Pathogen Effectors

Pathogenic virulence factors involved in virulence usually bind to certain host targets during their activity. Genetic modification of such host molecules targeted by pathogen can efficiently inhibit infection process. Modification/deletion of binding site residues in host target without compromising its other biological functions

can be an alternative strategy to obstruct infection. Pathogen-derived proteins either sustain pathogen growth in susceptible host or induce HR in resistant host, and some of them have proved to be effective in enhancing plant defence response while expressed in host systems and thus can serve as functional genes for creating resistant GM plants (Silva et al. 2018). Expression of such defence promoting effectors from pathogens may confer resistance in host plants. *N. benthamiana* expressing *P. sojae* CRN effector PsCRN115 was conferred with upregulated defence responses, such as ABC transporters, cytochrome P450 and PRRs, and increased the level of plant resistance to *P. capsici* and *P. parasitica* oomycetes (Zhang et al. 2015).

23.9.7 Silencing Pathogen Genes

RNA interference is a potential strategy to control microbial pathogens of all classes – viral, bacterial, fungal (bio-necrotrophic and oomycetes) and nematode – wherein expression of essential genes of pathogen required for proliferation and establishment of infection is silenced post-transcriptionally by siRNAs complementary to pathogen-derived mRNAs. A prerequisite for RNAi is a double-stranded DNA precursor derived from a replicating viral genome or viral RNA secondary structure, which will be cleaved by DICER-like proteins (ribonuclease III – DCL), and the resulting ssRNA is loaded into silencing complex (RISC complex). siRNA-guided activated complex further recognizes and degrades the target mRNA by Argonaut (Ago) protein in cytosol (Seo et al. 2013; Weiberg et al. 2014). Conserved domains of vital genes related to cellular processes, morphogenesis or pathogenesis can be potential target sites for host-induced gene silencing, which may confer broad-spectrum durable resistance. Similarly, host genes, which are inevitable for the infection process, whose silencing does not have any/have minimal negative fitness costs also, can be targeted. Transgenic plants expressing target-specific siRNAs successfully confer resistance by sequence-specific degradation of target mRNA.

23.9.8 Transgenic Expression of Detoxifying Pathogenic Toxins

Several pathogens produce toxins, which adversely affect various metabolic pathways/processes of host system. The expression of detoxifying enzymes, either native or transgenic, specific to each pathogen under native or heterologous promoter would considerably reduce/alleviate the detrimental effects of such pathogenic toxins. For instance, detoxification of phytotoxin oxalic acid from *Cryphonectria parasitica* causing chestnut blight disease was achieved in American chestnut transformed with wheat gene coding for the production of the degradative enzyme, oxalate oxidase, and the resultant GM chestnut plant showed considerable reduction in chestnut blight disease development (Zhang et al. 2013). Similarly, transgenic

wheat expressing a toxin-degrading enzyme from barley showed resistance to *Fusarium* head blight (Li et al. 2015).

23.9.9 Expression of Antimicrobial Compounds

Introduction of genes encoding antimicrobial compounds into host plant can confer efficient resistance to specific pathogens. Defensins, an antimicrobial peptide of plant origin, chitin-degrading enzyme from other microbial species, is a well-established example for this strategy. Resulting transgenic crops must display pathovar-/strain-/race-specific resistance to a respective pathogen in the field, while exhibiting normal growth and development pattern without compromising the yield and their responses to other biotic as well as abiotic stress stimuli. Spatio-temporally regulated expression of these genes using tissue-/development-specific promoters or pathogen-inducible promoter would be advantageous to minimize the negative fitness effects, if any. The effect of transgene encoded antimicrobial compound on animal/mammalian system should be assessed before employing this strategy for transforming edible crops. The manipulation of bacterial cry genes is an efficient strategy for controlling insect pests and thereby prevents vector-borne diseases (Mayee et al. 2003). A detailed understanding of the underlying molecular mechanisms of their mode of action would permit the development of much sophisticated strategies for the regulated expression of appropriate antimicrobial compounds to confer broad-spectrum durable resistance to crop plants.

23.10 Cisgenesis: Alternative to Transgenics

Cisgenesis is emerging as an efficient alternative strategy of crop improvement, which combines the beneficial aspects of classical breeding and modern biotechnology. In cisgenesis, desirable genes are transferred among crossable species/species capable of sexual hybridization (Schouten et al. 2006). In this method, the cisgene to be transferred is same as that in breeding procedure, and the resulting plant also is the same as a classically bred plant. Thus, cisgenesis eliminates biosafety concerns regarding transgenic plants, thereby overcoming the regulatory issues for field cultivation and marketing. As cisgenesis facilitates precise gene transfer, negative effects of linkage drag that associated with conventional breeding can be avoided. Additionally, cisgenesis is not time-consuming as the conventional breeding. Thus, cisgenesis can be adopted for specifically stacking resistance genes from crossable gene pools.

23.11 Conclusion

Genetic engineering technology has made tremendous contributions in developing disease-resistant crop plants. Transgenic technology has evolved multitude of options for precise alteration of crop genome, even at nucleotide level over a very short time span, and crop improvement programmes for disease resistance have taken advantage of these methods in order to circumvent the prevailing as well as expanding challenges/vulnerabilities posed by pathogenic organisms. From the incorporation of resistance genes or pathogen targeting genes and manipulation of plant's biochemistry for harming pathogens to editing of single bases to disrupt the entire plant–pathogen interaction and thereby disease development, genetic engineering techniques have opened up every possible way for crops to combat pathogens. Sequence databases and functional annotation information are expanding on a daily basis with the establishment of high-throughput sequencing and genotyping platforms. This further unravels novel molecular and genomic targets that can be manipulated for conferring robust – broad-spectrum – durable resistance to all crop plant species.

Despite the availability of transgenic technology for developing crops with desirable resistance traits, field-level cultivation and commercialization of produce are still a major challenge in the case of these plants due to stringent regulations. Although some transgenic crops have been commercialized, many are not going for field trials. Stability of resistant phenotypes without any negative fitness effects under field conditions needs to be ensured. Protocols for transformation and regeneration of farmer-preferred cultivars/local cultivars of crop of interest need to be optimized. Stigma associated with transgenic plants is another major issue that restricts the GE crops in laboratories or experimental glasshouses. Even in the case of engineering resistance, GE strategies, which modify or alter the endogenous immune components rather than those adding/introducing novel/foreign gene or sequence or metabolic pathways, are preferred, when such issues are concerned. Ecological and health concerns regarding possibilities of horizontal gene flow, especially the bacterial marker genes used in the transformation process and the loss of endogenous agrobiodiversity, are the prominent underlying causes of such regulations. The evaluation of transgenic crop products to ensure biosafety should be followed before releasing. For transgene-free editing options opened up by third-generation genome editing tool, CRISPR/Cas is a promising strategy to overcome regulatory barriers associated with transgenic crops. Recently, the definitions of genetically modified plants are getting revised, enabling field-level cultivation of many transgenic disease-resistant crops. However, the innovations that render transgenic technology free of any risks need to be emerged for better public acceptance of genetically modified crops. But lastly, the conventional breeding techniques, which created the large pool of disease-resistant cultivars we had all this time, cannot be replaced completely and are powerful to sustain crop production, despite its shortcomings. Genetic engineering can supplement and renovate at points where breeding techniques are struggling to prevail over and that will lead us to the prime goal, sustainable crop/food production.

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