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CRISPR/CAS9 SYSTEM TO TARGET PROVIRAL HOST GENES INVOLVED IN BEGOMOVIRUS INFECTION: OPPORTUNITIES AND CHALLENGES IN *Capsicum*

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REVIEW ARTICLE

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Abstract

Pepper (*Capsicum* spp., family Solanaceae) is an economically important spice in the world, due to its pungent taste and aroma. However, peppers are prone to a wide variety of viruses, including begomoviruses (family *Geminiviridae*), an emergent pathogen causing significant yield loss to a wide range of crops in the tropical and subtropical regions. Since plant viruses have limited coding capacity, enhancing plant recessive resistance is considered a promising approach for controlling plant viral diseases. This type of immune system, conferred by the absence or mutation of genes encoding critical host factors for the virus to complete its biological cycle, can be achieved through genome editing based on RNA-guided nucleases (CRISPR/Cas9). The CRISPR/Cas9 is a compelling system since it allows the development of transgene-free improved varieties of crops. Here we list and review some potential pro-viral host factors as targeted genetic resources for gene editing by CRISPR/Cas9, to establish recessive resistance gene-based breeding against begomovirus in pepper. We also address the challenges for the application of CRISPR/Cas9 system in pepper, particularly regarding to the high genotypic dependence and recalcitrant nature of genus *Capsicum* to genetic transformation and *in vitro* regeneration.

INTRODUCTION

Pepper (*Capsicum* spp., family Solanaceae) is an economically important crop that dominates the trade of hot spices worldwide. Genus *Capsicum* consists of more than 200 varieties grouped into more than 30 species, with five of which have been domesticated, including *Capsicum annuum* L., *C. baccatum* L., *C. chinensis* Jacq., *C. frutescens* L., and *C. pubescenns* Ruiz and Pav [1, 2]. Due to its pungent taste and aroma, pepper is widely used worldwide, and has been cultivated extensively with a large annual production rate. However, this genus is susceptible to many pathogens, including begomovirus.

Begomovirus is the largest genus of *Geminiviridae*, a family of single-stranded DNA (ssDNA) viruses. In fact, with more than 380 species recognized by the International Committee on Taxonomy of Viruses (ICTV), *Begomovirus* is the largest genus of all viral taxonomy [3, 4, 5]. Among all genus of geminiviruses, *Begomovirus* has the most complex genome organization. Their genomes can be either monopartite (containing a DNA A-like genome, about 2.9 kb in size) or bipartite (known as DNA-A and DNA-B, each of about 2.7 – 2.8 kb in size) [6]. In addition, three classes of circular satellite DNAs, known as alphasatellites, betasatellites, and deltasatellites, have been observed to be associated with begomovirus and enhance its pathogenicity

[7, 8, 9, 10]. Monopartite and bipartite begomovirus is widely spread in the Old World (OW) while bipartite begomoviruses are mostly distributed in New World (NW), with a few exceptions [11].

The infection of begomovirus is mediated by whitefly (*Bemisia tabaci*), which is distributed worldwide and colonizes a wide range of plants, causing significant yield loss to many crops, mostly in the tropical and subtropical regions [3]. The symptoms developed in plants affected by begomovirus are chlorotic mottle/yellow mosaic, curled and shrunken leaves, and acute stunting, and lead to a production loss range within 20 – 100% [12, 13]. Begomovirus species that attack pepper include TYLCV (Tomato yellow leaf curl virus), PYLCV (Pepper yellow leaf curl virus) [12], PepGMV (Pepper golden mosaic virus), and PHYVV (Pepper Huasteco yellow vein virus) [14].

Numerous attempts have been made to overcome begomovirus invasion, including integrated pest and disease management, excessive pesticide applications to the whitefly, and destruction of infected plants [15]. However, the complex epidemiological factors associated with begomovirus disease outbreaks have made it very difficult to develop effective long-term disease management strategies [16]. In addition, begomovirus is often found in complex diseases caused by various types of viruses [17] and possesses high recombination and mutation rates [18]. Therefore, preventing and controlling plant viruses effectively in agricultural production should involve virus-resistant or virus-tolerant crops.

Plant viruses have limited coding capacity hence they depend entirely on many host factors to multiply and invade their host [19, 20]. Subsequently, virus-resistant or virus-tolerant crops can be developed based on host-virus interaction at a molecular level. Based on their role in host–virus interactions, host factors can be divided into antiviral and proviral functional groups. Host factors with antiviral activities restrict the virus life cycle, such as virus replication, viral RNA translation, or virus movement, while host factors with proviral activity are necessary for essential steps of the infection process [21].

The presence of proviral factors in the host plant creates a permissive environment for virus infection. Therefore, the absence or disruption of those host factors will consequently impair virus infection, which is equivalent to loss of susceptibility in host plants [22, 23, 24, 25]. This type of plant resistance, conferred by the absence or mutation of genes encoding critical host factors for the virus to complete its biological cycle, is referred to as recessive resistance [15, 25, 26]. Taking advantage of this host plant's resistance machinery is considered as one of the most effective methods for controlling viral diseases due to its durability as an inherited characteristic [26].

Genome editing method based on RNA-guided nucleases allows site-specific reverse genetic engineering to be carried out on the targeted host genes efficiently [27, 28, 29]. The most widely used system is the type II clustered regularly interspaced short palindromic repeat (CRISPR)/Cas9 (CRISPR-associated), adapted from *Streptococcus pyogenes* resistance machinery against viruses [30]. This system has gained a lot of attention due to its simplicity, efficiency, and high reproducibility. Cas9 activity induces DNA double-strand breaks at a specific site [28, 31, 32], and DNA repair by non-homologous end-joining (NHEJ) results in the insertion or deletion of random base pairs, and if it happens in exon, causing gene knock-out by disruption [27, 30, 31, 33]. The Cas9 protein is guided by sgRNA, a short 20 RNA nucleotides that hybridize with specific sequence in the targeted gene, hence determining target specificity [28, 29, 30]. Not only providing the editing of specific target in genome, CRISPR/Cas-based genome editing is a compelling system for crops since following genome modification, the CRISPR/Cas9 system can be segregated out through the breeding program so that the end-product of mutated plant does not contain any transgenes [22, 25].

Host factors identified from naturally occurring resistant cultivars are important genetic resources for recessive resistance [26]. However, in the absence of such cultivars, it is important to introduce mutation in potential recessive resistance gene(s). Therefore, for genus *Capsicum* that lacks naturally occurring begomovirus-resistant cultivars, more susceptible genes to begomovirus need to be revealed to enable recessive resistance-based antiviral breeding.

Despite its extensive use as well as its improved efficiency and portability in the model plants, designing an appropriate and effective CRISPR/Cas9 system in crops is still a challenge for pepper. Unlike the other genera of Solanaceae, genetic manipulation in this genus is still lacking because *Capsicum* is known to be recalcitrant to genetic transformation and *in vitro* regeneration. This review focuses on our current understanding of the genetic resources for recessive resistance against begomovirus, and the opportunities and challenges for the application of CRISPR/Cas9 system to enhance recessive resistance gene-based breeding against begomovirus in *Capsicum*.

PLANT-VIRUS INTERACTION: THE BASIS OF ESTABLISHING PLANT RECESSIVE RESISTANCE

Begomovirus genomes are arranged in several overlapping viral transcripts under the control of two RNA polymerase II promoters generally located within the intergenic region, which is also contained the origin for rolling-circle replication [34]. There are 6 genes in the DNA A component of OW begomoviruses, *C1-C4* in the complementary strand, and *V1-V2* in the virion strand [6, 35]. The four genes in the complementary strand encode a replication-associated protein (C1, Rep), a transcription activator protein (C2, TrAP), a replication enhancer protein (C3, REn), and a C4 protein, while *V1* and *V2* encode for a coat protein (CP) and a pre-coat protein, consecutively [6, 34, 36]. However, the DNA A component of NW bipartite begomoviruses lacks a V2 ORF [3, 35]. The protein encoded

by *V1* and *V2* are involved in intra- and inter-cellular macromolecular movement, encapsidation, and mediates vector transmission [37, 38]. CP also functions as the nuclear shuttle protein (NSP) for monopartite viruses [36]. On the other hand, the DNA B of bipartite viruses encodes a nuclear shuttle protein (BV1, NSP) and a movement protein (BC1, MP) to facilitate virus transport within and across cells [6, 11].

Due to their small genomes and limited coding capacities, Begomovirus depend exclusively on host machinery for completing their life cycles. They need to interact with a wide range of host proteins, redirecting and reprogramming plant processes to facilitate viral DNA replication, gene expression and movement, and at the same time, counteract the innate immune response of the host plant [34]. Therefore, the establishment of infection is determined genetically by the availability of host factors essential for the virus life cycle, in addition to the balance between plant immune system and the suppression of host defense by the virus [21]. The absence or alteration of genes encoding critical host factors required for virus infection appoints to plant defense mechanism [21, 26]. This type of plant defense mechanism is referred to as recessive resistance. In fact, about half of the resistance genes targeting plant viruses are inherited recessively [15], indicating that defense systems involving recessive genes are more generally aimed at viruses than other types of pathogens [26].

To understand how begomovirus manipulates its host, the protein network of host-viral interactions should be identified. Host factors are identified either through forward or reverse genetic approaches using model plants [23, 39], screening for interactors with viral proteins [4, 40], or the insight from the differentially expressed genes and proteins between susceptible and resistant cultivars [41, 42]. Furthermore, the interacting proteins need to be functionally characterized to provide with potential host factors for resistance against viruses.

Several studies showed that a viral protein can interact with many host factors [4, 40, 43]. The interaction studies of MYMIV-Rep with the host peptide library in phage and host cDNA library in yeast have demonstrated that Rep might interact with a wide range (about 150) of host factors [43]. Maio et al. [4] identified the interactors of TYLCV Rep proteins in tomato by affinity purification-mass spectrometry (AP-MS) analysis to build a protein network of the Rep tomato interactome. From the 427 candidate interactors, a total of 54 high-confidence tomato proteins was obtained, of these, 27 proteins interacted with Rep alone, 40 proteins were expressed together with Rep-PCNA, and 13 proteins interacted with Rep and Rep-PCNA [4]. Similarly, Wang et al. [40] used the AP-MS analysis to identify protein-protein interactions between TYLCV-encoded proteins (C1/Rep, C2/TrAP, C3/REn, C4, V2, and CP) and its host *N. benthamiana*, and later, to infer the interaction network of each viral protein. A total of 728 high-confidence plant interactors were identified: 284 proteins interacted with Rep, 345 proteins interacted with TrAP, 264 proteins interacted with REn, and the host proteins interacted with C4, CP, and V2 proteins were 31, 25, and 24, respectively.

The insight of plant host factors involved in begomovirus infection can be obtained from transcriptomic and proteomic studies, through identifying the differentially expressed genes and proteins between resistant and susceptible cultivars to begomovirus. The transcriptomic studies carried by Chen et al. [41] showed that of the total of 34831 mapped transcripts, 209 and 809 genes were differentially expressed in the TYLCV-resistant tomato breeding line CLN2777A and TYLCV-susceptible tomato breeding line TMXA48-4-0, respectively. On the other hand, Huang et al. [42] conducted proteomic studies to investigate the molecular mechanisms involved in tomato leaf defense against TYLCV infection and to build a putative TYLCV infection response network. Eighty-six differentially expressed proteins were identified between resistant tomato cultivar 'Zheza-301' and susceptible cultivar 'Jinpeng-1' after TYLCV infection [42].

The identification of plant genes involved in infection and in resistance to begomoviruses has also been done using a reverse genetic approach. Lozano-Durán et al. [39] used TRV-induced gene silencing in combination with TYLCSV for reverse genetic studies and identified eighteen genes potentially involved in begomovirus infection. Seven of which, promote earlier infection after silencing thus have a potential anti-viral effect, whereas the expression of eleven genes is required for TYLCSV infection [39]. Also, Czosnek et al. [44] discovered five host genes involved in the resistance network against Tomato yellow leaf curl virus (TYLCV) infection using Tobacco rattle virus-based Post-Transcriptional Gene Silencing (PTGS).

Further functional characterization of identified host factors by reverse genetic approach revealed two key findings in host factors-viral protein interactions as the basis to develop recessive resistance mechanisms in the host plants. First, even though one viral protein interacts with many host factors, knock-out or silencing on a single gene encoding proviral host factor can significantly increase plant resistance to the corresponding virus [26, 25]. Pyott et al. [25] showed that mutation in *eIF(iso)4E* locus resulted in TuMV-resistant *Arabidopsis thaliana*. Further, mutation in *eIF4E* isoforms of cassava [45] and *eIF4E1* gene of *A. thaliana* [46] increased plant resistance against Cassava brown streak virus (CBSV) and Clover yellow vein virus (ClYVV), respectively. Another example, silencing of *NSI, HSC70-1* or *SK41/SKK* gene negatively affects TYLCSV infection in *N. benthamiana* [39].

Second, knock-out or silencing of one gene encoding proviral host factor can reduce the severity of infection caused by several types of viruses [22, 24]. For instance, the disruption of the *eIF4E* gene in *Cucumis sativus* gives rise to plant resistance against Cucumber vein yellowing virus (CVYV, *Ipomovirus*) and Papaya ring spot mosaic virus-W (PRSMV-W, *Potyvirus*) [22]. Also, mutation in the *nCBP* gene can reduce infection severity in *A. thaliana* caused by

PIAMV (Plantago asiatica mosaic virus), AltMV (Alternathera mosaic virus) and CymMV (Cymbidium mosaic virus) from genus *Potexvirus*, LoLV (Lolium latent virus, genus *Lolavirus*), and PMV (Panicum mosaic virus, genus *Carlavirus*) [24].

To date, eukaryotic translation initiation factor (eIF4) is the proviral host factor most studied for developing plant recessive resistance to viruses. Accordingly, eIF4-based resistance to viruses could help in defining a general approach applicable to other host factors. The first step is using high throughput techniques such as AP-MS, transcriptomics or virus-induced gene silencing to identify novel interactors of viral proteins in the host plants, and the second step is evaluating the biological importance of protein-protein interaction for virus infection [47]. In addition, essential genes required for host survival cannot be disturbed. Therefore, the possibility of host genes disruption upsetting plant health, growth, development, and yields, as well as causing pleiotropic effects such as nutrient deficiency and dwarfness, should also be evaluated when the host gene is manipulated [47, 48]. For example, HSP90 is required for viral replication of Red clover necrotic mosaic virus (RCNMV, *Dianthovirus*) [49] and Bamboo mosaic virus (BaMV, *Potexvirus*) [50]. However, cytosolic HSP90 is also important for disease resistance against pathogens other than viruses [51, 52]. Further evaluation should also be carried out on the effect of knock-out or silencing of host gene to the plant since it may affect plant's agronomic traits differently. For example, silencing of *eIF4E* genes impairs growth and fertility in tomato [53] but not in potato [54].

PROVIRAL HOST FACTORS: GENETIC RESOURCES FOR ESTABLISHING PLANT RECESSIVE RESISTANCE TO BEGOMOVIRUS

Over the last few decades, a large number of plant host factors have been identified and functionally characterized to generate a better understanding of virus life cycles and the molecular basis of plant-virus interactions. Cao et al. [55] further classify proviral host factors into four groups: (1) the negative regulators of plant defenses, (2) the susceptible factors involved in viral life cycles, (3) the host factors participating in viral proteins modification to enhance their effective function, and (4) the factors involved in the cellular processes beneficial for virus behaviors. Table 1 lists several host factors that have been identified to interact with Begomoviral proteins and functionally characterized, as potential recessive resistance targets for gene editing by CRISPR/Cas9 system.

Table 1. The genetic resources for developing recessive resistance against begomovirus

Begomovirus	Viral protein	Host plant	Host gene	Begomovirus-host interaction	References
MYMIV	Rep	*N. xanthi*	*RPA32*	Viral DNA replication	56
MYMIV	Rep	*A. thaliana*	*Rad51, Rad54*	Enhances viral replication	57, 58
MYMIV	Rep	*A. thaliana*	*MCM2*	Enhances viral replication	34, 59
TGMV CaLCuV	Rep	*N. benthamiana*	*RBR*	Enhances viral replication and cell division	60, 61, 62
TYLCSV	Rep	*N. benthamiana*	*GRAB2*	Enhances viral replication	39
MYMIV	Rep	*A. thaliana*	*NAC083*	Enhances viral replication	63
ChiLCV	Rep	*N. benthamiana*	*UBC2* *HUB1*	Enhances viral genes transcription	64
TYLCSV TGMV	Rep	*N. benthamiana*	*SCE1*	Alters cellular process through modifying sumoylation	65, 66
TYLCV TYLCSV TGMV MYMIV	Rep, REn	*A. thaliana* *N. benthamiana* *S. lycopersicum*	*PCNA*	Enhances viral replication	4, 34, 67, 68, 69
TYLCSV TYLCV	TrAP	*N. benthamiana*	*CSN3*	Alters cellular process through modifying ubiquitination	39
TYLCSV	TrAP	*N. benthamiana*	*ASK2*	Alters cellular process through modifying ubiquitination	39

TGMV	TrAP	*A. thaliana* *N. benthamiana*	*Rgs-CAM*	Suppress post-transcriptional and transcriptional gene silencing	70
TYLCSV	REn	*N. benthamiana*	*deltaCOP*	Vesicular transport of viral components	39
TLCV	REn	*S. lycopersicum* *N. benthamiana*	*NAC1*	Enhances viral replication	71
TYLCSV ToLCGdV	C4	*N. benthamiana*	*BAM1*	Suppress post-transcriptional gene silencing	39, 72
TYLCSV	C4	*N. benthamiana*	*SK4-1/SKK*	Suppress gene silencing and trigger disease symptoms	39
TYLCCNV TLCYnV	C4	*N. benthamiana*	*SKη*	Induction of abnormal cell division	73
TLCV	C4	*S. lycopersicum*	*SK*	Suppress gene silencing	74
TYLCV	CP	*S. lycopersicum*	*HSP70*	Localization of viral proteins into nucleus	75, 76
CaLCuV	NSP	*A. thaliana*	*AS2*	Suppress post-transcriptional gene silencing (PTGS)	77
CalCuV TGMV	NSP	*A. thaliana*	*NsAK*	Regulate NSP function and enhance virus infection	78
TGMV TCrLYV CaLCuV	NSP	*N. tabacum*	*NIG*	Redirects the viral protein from the nucleus to the cytoplasm	79
TYLCSV CaLCuV	NSP	*A. thaliana* *N. benthamiana*	*NSI*	Facilitates the ss-viral DNA-NSP nuclear export	39, 80, 81
BDMV TYLCV	NSP MP	*N. benthamiana* *S. lycopersicum*	*Histone H3*	Facilitates DNA viral trafficking intra- and intercellularly	82
CaLCuV	MP	*A. thaliana*	*SYTA*	Endocytosis and virus movement protein cell-to-cell transport	83
TYLCV	-	*S. lycopersicum*	*Pelo*	Involved in ribosome recycling-phase of protein synthesis	85, 86

Abbreviation of virus: BDMV, Bean dwarf mosaic virus; CaLCuV, Cabbage leaf curl virus; ChiLCV, Chilli leaf curl virus; MYMIV, Mungbean yellow mosaic India virus; TGMV, Tomato Golden Mosaic Virus; TLCV, Tomato leaf curl virus; TLCYnV, Tomato leaf curl Yunnan virus; ToLCGdV, Tomato leaf curl Guangdong virus; TYLCCNV, Tomato yellow leaf curl China virus; TYLCSV, Tomato yellow leaf curl Sardinia virus; TYLCV, Tomato yellow leaf curl virus.

Abbreviation of gene: *RPA2*, Replication Protein A2; *MCM2*, Minichromosome Maintenance Complex Component 2; *RBR*, retinoblastoma-related; *GRAB2*, geminivirus Rep A-binding; *NAC083*, NAC domain-containing protein 83; *UBC2*, Ubiquitin-Conjugating Enzyme2, *HUB1*, Histone Monoubiquitination1; *SCE1*, SUMO, conjugating enzyme E1; *CSN3*, COP9 signalosome subunits 3; *ASK2*, Apoptosis signal-regulating kinase 2; *rgs-CaM*, regulator of RNA silencing, calmodulin-like protein; *delta COP*, coatomer delta subunit; *NAC1*, NAC domain-containing protein 1; *PCNA*, Proliferating Cell Nuclear Antigen; *BAM1*, Barely any meristem 1; *SK4-1/SKK*, Shaggy-related kinase proteins; *SKη*, Shaggy-like kinase η; *SK*, Shaggy-like kinase; *HSP70*, Heat shock protein; *AS2*, Asymmetric Leaves2; *NsAK*, a prolinerich extensin-like receptor protein kinase (PERK); *NIG*, NSP-interacting GTPase; *SYTA*, synaptotagmin A; *NSI*, nuclear acetyltransferase I.

Rep is the only viral protein strictly required for viral DNA replication to occur *in planta* [4]. This multifunctional, oligomeric protein is required for initiation, elongation as well as termination of the viral replication process [84]. Rep contains site-specific and conserved DNA-binding, also has nicking and ligation, ATP-dependent topoisomerase and ATPase activities [56, 68, 84]. Although the Rep is a crucial protein for viral replication, it requires support from various host factors for efficient viral DNA replication.

Rep protein recruits host factors to form viral replisomes, a complex of viral and host proteins that carry out viral DNA replication [4], such as RPA32 [56], Rad54 [57], Rad51 [58], and MCM2 [59]. RPA32 interacts directly with MYMIV-Rep through a novel interacting site at the C-terminus of the Rep, and modulates the Rep functions by enhancing its ATPase activity, downregulating its nicking and ligation activity, and upregulating the transient replication of the MYMIV-amplicon [56]. Rad51 and Rad54 also interact

directly with MYMIV-Rep, enhancing MYMIV-Rep nicking, ATPase, and helicase activities *in vitro* [57, 58]. *In planta* replication assay further confirms the role of these proteins to enhance MYMIV replication through the presence of higher Rad51 and Rad54 transcripts in MYMIV-infected plants than in uninfected, healthy plants. Suyal et al. [59] revealed the interaction between *A. thaliana* MCM2 and MYMIV and further confirmed the role of MCMC2 in MYMIV replication *in planta* through transient replication assay in both wild-type and mcm2Δ mutant arabidopsis plants. However, the mechanism of MCM2 involvement in begomoviral replication has not been clarified.

In addition, Rep reprograms the host cell cycle by interacting with transcriptional regulators of the cell cycle, such as plant RBR [60, 61, 62], GRAB2 [39], and NAC083 [63]. The binding of Rep protein to pRBR releases the transcription factor E2F, probably turning on the expression of many genes involved in G1/S phase transition and S phase progression, and forcing the cell to enter into S phase [56, 62]. GRAB2 and NAC083 are transcription factors that belong to the NAC-domain containing protein family [63, 87]. Xie et al. [87] suggested that GRAB proteins participated in cellular pathways related to viral DNA replication by acting directly or indirectly. Lozano-Duran et al. [39] showed that silencing of the *GRAB2* during TYLCSV infection inhibited viral propagation, even though the opposite effect was observed when interacting with WDV-RepA [87]. Interaction between *A. thaliana* NAC083 and MYMIV-Rep was observed in study by Suyal et al. [63] and there was a possibility that AtNAC083 involved in geminiviral DNA replication by altering the nicking, ATPase, or helicase activities of Rep.

Rep is also interacted with host factors involved in post-translational modification mechanisms, such as ubiquitination [64] and sumoylation [65, 66]. Ubiquitin facilitates protein-protein interactions, alters the molecular conformation of the target protein, hence promoting its interaction with other proteins. Kushwaha et al. [64] revealed that the interaction between ChiLCV-Rep and two major components of the monoubiquitination machinery, UBC2 and HUB1 from *N. benthamiana*, resulted in the monoubiquitination of histone 2B and finally enhances transcription of the viral genes. On the other hand, SUMOylation is a posttranslational process that modifies the function, activity, or localization of the target protein by a ubiquitin (Ub)-like polypeptide (Ubl) called SUMO [88], catalyzed by different enzymes: the activating enzyme (E1) and conjugating enzyme (E2), respectively [65]. Rep interacts with SCE1 and modifies the sumoylation state of selected host proteins to create an environment suitable for viral DNA replication [66].

Rep protein also interacts with other viral proteins such as REn and recruited several host factors such as PCNA [3, 34, 67, 68, 69]. PCNA is a highly conserved protein in eukaryotes that plays an essential role in the cell cycle, DNA replication, and DNA repair machinery [4]. PCNA is recruited as a part of viral replisomes and acts as a DNA clamp for processive DNA synthesis [67, 68].

TrAP is a multifunctional protein encoded by gene *C2* that involved in gene activation, virus pathogenicity, and suppression of gene silencing [89]. Several host factors interacted with Begomoviral-TrAP including CSN3, ASK2 [39], and Rgs-CAM [70]. CSN3 is a part of CSN complex, which regulates the activity of ubiquitin Cullin RING Ligases (CRLs), an essential component of SCF ubiquitin E3 ligase complex [90]. Similarly, *ASK2* is a member of a gene family encoding SKP1-like protein, a component of SCF ubiquitin-protein ligase complexes [91]. CSN3 and ASK2 are probably recruited by TrAP to redirect and modify ubiquitination of certain host proteins, thus altering the host cellular processes regulated by SCF complexes and creating a permissive environment for virus infection [39, 89]. On the other hand, rgs-CaM is a regulator of gene silencing and its interaction with TrAP protein negatively regulates RNA silencing and suppresses antiviral response of the host plant [70].

REn is a protein encoded by *C3* gene that can enhance symptom development in plants infected by begomoviruses [89]. REn is also involved in virus replication, although not essential, through interaction with Rep and PCNA [67]. Not many host factors have been identified to interact with begomoviral-REn protein. Besides PCNA, other host factors that interacted with the begomoviral-REn are deltaCOP [39] and NAC1 [71]. deltaCOP is a component of the polymeric coatomer coat complexes COPI, which has been associated with intracellular vesicular transport between the ER and Golgi [92]. The study by Lozano-Durán et al. [39] showed that *deltaCOP* silencing completely eliminated TYLCSV infection, suggesting the importance of vesicular trafficking in viral infection.

C4 protein involves in symptom development, virus movement, and is able to suppress RNA silencing, although conserved for several bipartite and monopartite geminiviruses [89]. Several studies revealed that C4 protein interacted with protein kinases, including BAM1 [39, 72], SK4-1/SKK [39], SKη [73], and SK [74] with various biological importance to virus infection. Li et al. [72] revealed that ToLCGdV-C4 interacts with BAM1 suppressed post-transcriptional gene silencing in *N. benthamiana* while silencing of *BAM1* delayed or suppressed TYLCSV replication [39]. Similarly, the interaction between ToLCV-C4 and SK from *S. lycopersicum* also suppressed host RNA silencing [74]. In addition, silencing of SK4-1/SKK that negatively impacts TYLCSV infection as shown by Lozano-Durán et al. [39], can also be associated with C4 function to suppress gene silencing [74]. On the other hand, the TLCYnV-C4 hijacks NbSKη to induce abnormal cell division in plants and eventually, enhances viral DNA replication [73].

CP is the only structural protein-encoding by begomovirus genes. In addition to virus genome packaging, the CP has been associated with several other functions,

including insect transmission of monopartite begomovirus, intracellular trafficking of viral DNA into and out of the nucleus, cell-to-cell movement, and essential for host plant infection [89]. Gorovits et al. [75, 76] reported that TYLCV-CP interacts with the HSP70 and enhances TYLCV multiplication in *S. lycopersicum* by promoting TYLCV DNA-CP complexes and infectious virions intracellular movement.

NSP, one of the two proteins encoded by DNA B of bipartite begomovirus, is required for viral ssDNA transport between the nucleus and the cytoplasm in the form of a viral DNA–NSP complex [11, 89]. The host factor involved in trafficking Begomoviral DNA in and out of the nucleus upon its interaction with NSP is NIG, a cytosolic GTP-binding protein that possesses GTPase activity [79], and a nuclear protein NSI [39, 80, 81]. According to Carvalho et al. [79], NSP interacts with NIG to redirect the movement of viral protein from nucleus to cytoplasm and facilitates MP-NSP interaction in cytoplasm, hence contributing to begomovirus infection. On the other hand, NSP binds to ssDNA-CP complex, recruits NSI to acetylate genome-bound CP [81], disrupts CP-ssDNA binding, and allows NSP to displace CP binding to the viral genome for nuclear export [80]. Silencing of *NSI* also negatively affects TYLCSV infection [39]. Besides NIG, modulation of NSP function also happens following interaction between NSP and NsAK, an authentic serine/threonine kinase [78]. NsAK enhanced the phosphorylation level of CaLCuV-NSP, increasing the efficiency of CaLCuV infection and elevating symptom development [78]. In addition to facilitating the transport activity of viral components, it turns out that NSPs also modulate host gene silencing. Ye et al. [77] showed that CaLCuV-NSP induced the expression of *AS2*, an endogenous suppressor of virus silencing, which activated the mRNA decapping activity and weakened antiviral defenses in host plants.

NSP facilitates trafficking of viral DNA components through the nuclear pore complex, while MP facilitates DNA viral transport through plasmodesmata [82, 89]. However, Frischmuth et al. [93] also suggested the NSP-MP cooperation to enable the transfer of viral DNA to adjacent cells, in the form of MP-DNA-NSP complex. To facilitate this intra- and intercellular transport, MP, NSP, and viral DNA form complex with histone H3 [82]. MP also interacts with host factors SYTA to facilitate cell-to-cell movement and systemic spread of CaLCuV-DNA, since SYTA regulates endosome recycling and mediates CaLCuV-MP trafficking of plant virus genomes through plasmodesmata [83].

Another susceptible gene to begomovirus identified in tomato is *Pelo* gene. Located in Ty-5 locus of *S. lycopersicum*, this gene synthesizes Pelota (PELO) protein, an mRNA surveillance factor that play important role in ribosome recycling during protein synthesis [85]. Although its interaction with begomoviral is still unknown, the gene knockout suppressed TYLCV proliferation in tomato [85, 86]

Even though the establishment of efficient, recessive resistance-type antiviral control strategies against plant viral diseases requires many more genetic resources, the most widely exploited recessive resistance genes in several crop species to date are *eIF4E*, *eIF4G*, and their isoforms [26, 94]. In fact, the identification and characterization of host factors involved in DNA virus infection such as begomovirus are still underrepresented compared to those involved in RNA virus infection. Therefore, identification and functional characterization of novel proviral host factors involved in the begomovirus life cycle are imperative to a better understanding of begomovirus infection in crops, including pepper.

CRISPR/CAS9 MEDIATED VIRUS RESISTANCE IN PLANTS

CRISPR/Cas systems are part of the adaptive immune system against viruses in archaea and bacteria, by cleaving the foreign DNA in a sequence-dependent manner [95]. The CRISPR locus was first identified by Ishino et al. [96] in the *Escherichia coli* genome as a direct repeat sequence of 29 bp, interspersed by a 32 bp spacer sequence, in an almost palindromic pattern. In 2002, homologous *CRISPR-associated* (*Cas*) genes were found around the locus, and for the first time, it was named clustered regularly interspaced short palindromic repeats (CRISPR) [97]. The biological function of CRISPR began to be understood in 2005, when it was discovered that the spacer sequences were homologous to viral and plasmid sequences [98, 99, 100]. The hypothesis that CRISPR may play a role in prokaryotic cell immunity against viruses was confirmed by Barrangou et al., [101] in *Streptococcus thermophilus*. After that, several studies reported that the mechanism of CRISPR system is guided by RNA molecules, known as crRNA [103].

Cas protein is a key molecule in CRISPR system due to its role in crRNA synthesis as well as the recognition and degradation of foreign nucleic acids [104]. Therefore, the molecular mechanism of all types of CRISPR was determined based on the type of Cas protein involved. The CRISPR/Cas system is divided into two main classes, 6 types and 33 subtypes [105]. The class 1 consist of Type I, Type III and Type IV. This class uses an effector module consisting of several Cas proteins to form a protein-crRNA complex that acts to bind and cut the DNA target. Meanwhile, class 2 that comprises Type II, Type V, and Type VI, only uses a single effector protein containing multiple domains, whose functions are analogous to all effector complexes in the class 1 CRISPR system [105].

The CRISPR type II system is the first CRISPR system used in various genome editing studies in eukaryotic cells [106]. In addition to being the most studied mechanism, the type II system only requires one Cas protein to recognize and

cleave the targeted DNA [107], hence simplifying its uses as a tool for genome editing. The transition of CRISPR/Cas9 system from a biological phenomenon to a biotechnological tool for genome editing happened following the discovery that the target DNA can be simply reprogrammed by replacing 20 nucleotides of crRNA with nucleotide sequences complementary to the target DNA sequence [28]. In addition, the 3' end of the crRNA is fused with the 5' end of the tracrRNA to form a single guide RNA (sgRNA) chimera [28].

A requirement for cleavage by Cas9 is the presence of a conserved protospacer-adjacent motif (PAM) downstream of the target DNA, which usually has the sequence 5′-NGG-3′ or less frequently NAG [31]. Cas9 activity leads to the formation of double-stranded breaks (DSB) in the target DNA [28, 31, 32] resulting from cleavage by the HNH and RuvC domains of Cas9 [108]. DSB can be repaired at least through two different pathways found in almost all cell types and organisms: non-homologous end-joining (NHEJ) and homologous recombination (HR) [27, 30, 33]. Repairment through the inaccurate NHEJ pathway can produce insertion and deletion of varying lengths at DSB sites, while repairment via the HR pathway can be exploited to achieve precise gene modifications or gene insertions [27, 31].

The CRISPR/Cas9 technology enables precise genomic modification in various organisms and has been used recently for enhancing disease resistance in plants. The CRISPR/Cas9 system has also been used to improve plant resistance to viruses, either by targeting direct modification to the viral genome or modifying the host plant genome [16, 22, 25, 109, 110, 111, 112]. However, targeting virus mutation may lead to unpredicted evolution of the virus that results in viral variants which could evade the plant resistance machinery [110]. In addition, modifications to the viral genome require the generation of stable transgenic plants overexpressing Cas9 protein to provide durable resistance to virus and inevitably, raise the ethical issues of genetically modified crops [113]. On the contrary, by targeting modification in the host genome, the CRISPR/Cas9 device can be segregated out of the plant through a breeding program so that the engineered plant products obtained are non-transgenic plants [22, 25].

The antiviral engineering in plants by CRISPR/Cas9 technology has been carried out through disruption of essential host genes encoding proviral host factors, and therefore, give rise to loss-of-susceptibility phenotypes in plants. The earliest application of CRISPR/Cas9 system to target susceptible host genes and increase plant resistance against viruses has been demonstrated by Pyott et al. [25] and Chandrasekaran et al. [22]. Both targeted the eukaryotic translation initiation factors and their isoform, which play an essential role in the plant's translational mechanism and an important susceptible factor for various viral infections [94]. According to Pyott et al. [25], point mutation by deletion in *eIF(iso)4E* locus resulted in TuMV-resistant *A. thaliana*. Likewise, the disruption of the *eIF4E* gene in *C. sativus* has increased plant resistance against CVYV and PRSMV-W [22]. Further, *eIF4E* isoforms of cassava [45] and *eIF4E1* gene of *A. thaliana* [46] were targeted using CRISPR/Cas9 and found that the mutation increased plant resistance against CBSV and ClYVV, correspondingly. Likewise, a mutation in *eIF4G* gene of rice also improved the resistance against Rice tungro spherical virus (RTSV) and Rice tungro bacilliform virus (RTBV) [114].

Although the published research is still scarce, CRISPR/Cas9 system for gene editing has also been successfully applied recently in several members of Solanaceae family to confer plant resistance against viruses (Table 2). In *Capsicum*, CRISPR/Cs9 technology has been used at various states of plant stages. Kim et al. [119] successfully edited *C. annuum MLO2* (*CaMLO2*) gene in protoplasts derived from leaf-induced callus with the highest indel frequency of 11.3% and 17.5% in Dempsey and CM334 cultivars, respectively. They showed that protoplasts derived from leaf-induced callus are a useful system for screening of efficient guide RNAs for CRISPR/Cas9 [119]. The same *C. annuum* cultivars were also subjected to *Agrobacterium*-mediated gene editing using AGL1, EHA101, and GV3101 strains to edit *CaMLO2* gene in callus system [120]. The results showed different indel frequencies from the non-transformed calli, with an average frequency of 0.028% and the highest frequency of 0.07% in Dempsey, and an average frequency of 0.035% and the highest frequency of 0.09% in CM334 with EHA101 [120]. Although the indel frequencies in the target *CaMLO2* gene of transformed calli were not as high as those shown in protoplast-based systems at more than 10%, the editing of 1-bp deletion at the target locus occurred very precisely and was reproducible [119, 120]. Another study by Kurniawati et al. [118] has successfully implemented CRISPR/Cas9 system to edit *PCNA* gene in var. Lingga and Chiko of *C. annuum* to confer resistance to yellow leaf curl disease. Unlike the previous studies, Kurniawati et al. [118] delivered CRISPR/Cas9 component to the seeds using *Agrobacterium*-mediated *in planta* transformation and attained a primary indel pattern of a 1-bp deletion at the target locus of *PCNA* gene. These results demonstrate that generating a gene-edited *Capsicum* cultivar using CRISPR/Cas9 system to develop plant resistance against viruses is challenging but feasible.

Table 2. Application of CRISPR/Cas9 targeting susceptible plant genes to confer viral resistance in family Solanaceae

Species	Target Gene	Mutation Type	Mutation position	Affected phenotype	References
Tomato	*eIF4E1*	Base deletion, frame shift mutation	Exon 1	Enhanced resistance to Pepper mottle virus	115
Tomato	*Pelo*	Base insertion	Domain eRF1_1 (multiplex CRISPR/Cas9)	Suppressed the accumulation of TYLCV	86
Potato	*Coilin*	Large deletion (up to 600 bp)	Allele A1 and A2 of *Coilin* gene (bombardment with gold microparticles)	Increased resistance to Potato virus Y	116
Tobacco	*Va*	Mostly base deletion	Exon 1	Increased resistance to Potato virus Y	117
Pepper	PCNA	Base deletion	Exon 1	Increased resistance to TYLCV	118

RECALCITRANCY AND GENOTYPE DEPENDENCY OF CAPSICUM: BOTTLENECK FOR GENETIC TRANSFORMATION AND *IN VITRO* REGENERATION

The general pipeline of CRISPR/Cas9 includes delivery of CRISPR/Cas9 components into the plant, followed by callus induction and plant regeneration via organogenesis [121]. Therefore, the successful application of the CRISPR/Cas9 system is closely supported by the efficiency of genetic transformation and *in vitro* regeneration methods in host plants. An effective transformation system allows for a high success rate of plant mutation, while *in vitro* regeneration is essential to produce transgenic plants and carried out stable inheritance of mutated alleles or genes. Transient transformation is used to evaluate the activity of sgRNA design to edit targeted sequence or gene and a part of screening system for the design of CRISPR/Cas9-mediated gene editing. Even though transient expression of CRISPR/Cas9 components can produce DNA-free genome editing in plants, the stable expression of CRISPR/Cas9 components in germline cells is preferable since all plant cells will express the CRISPR system, which may not occur in transient expression [122]. Therefore, CRISPR/Cas9 components need to be introduced into germline cells for allowing mutations to be inherited by the next plant generation without the need of tissue culture.

Several methods have been applied to deliver CRISPR/Cas9 components in plants, including *Agrobacterium*-mediated, PEG-mediated transformation, and biolistic or bombardment transformation, [122, 123]. The *Agrobacterium*-mediated transformation method is still one of the most widely applied methods. A binary vector containing Cas9 and gRNA is transformed into *Agrobacterium* strains and transferred into the desired explants such as callus, leaves, and flowers [124]. The significant advantage of this method is its high editing efficiency compared to other delivery methods, such as biolistic method [125]. Also, stable transgene integration can be achieved, most of which are integrated as many as one copy [125]. On the other hand, PEG-mediated transformation required plant cells to firstly remove their rigid walls, and the regeneration process of protoplast is arduous with the possibility of obtaining many somaclonal variations [123].

CRISPR/Cas9 components can be introduced in plan cells using plasmid, ribonucleoproteins (RNPs) or viruses. The use of plasmid containing T-DNA is the most common, but recently, RNP has become an attractive approach due to several advantages, such as allowing DNA/transgene-free editing, off-target effects are minimal, and having low toxicity due to the rapid degradation of RNPs [126]. Although this method has been applied in many plant species, the editing efficiency is relatively low, and the use of protoplast complicated the plant regeneration and selection [126]. Viruses have also been used recently as mediators to improve the efficiency of genome editing in plants, mostly from family *Geminiviridae* [127] and TRV from family *Virgaviridae* [128]. However, due to the small sizes of their genomes, the viruses are mainly used for the high production of guide RNA in transgenic plants expressing the *Cas9* gene [129].

For the disease-prone peppers, plant improvement through tissue culture and genetic engineering is becoming a functional aspect of breeding programs to produce disease-resistant cultivars in pepper. However, this approach is still difficult to apply in pepper because unlike other members of the Solanaceae family, the genus *Capsicum* is naturally unresponsive or recalcitrant to genetic transformation and *in vitro* regeneration [130, 131, 132, 133].

The transformation method mostly used for *Capsicum* is mediated by *Agrobacterium*. However, the success rate of this method in *Capsicum* cultivars is generally low. For example, Pusa Jwala with an efficacy level of 5-12.2% [133],

Bryza, 13.3% [134], Nockkwang, 0.6% [135], and California wonder, 1.3-2.9% [136]. Other than the recalcitrant nature of *Capsicum*, low efficiency of *Agrobacterium*-mediated transformation is partly due to the low incidence of stably transformed plants, the long period of tissue culture, limited success of transformation to only a few genotypes, tissue browning, and necrosis induced by *Agrobacterium*, induction of somatic mutations, and low amount of DNA transferred insufficient for efficient genetic transformation [124, 137]. In addition, there is no universal transformation protocol that can be applied to various cultivars [131, 132]. Therefore, despite of its extensive use as well as its improved efficiency and portability in the model plants, delivering CRISPR/Cas9 components to the host cells via *Agrobacterium*-mediated transformation is still a challenge for pepper.

Previous studies have revealed various problems associated to the difficulty of carrying out *in vitro* regeneration in *Capsicum*, such as severe recalcitrant morphogenic nature [132]. Even though there are several reports of relative success [130, 131, 133, 134, 136, 138], genetic engineering is still limited by the low morphogenetic potential of *Capsicum* species [139]. Also, *Capsicum* tends to form rosette shoots or ill-defined shoots structures, which are resistant to the elongation process [132, 136]. The difficulty of elongation in regenerated pepper shoots seems to be due to the development of degenerative meristem and teratological development processes [140]. The histological study by Mezghani et al. [140] revealed a large number of directly induced teratological protrusions, whereas shoot meristems with a clear organization were rare.

High genotype dependence is another major factor inhibiting organogenesis in *Capsicum* cultures. *In vitro* conditions designed for the regeneration of a specific cultivar have been shown to be unsuitable for the propagation of other cultivars [134, 141]. The existence of high genotype specificity to the various regeneration capacities of different cultivars is an important inhibiting factor that makes it necessary to develop a standard regeneration protocol for each cultivar [132].

Various modifications to the protocols that could help alleviate recalcitrance including the selection of proper explants at specific responsive stages, modification of various components in the media, and the addition of growth regulators at various compositions and doses [130, 131, 133, 134, 136]. Developing a combination of organic and inorganic nutrients important for elongation and treatment of variations in growth regulators are usually carried out to overcome the formation of ill-defined shoots with various results [130, 133, 136, 138]. Grozeva and Todorova [142] analyzed the influence of various factors on *in vitro* regeneration of *C. annuum* and revealed that the influence of culture medium was the highest, at 38.13%, followed by the interaction between genotype and culture medium, accounted for 21.57%, and the interaction between explant type and culture medium (14.95%).

Several studies have reported the success of genetic transformation and *in vitro* regeneration in *Capsicum*. However, different cultivars call for different adjustments, since in most cases, the protocol is not reproducible or results in lower efficiency when applied to different cultivars [130, 131]. Thus, optimization of the transformation and regeneration protocols for different cultivars is always required to get benefits from transgenic-based studies [136]. Due to its recalcitrant and high genotype-dependent nature, *Capsicum* has been a hard to work with plant. Though challenging, the CRISPR/Cas9 is a compelling system since it allows the development of transgene-free improved varieties of *Capsicum*.

STRATEGIES FOR RECALCITRANT PLANTS: ADVANCING GENOME EDITING IN *CAPSICUM*

Recalcitrance can be defined as the inability of plant cells, tissues, and organs to respond to *in vitro* manipulations. During shoot induction of callus, the growth of recalcitrant plants can remain in an undifferentiated callus state. Thus, recalcitrant nature can be a major restricting factor for the biotechnological exploitation of economically important crops. Several approaches have been studied to break recalcitrancy, including for genome editing purposes, such as *de novo* meristem induction and *in planta* transformation methods.

De novo induction of meristem involves overexpressing developmental regulators (DRs), such as BABYBOOM (BBM), WUCSHEL (WUS), LEAFY COTYLEDON (LEC) LEC1 and LEC2, GROWTH-REGULATING FACTOR 4 (GRF4) and GRF-INTERACTING FACTOR 1 (GIF1), SHOOT MERISTEMLESS (STM), and ISOPENTENYL TRANSFERASE (IPT) to induce somatic embryogenesis in recalcitrant plants and promoting genetic transformation as well as plant regeneration [143, 144, 145, 146]. DRs can act through a wide variety of developmental mechanisms to promote regeneration of plant cells, also effective in various non-model plant species, such as rice, coffee, cotton, maize, and forest species [147]. The ectopic expression of *Wus2*, and *ipt* on tomato seedlings induced shoot-like formations and whole plants can be recovered [146]. In *Capsicum*, overexpression of WUSCHEL in *C. chinese* causes ectopic morphogenesis [148], but the gene has not been applied as a tool for transformation improvement in *Capsicum*.

The utility of these DRs as the basis of a robust transformation system for gene-editing in plants has been demonstrated in several studies. Maher et al. [146] transiently delivered guide RNAs targeting *PDS* (*phytoene desaturase*) gene and combination of DRs (WUS2, STM, and IPT) to transgenic *N. benthamiana* that constitutively express Cas9, through *Agrobacterium* injection. Approximately 15% of the generated shoots showed photobleaching and the mutation was transmitted to the next generation, thus showing that gene editing reagents can be co-delivered with DRs to promote plant regeneration. Maher

et al. [146] also showed that this method can also be applied to induce genetically modified meristem on soil-grown plants. Transgenic *N. benthamiana* that constitutively express Cas9 were pruned to remove all shoot meristems and the cut sites were perfused with *A. tumefaciens* expressing a combination of DRs and luciferase reporter gene. The formed meristem carried all modifications, and the mutations were transmitted to progeny without the use of tissue culture method. Similarly, the combination of GRF-GIF and CRISPR/Cas9 through *Agrobacterium*-mediated transformation increased the frequency of genome-edited plants in wheat and improved regeneration efficiency in citrus, in tissue culture conditions [145]. Therefore, *de novo* meristem induction by DRs combined with the delivery of CRISPR/Cas9 components via *Agrobacterium* is a feasible approach to facilitate gene editing in recalcitrant species.

Nevertheless, the application of genome editing method using CRISPR/Cas9 system for recalcitrant crops that is free from any tissue culture step is more appealing. Although transformation and regeneration of plant cells by tissue culture has been successful in some pepper cultivars, tissue culture can be laborious and often creates unintentional changes to the genome of regenerated plants. A simple, easy, cost-effective, and efficient transformation method, such as *in planta* transformation, could be the appropriate approach for genome editing in *Capsicum*.

In planta transformation refers to the direct transformation of the plant without involving *in vitro* culture and regeneration of plant cells or tissue. This method still uses *Agrobacterium* to mediate the transfer of transgene to host cells. *Agrobacterium* with the required transgene is allowed to infect the meristematic tissue of the plant directly, thus eliminating the optimization phase of tissue culture as well as several stages of selection and *in vitro* regeneration of transgenic plants [149, 150], which is suitable for recalcitrant plants. A few important crops have been successfully transformed with *in planta* transformation methods, including soybean [151], sugarcane [152], tomato [154, 154], and pepper [118, 156, 157, 158]. This can be achieved in various ways such as the floral dip method [149], and mechanical injury to the seed meristem [156, 157].

CRISPR/Cas9 genome editing through *in planta* transformation using floral dip method has been implemented in *A. thaliana* [159, 160, 161], *Camelina sativa* [161] and *Thlaspi arvense* [162]. However, the floral dip-mediated genome editing is limited to few plants such as *Arabidopsis*, *Camelina*, etc., with less efficiency due to limited flower and seed formation [123]. For pepper, mechanical injury to shoot apical meristem followed by incubation [156, 157], submerging seed in *Agrobacterium* culture [118], and piercing on hypocotyls, cotyledons, radicles [158] have been applied to generate transgenic plants with various results.

Kumar et al. [157] and Arthikala et al. [156] carried out *A. tumefaciens*-mediated *in planta* transformations on bell pepper varieties using piercing on shoot apical meristem and incubation in *Agrobacterium* culture containing wounded tobacco leaf filtrate as phenolic source for *Agrobacterium Vir* gene induction. Kumar et al. [157] used two varieties viz., Arka Gaurav and Arka Mohini, while Arthikala et al. [156] only used Arka Gaurav variety. In both studies, *in planta* transformation was mediated by *Agrobacterium* EHA105 harboring the binary vector pCAMBIA1301 that carries the gene of interest. The 3-4 days old seedlings with just emerging plumule (apical meristem) were infected by pricking at the meristem with a sterile needle followed by submerging in the *Agrobacterium* culture containing wounded tobacco leaf filtrate for 40 min, 40-50 rpm, at 28° C. Co-cultivation was conducted at 28° C for 48 h in dark. In the study by Kumar et al. [157], the method resulted in 11.4% and 17.8% of the T0 plants being chimeric in Arka Mohini and Arka Gaurav and 29.7% and 35.0%, correspondingly, were identified in the T1 generation as stable transformants [157]. In Arthikala et al. [156], the method resulted in 26.4% and 24.2% transgenic plants in primary transformants and T1 generation, respectively. Both studies showed that the growth of the transformed seedlings was slower than the untransformed controls, but the T1 generation plants set the flowers and seeds similar to controls [156, 157].

On the other hand, Toth et al. [158] used *A. rhizogenes* to mediate *in planta* transformation on radicles, hypocotyls, and cotyledons of the *C. annuum* Global variety seedlings. *A. rhizogenes* ARqua1 were applied with a thrust of a tungsten needle and co-cultivated for 4 days in MS media. The cotyledon and hypocotyl showed a high transformational efficiency at 70% and 60%, respectively, while radicle has the lowest transformational efficiency (50%). However, due to the rooting on the cotyledon, the plants became distorted and difficult to handle, hence Toth et al. [158] suggested that this method is most effective applied for hypocotyl transformation. Toth et al. [158] highlighted that too low amounts of bacteria resulted in a mosaic transformation and weak gene expression, while too much bacteria resulted in high plant material loss. However, it is really hard to measure the right amount of bacteria for the transformation with the tungsten needle. Toth et al. [158] suggested that efficient transformation was obtained when the tungsten needle tip was gently pinched to a 1-day old bacterial lawn.

Both *de novo* meristem induction and *in planta* transformation methods can offer feasible alternative methods to improve the efficiency of gene editing by CRISPR/Cas9 in recalcitrant *Capsicum* and bypass arduous tissue culture steps for regeneration of edited plants. However, some adjustments will still be needed in transformation methods since the efficiency of *Agrobacterium*-mediated transformation in *Capsicum* is still relatively modest.

CONCLUSION AND FUTURE PERSPECTIVE

As an economically important plant, the loss of pepper production caused by begomovirus is still difficult to control. Moreover, gene pool containing naturally occurring resistance alleles to begomovirus is few to none in *Capsicum*. Hence, the plant biotechniques encompassing plant tissue culture and genetic engineering are becoming a functional aspect of disease-resistance breeding programs in pepper. The CRISPR/Cas9 technology enables precise genomic modification in various organisms and has been used recently for enhancing disease-resistance in plants through disruption of essential host genes encoding proviral host factors, and therefore, give rise to loss-of-susceptibility phenotypes in plants. CRISPR/Cas9 system is also a valuable tool to identify host factors that determine susceptibility to plant viruses. As more host factors interact with viral protein are revealed, the understanding of basic mechanisms governing begomovirus–host interactions can also be advanced.

Despite its agricultural importance, host factors necessary for DNA virus infection such as begomovirus are underrepresented compared to those involved in RNA virus infection, both in model plants and crops. This knowledge gap is a research opportunity with important practical applications to develop antiviral control strategies against begomovirus. The previous studied host factors for recessive resistance and the application of CRISPR/Cas9 system in model plants could help in defining a general approach applicable to antiviral engineering in pepper. Identification and functional characterization of novel proviral host factors in *Capsicum* are imperative and evaluating the biological importance of interaction between host factor and begomovirus protein is important to assess the possibility of host gene modification to develop improved varieties of pepper that show resistance against begomovirus infection. Also, the possibility of host gene mutation affecting important agricultural traits in pepper needs to be evaluated.

Successful implementation of the CRISPR/Cas9 system in engineering plants to confer resistance to biotic stresses has proven the concept to be effective and plausible. If the CRISPR/Cas9-based tools can be effectively applied to edit host gene involved in begomovirus life cycle, it will be an important breakthrough to establish genetic resources in pepper cultivars that show resistance to begomovirus. Though challenging due to the recalcitrant nature of *Capsicum*, the CRISPR/Cas9 is a compelling system since it allows the development of transgene-free improved varieties of pepper. Both *de novo* meristem induction using developmental regulators and *in planta* transformation system offers superiority over the tissue culture-based transformation method for recalcitrant plant because both methods can eliminate any tissue culture steps for plant regeneration. In addition, this method can generate a large number of transgenic plants using less time, money and effort. Therefore, for recalcitrant *Capsicum*, involving developmental genes as a transformation tool and *in planta* transformation method to deliver CRISPR/Cas9 components in plants may increase the successful application of antiviral engineering by genome editing. Further improvement in the optimization of this method for more beneficial effects in pepper is needed, particularly to alleviate the recalcitrant nature of *Capsicum* to genetic transformation and to develop a universal transformation protocol for all pepper cultivars.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this manuscript.

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