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Seed Transmission of Tobamoviruses: Aspects of Global Disease Distribution

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Abstract

Global seed trade contributed to development and improvement of world agriculture. An adverse effect of global seed trade is reflected in disease outbreaks in new growing areas, countries, and continents. Among the seed-borne viruses, *Tobamovirus* species are currently considered a peril for crop production around the world. The unique tobamoviral particles confer stability to the RNA genome and preserve their infectivity for years. High titer of *Tobamovirus* species accumulates in reproductive organs leading to viral particles adsorbed to seed coat, which potentially establish a primary infectious source. *Tobamovirus*-contaminated seeds show very low virus transmission in grow-out experiments as detected by enzyme-linked immunosorbent assay (ELISA) and reverse transcription polymerase chain reaction (RT-PCR) analysis. Interestingly, in situ immunofluorescence analysis of *Cucumber green mottle mosaic virus* (CGMMV) reveals that the perisperm-endosperm envelope (PEE) is contaminated as well by the *Tobamovirus*. Indeed, chemical seed disinfection treatments that affect primarily the seed coat surface are efficient for several *Tobamovirus* species but apparently do not prevent seed transmission of CGMMV to occur. *Tobamovirus* infection of the seed internal layers, which rarely includes the embryo, may partially follow the direct invasion pathway of *Potyviruses* such as *Pea seed-borne mosaic virus* (PSbMV) to pea embryo.

Keywords: contaminated seeds, perisperm-endosperm envelope (PEE), seed coat, seed disinfection, seed testing, size exclusion limit (SEL), seedlings, symplast, phloem

1. Introduction

The contribution of global seed trade to modern agriculture is clearly emphasized in breeder's broad range of crop species that contribute to increased yield, long shelf life, pathogen resistance, and tolerance to extreme circumstances (e.g., salinity, drought, and arid climate

conditions) around the world. However, spread of seed-borne viruses is an unfortunate side effect of this industry. Seed transmission of plant viruses has a great epidemiological significance causing disease outbreaks worldwide. Seed trade globalization has opened new pathways for the mobilization of crop produce between continents and countries. In the modern agriculture, new crop varieties are introduced into new growing areas and are cultivated in proximity to indigenous crops, which are exposed to the imported remote endemic diseases. Seed-borne plant viruses are a threat to world agriculture. Among them, species that belong to the genus *Tobamovirus* are considered a major peril to a range of cultivars especially to those belonging to the *Solanaceae* and *Cucurbitaceae* (cucurbit) families. These viruses have long been a threat to agriculture. For more than a century, tobacco, tomato, and pepper plants that belong to the *Solanaceae* family are infected by the *Tobacco mosaic virus* (TMV) [1, 2], *Tobacco mild green mosaic virus* (TMGMV) [3], *Tomato mosaic virus* (ToMV), [4] and *Pepper mild mottle virus* (PMMoV) [5]. Similarly, cucurbits are infected by the *Cucumber green mottle mosaic virus* (CGMMV) that was discovered in England in 1935 [6] and spread into neighboring countries in Europe and to the Middle East, Asia, and the Far East. In the last decade, CGMMV was detected in new countries and continents—Canada [7] and the USA [8] in North America and Australia [9]—recently reviewed in [10], achieving a global distribution and becoming a major threat to cucurbit industries worldwide. Among the main cucurbit-infecting tobamoviruses, CGMMV is the most economically important and currently considered a significant threat for the produc-tion of cucumber, melon, watermelon, gherkin, and pumpkin, which has been endangered by the globalized spread of the *Tobamovirus* (**Figure 1**). Additional *Tobamovirus* species that infect cucurbits need to be considered in seed transmission tests in countries that commercially produce seeds: the *Kyuri green mottle mosaic virus* (KGMMV) [11, 12], the *Zucchini green mottle mosaic virus* (ZGMMV) [13], and the *Cucumber fruit mottle mosaic virus* (CFMMV) [14].

The tobamoviruses are seed-borne, mechanically transmitted stable viruses. Infectious par-ticles are primarily attached to the seed coat [4]. Indeed, viral inoculum is efficiently trans-mitted when it enters the embryo, and viruses attached to the seed coat may not survive germination when seed coat separates from the seedlings [15]. However, in nursery seed-lings, the *Tobamovirus*-contaminated seed coat may affect the wounded roots, which occur upon transplantation [4]. Furthermore, low transmission rate to seedlings does occur when tobamoviruses contaminate the seeds. Seeds or seedlings are used in large scale continuously in regular farming. Therefore, even a low percentage of contaminated seeds can cause a mul-titude of infection foci [16]. Consequently, the primary infectious source can be spread rap-idly by mechanical contacts, workers' hands, tools, greenhouse structure, and trellising ropes [17, 18] and the tractor path in open fields [19]. The infectivity of tobamoviruses is preserved in plant debris and in the contaminated soil and clay for months up to years [20].

The entry and the establishment of new *Tobamovirus* diseases inflict a major concern for grow-ers around the world. Seed nurseries, especially those that produce grafted seedlings (a pro-cedure that requires cutting and hand handling), and protected structures (e.g., greenhouses, net houses, walk-in tunnels), which are commonly used worldwide, mostly with monoculture

Figure 1. *Cucumber green mottle mosaic virus* (CGMMV)-infected cucurbit species. (**a, b, e, i**) Mild mottling and mosaic on infected leaves of cucurbits: (**a–b**) cucumber (*Cucumis sativus*), (**e**) melon (*Cucumis melo*), and (**i**) watermelon (*Citrullus lanatus*) plants. (**c–d**) Mosaic pattern developed on cucumber fruits. (**f–h**) Mosaic pattern and mottling developed on melon fruits. (**j–k**) Yellowing fruits and spongy flesh (double-headed arrows) accompanied by necrotic lesions on stems and peduncles (single-headed arrows) of watermelon fruits.

crops (without crop rotations between growing cycles), are highly susceptible to *Tobamovirus* infection. When a new *Tobamovirus* disease emerges in a new country, the first response is to try to eradicate the disease, a strategy that is unfortunately prone to failure in most of the scenarios. The major reasons for this failure may be attributed to multiple contaminated seed entry events that occur in parallel, in multiple growing areas/farms, and late detection and response. The second strategy upon the emergence of a new *Tobamovirus* disease is based on management of the disease, which combines several approaches to contain the disease and reduce the disease damage below the economic threshold. A proper management needs to cover all aspects of plant growth that are involved in disease spread: disinfection of contaminated soil from the previous growing cycle, trellising ropes and the greenhouse structure, worker hands and clothes, etc. This approach however, cannot ensure low disease damage and further emphasizes the importance of proper management of commercial seed production, accompanied by the appropriate diagnostic approaches in order to eliminate *Tobamovirus*-contaminated seed lots.

2. Resistance-breaking tobamoviruses

Plant viruses belonging to the genus *Tobamovirus* (currently comprised of 37 species) are rod-shaped particles encapsulating a single-stranded RNA (+ssRNA) genome of ~6.4 kb. The genome encodes four ORFs. ORF1 and ORF2, separated by a leaky stop codon, encode the 126 and 183 kDa replicase protein complex (recently reviewed in [21]). ORF3 encodes the 30 kDa movement protein (MP), and ORF4 encodes the 17.5 kDa coat protein (CP). The tobamoviruses endanger cultivars in the world. For more than a century, tobacco and tomato plants that belong to the *Solanaceae* family are infected by TMV [1, 2] and ToMV [4]. Resistance to these viruses was introduced to tomato plants by introgression [22]. However, the durability of resistance genes is compromised by the pathogen selection pressure that gradually breaks the plant defense system. In tomato plants, the durability of *Tm-1*, *Tm-2*, and *Tm-2²* [23] resistance genes has recently been jeopardized by the newly discovered tobamoviruses *Tomato mottle mosaic virus* (ToMMV), reported in Brazil [24], Mexico [25], and the USA [26], and the *Tm-2²* resistance-breaking *Tomato brown rugose fruit virus* (ToBRFV) reported in Jordan [27] and Israel [28] (**Figure 2**). Within the *Solanaceae* family, pepper crops were also affected in the last decades by *Tobamovirus* species, mostly by PMMoV that overcame the resistance conferred by the L^{1-3} genes [29–31] and rarely the L^4 gene in Japan [32] and Israel [33]. In cucurbits, the production of cucumber, melon, and watermelon has been endangered by the globalized spread of CGMMV. Since its discovery by Ainsworth in 1935 [6], no commercial cultivars fully resistant to CGMMV are available although temperature-sensitive strains with specific resistance were reported.

Figure 2. *Tomato brown rugose fruit virus* (ToBRFV)-infected tomato (*Solanum lycopersicum*) plants. (**a, d**) Brown rugose symptoms developed on fruits. (**b, c**) yellow spots on fruits. (**a, e–g**) Mosaic pattern developed on leaves and narrowing accompanied by mottling leaves. (**g**) Necrotic symptoms on pedicle, calyces, and petioles.

3. Virus preservation in seeds

The viral particles of tobamoviruses are extremely stable (**Figure 3(a)**), and infectivity is preserved in seeds for up to several years. Most of the *Tobamovirus* species display low percentage of seed transmission, but even very low occurrence of seed transmission is enough to start a spread of the disease [16]. Seed transmission of viruses occurs primarily via infected embryos through paternal or maternal pathways. However, tobamoviruses mostly infect the seed coat (testa) and the endosperm [4, 34, 35]. Transmission of the virus occurs primarily by mechanical means through transplanting the seedlings and causing cuts in the roots that are then susceptible to infection by the contaminated seed coat. The rout of *Tobamovirus* transmission to the seeds is not quite clear. While the testa is of maternal origin, the endosperm is the outcome of fertilization. It is possible that tobamoviruses partially follow the rout of the symplasmic pathway suggested to occur in seed infection of *Pea seed-borne mosaic virus* (PSbMV) without the accomplishment of embryo invasion.
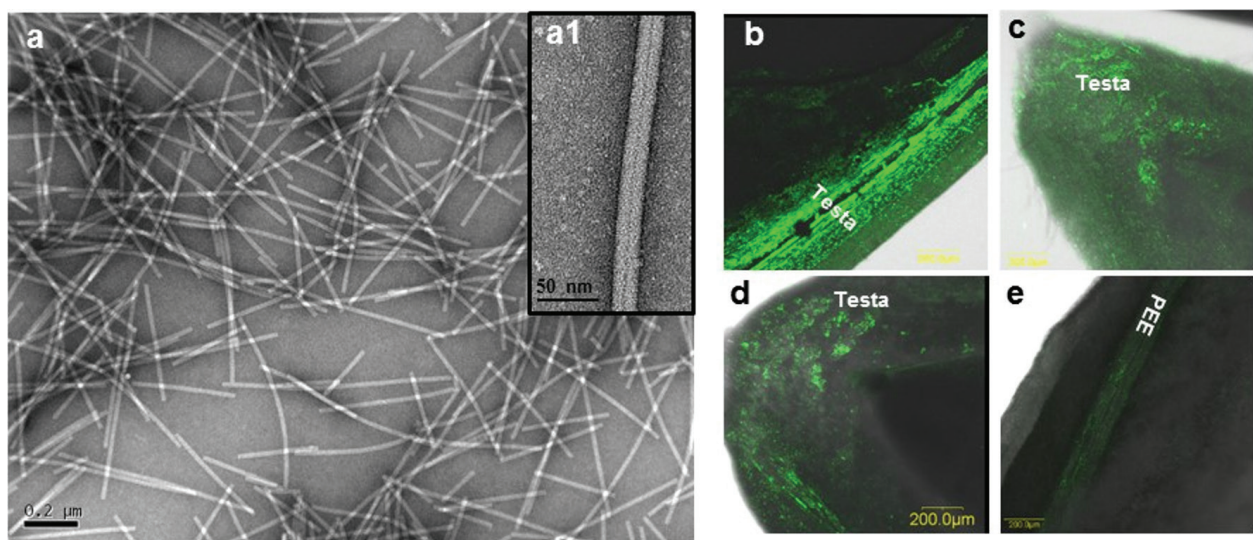

Figure 3. *Cucumber green mottle mosaic virus* (CGMMV) morphology and localization in cucurbit seeds. (**a**) Electron micrograph illustration of viral particles, (**a1**) high resolution of a single viral particle. (**b–e**) In situ immunofluorescence of CGMMV in infected melon (*Cucumis melo*) seeds using CGMMV-specific antibodies and secondary antibodies conjugated with Alexa Fluor 488. The fluorescent signal indicates the presence of CGMMV coat protein. Bars, 200 μm.

4. Symplasmic viral movement

The symplast is the conduit for virus movement from cell to cell and through the phloem vasculature. They follow source to sink transportation of photoassimilates albeit with a slower rate [36]. At early stages, seeds are sinks. Nutrients enter the seed through the vascular bundle in the funiculus that ends up in the chalazal vein, which consists of xylem strand surrounded by phloem elements [37]. The seed coat (testa) is of maternal origin and mediates the release of nutrients to the embryo. It is a development of the integument/s. Symplasmic transport that can be exploited by viruses occurs from the sieve elements of the chalazal vein into the parenchyma layer [37].

4.1. Cell-to-cell movement

Viruses exploit the plasmodesmata (PD) for their movement from cell to cell. PD are membrane-bound tunnels that interconnect the cytoplasm of adjacent cells. Flattened endoplasmic reticulum membrane or desmotubule, which is surrounded by actin and myosin molecules, traverses PD in the center and is continuous with the cortical endoplasmic reticulum (ER) [38]. The actin and myosin molecules connect the desmotubule to the plasma membrane [39]. There are simple PD and branched PD; the latter are more common in mature tissues. Conversion of simple PD to branched PD presumably involves formation of bridges between simple PD. Trafficking between cells occurs via the "cytoplasmic sleeve," which is the area of the cytoplasm between the desmotubule and the plasma membrane, and via the desmotubule that bridges between the ER of adjacent cells. The desmotubule exists in both appressed and dilated states. The neck region at both ends of the PD is frequently restricted. In experiments studying the transport of small dye molecules through the cytoplasmic sleeve, it was observed that the transport is not interrupted by cytochalasin B, an inhibitor of actin polymerization [40], indicating that this intercellular movement occurs by diffusion and the PD is rate limiting [38].

Actin and myosin localized in PD may serve as scaffold for active transport of proteins. Actin filaments are also closely associated with the ER [41, 42]. Directionality of the movement of ER-localized proteins may be attributed to myosin.

The PD has size exclusion limit (SEL) [43], which changes during development and in response to environmental stress [38]. The mobility of molecules through PD is determined by Stokes radius (Rs), which is the hydrodynamic radius that is influenced by both the molecular weight of a molecule and its side group [44]. The rate of diffusion is directly correlated to the radius of a molecule [38]. SEL is measured by injecting labeled size markers such as F-dextran [45]. Transient increases in SEL were observed, and it is commonly reduced during maturation [46, 47]. SEL differs between tissues in a plant and between plant species [38]. Actin cytoskeleton may participate in regulation of SEL. Depolymerization of actin cytoskeleton by cytochalasin widened the neck region of PD and increased SEL of tobacco PD from 1 kDa to over 20 kDa [48]. Actin filaments attached to the ER participate in controlling intracellular movement of TMV viral particles [38, 49]. The integrity of the cytoskeleton is apparently necessary for viral spread. Virus movement protein (MP) is necessary for viral spread from cell to cell [50]. Cytoskeletal components were suggested to be involved in the transport of MP to the PD site and in viral cell-to-cell movement via the PD [49, 51, 52]. However, study of TMV MP and CMV MP showed that these viral proteins have F-actin depolymerization activity that causes increase in PD SEL [53]. Since actin is required for MP targeting to PD [49], it was suggested that MP-induced F-actin degradation occurs at the orifice area [53].

The MP of many viruses and of TMV is an endoplasmic reticulum membrane protein [54, 55]. Exogenously expressed MP target the PD autonomously [56–58].

Microtubules participate in cellular distribution and long-distance movement of MP and viral replication complexes [59]. However, cytoskeleton involvement in active movement of viral particles, when the PD is dilated, predicts a fast spread of the particles between cells, in the order

of seconds, which does not occur [51]. The model also predicts that in the presence of MP, cytoplasmic and ER membrane proteins will show enhanced cell-to-cell movement. This apparently did not occur when movement of cytoplasmic and ER membrane-fluorescent protein probes was studied [60], which may suggest the presence of passive diffusion of the viral complexes in the desmotubule [60].

Callose, a beta 1,3-glucan [61] deposition between the plasma membrane and the cell wall in the neck region of PD, participates in determining SEL [62, 63]. Class I beta-1,3-glucanase-deficient mutants have lower SEL and show decreased susceptibility to virus infection [64]. And targeted expression of class I beta-1,3-glucanase enhances TMV infection in tobacco plants [65].

The callose accumulation is often induced in the plants under stress conditions [64]. There is a controversy regarding the effect of TMV MP on increasing SEL [66]. It has been suggested that results showing the increase in SEL by MP depend on the technique of introducing the MP [67]. Apparently under conditions of unmodified SEL by MP, TMV-derived replicase supports the activity of the viral MP on induction of callose degradation [60] and increased the conductivity via the desmotubule. The involvement of RNA replicase in cell-to-cell movement of TMV has been observed [68]. RNA helicase domain in 126 kDa replicase protein is involved in execution of cell-to-cell movement independent of MP activity.

There are several other factors that are apparently involved in MP-induced dilation of PD and may shed a light on the mechanism of SEL modification by MP. Induction of callose degradation by the viral proteins may be mediated by plant cell proteins such as TGB12K interacting protein (TIP) that interacts with both the *Potato virus X* (PVX) movement protein TGB12K and beta-1,3-glucanase [69]. TMV MP has been shown to interact with pectin methyl transferase, which is essential for dilating PD [70, 71]. The carboxy terminus of TMV MP enhances cell-to-cell movement in *N. tobacco* cv. Xanthi plants [72]. Phosphorylation at the carboxy terminus is necessary for PD gating by MP [73]. MP dilation of PD may also be mediated by interacting with the *N. tabacum* noncell autonomous pathway protein 1 (NtNCAPP1) that its mutation interfered with TMV MP-induced SEL increase [74]. It has also been shown that mutation in Arabidopsis synaptotagmin, a Ca^{2+}-binding protein that is involved in endocytic recycling, interferes with TMV MP intercellular movement [75]. TMV MP also interacts with calreticulin, which resides in PD and sequester Ca^{2+} [76]. This binding may indicate the involvement of local Ca^{2+} levels in the control of TMV MP-induced PD dilation.

The above-illustrated role of *Tobamovirus* MP in dilating PD differs from that of MP of tubule-forming viruses that modifies PD by assembling tubules that mediate cell-to-cell viral transfer [77, 78]. And the tobamoviruses differ from the filamentous viruses that engage a homologue of Hsp70 and a complex of viral components for transport through PD [79].

4.2. Phloem movement

PD that mediate cell-to-cell movement partially mediate transport of viruses into the phloem vasculature for systemic infection. PD mediate symplasmic transport between epidermal or mesophyll cells through bundle sheath, vascular parenchyma to companion cells (CC). Between CC and the enucleated sieve elements (SE), specialized PD exist named pore

plasmodesmal units (PPU), which consist of multiple channels on the CC side and a single channel facing the SE [80]. PPU have higher SEL than other PD in the phloem vasculature, but it is still not enough for viral particles to cross the boundary between CC and SE, and ribonucleoproteins cross this boundary [81]. Analyzing the form of the *Tobamovirus* CGMMV that is transported through the phloem exudate showed that the virus is in the form of virus particles [82]. Apparently particle formation is essential for long-distance movement of viruses [83]. Once in the SE, the viruses are transported by diffusion.

It has been suggested that different mechanisms are employed for virus loading and unloading from the phloem since viruses enter the phloem vasculature through all classes of veins, but virus exit is limited to major veins [84–87]. Accordingly, various mechanisms in the host plants, hindering or promoting viral long-distance movement, are differentially located in the loading and unloading sites of the phloem vasculature. The cadmium ion-induced glycine-rich protein is localized to the cell wall of CC and SE, and its blocking capacity of TMV and *Turnip vein clearing virus* (TVCV) long-distance movement is attributed to callose deposition. Mutation at the host VSM1 gene blocked the entry of TVCV into the phloem indicating a role of a host gene in promoting *Tobamovirus* loading into SE [88, 89].

On the other hand, at the phloem exit, protein degradation occurs and results in viral restriction to the phloem [90]. The 26S proteasome was found in pumpkin sap and Arabidopsis thaliana [91] and aminopeptidases and proteases found in pumpkin SE sap [91, 92]. Promotion of viral exit from the phloem vasculature occurs by the *Tobamovirus*-infected plant protein pectin methyl esterase (PME) [93].

The CP of many viruses, including tobamoviruses, is required for systemic movement of the viral particles in the host plants. CP ability to assemble the viral particles is important for the long-distance movement as well as the origin of assembly, indicating the importance of virion formation for transport through the phloem [94]. Analysis of CP mutants revealed that retaining the C-terminus is essential for high particle accumulation [95].

The RNA-dependent RNA polymerase activity by the 126 and/or 183 kDa proteins of TMV is necessary for the accumulation of viral particles in the phloem vasculature. Mutations occurred in the 126 kDa protein, and the 183 kDa protein, which is the read through of the amber termination of 126 ORF, prevented the accumulation of the virus in the phloem parenchyma and CCs, and systemic invasion of the virus was hindered [96]. Comparison between the systemic infection of TMV in tobacco plants and the nonsystemic infection of *Sunn-hemp mosaic virus* (SHMV) in these plants showed as well that replicase proteins are necessary for long-distance viral movement in the phloem vasculature [72, 97]. The 126/183 replicase is active also as a suppressor of silencing, which correlates with the ability of the virus to move systemically and to cause symptoms [98].

5. Symplasmic conductivity in seeds

Compartmentalization of symplasmic conductivity may differ between seeds of different plant varieties. The funiculus phloem reaches a phloem-unloading domain at the chalazal area of the outer integument, which either alone or together with the inner integument evolves to

the seed coat, which differs between seed species as well. Using fluorescent probes, it has been shown that the entire outer integument at the end of the vascular bundle sheath of the funiculus is an extended symplasmic domain in *Arabidopsis thaliana* [99], and in *Crassulaceae* seeds [100], the inner integument and the embryo are separated symplasmic domains. In legumes, ^{14}C-labeled photosynthates showed PD connections between SE of the chalazal vein and the parenchyma cells [101] that comprise most of the seed coat layers [37] and consist of chlorenchyma, ground parenchyma, and branched parenchyma. In peas (*Pisum sativum* L) phloem unloading occurs in the ground parenchyma [37]. The phloem mobile molecule pyranine was transported to the chlorenchyma but not to the branched parenchyma or to the epidermis [37]. Compartmentalization of the symplasmic domains raises the important question regarding transport of viruses in seeds. This question was primarily addressed in the study of seed transmission in pea cultivars susceptible to PSbMV infection.

Apparently, incidences of seed transmission of a specific virus vary between plant species and between cultivars. Comparison between cultivars allowed to delineate the sequence of events necessary for seed transmission. Infection of the vegetative tissues and of the maternal testa occurred irrespective of the capability of the virus to be transmitted via seeds. High incidence of seed transmission occurred in direct relation to virus invasion of an immature embryo.

PSbMV embryo invasion occurred at a post-fertilization stage. Pollen grains and maternal integuments were devoid of the virus. It has been suggested that the embryo, at early developmental stage, may have the symplasmic connections necessary for viral seed transmission. Immediately after fertilization zygotic divisions form a globular terminal cell and a suspensor. The integuments of the ovule develop into the testa. In the testa there are vascular strands that are continuous with the vascular bundle sheath of the funiculus. The suspensor consists of globular cell/cells that support the embryo and elongated basal cells that are in contact with the testa at the micropylar region. Using immunohistochemistry technique with antibodies raised against PSbMV particles, and in situ hybridization technique with an RNA probe specific for the viral RNA, it was shown that before fertilization PSbMV was localized in vascular tissues. Fertilization triggered invasion of the virus into the ovule along the vascular strand in both seed-transmitting and seed-un-transmitting cultivars. However, early developmental stages in seed-transmitting cultivars characteristically showed PSbMV release from vascular tissues to neighboring tissues infecting the micropyle area, concomitant to diminishing presence in the earlier infected tissues. This observation raises the question regarding the possible symplasmic connections between the maternal and filial tissues at the micropyle area. PSbMV cylindrical inclusions, which are commonly found at PD openings and were observed at the boundary between the testa and endosperm [102, 103], may only indicate the possible presence of such symplasmic connections. Crossing this boundary however does not ensure embryo invasion. It has been suggested that the suspensor is the conduit for viral transmission to the embryo [104], and pore-like structures that exist in the suspensor sheath wall at the boundary between the endosperm and the suspensor allow this transmission to occur [103].

High efficiency of PSbMV seed transmission occurred early in embryo development [105]. At this stage, the suspensor may establish continuity between virus present in the embryonic sac fluid, as was observed at late heart stage of embryo development, and the embryo [104, 105]. Viral contamination of the suspensor occurred in embryos at the globular to early heart

stage [104]. The site of contact between the suspensor middle cell and the embryo became infected, further supporting the role of the suspensor in viral transmission to the embryo. The short time span the virus has before the suspensor degenerates and the fact that only passive accumulation of the virus and not its replication determines the amount of virus ready for transmission to the embryo [103, 104] are limiting conditions that hinder direct viral invasion of the embryo.

Direct viral invasion of the embryo differs from indirect embryo invasion in which the viruses infect gametes. Unlike the distribution of viruses that directly invade the embryo, such as PSbMV, viruses indirectly invading the embryo, e.g., *Pea early-browning virus* (PEBV), cause infection to the entire embryo [106, 107]. Apparently, indirect embryo invasion depends on the virus capability to invade the meristem [16]. In the plant meristem, there is a defense mechanism that protects the plant from viral RNA invasion to the germline [108]. Suppression of RNA silencing by the virus could allow meristem entry [108]. However, interfering with the plant systemic posttranscriptional gene silencing does not ensure meristematic invasion of the virus. Moreover, viral induction of DNA methylation associated with induction of posttranscriptional gene silencing does not correlate with viral invasion to meristematic cells [109]. Apparently, the signaling pathway for meristematic exclusion of viruses is not quite clear. Interestingly, the possibility that tobamoviruses could be transmitted indirectly to seeds through gamete infection was shown by mechanically applying CGMMV-infected pollen onto healthy flowering plants [110]. Under those specific experimental conditions, virus transmission rate via seeds was ~70%, which is not typical to seed transmission of tobamoviruses (**Table 1**).

Species/ acronym	Hosts	Seed				Reference
		Contamination %		Transmission %		
Cucumber green mottle mosaic virus (CGMMV)	*Cucurbitaceae*	+	84%	+	2%	[141]
		+	NT	+	NT	[148]
		+	NT	+	8%	[144]
		+	95%	+	<1%	[34]
		+	100%	+	0.9%	[147]
Kyuri green mottle mosaic virus (KGMMV)	*Cucurbitaceae*	+	NT	+	NT	[148]
		+	NT	NT	NT	[144]
Odontoglossum ringspot virus (ORSV)	*Orchidaceae, Solanaceae*	+	NT	+	NT	[148]
Paprika mild mottle virus (PaMMV)	*Solanaceae*	+	NT	NT	NT	[3]
Pepper mild mottle virus (PMMoV)	*Solanaceae*	+	NT	+	NT	[148]
		+	NT	NT	NT	[3]
		+	NT	+	(0.8–7.5%)	[123]
Ribgrass mosaic virus (RMV)	*Plantaginaceae*	+	NT	−	0%	[145]
		+	NT	+	NT	[148]

Species/ acronym	Hosts	Seed		Transmission %		Reference
		Contamination %				
Sunn-hemp mosaic virus (SHMV)	*Solanaceae*	+	NT	+	NT	[148]
		+	NT	−	0%	[130]
	Fabaceae	+	NT	+	18%	[130]
Tobacco mild green mosaic virus (TMGMV)	*Solanaceae*	+	NT	NT	NT	[3]
Tobacco mosaic virus (TMV)	*Solanaceae*	+	NT	−	0%	[4]
		+	NT	+	21.8%	[134]
		+	100%	−	0%	[133]
		+	NT	−	0%	[142]
		+	50%	NT	NT	[149]
Tomato mosaic virus (ToMV)	*Solanaceae*	+	NT	+	NT	[35]
		+	NT	−	0%	[4]
		+	NT	−	0%	[143]
		+	NT	+	NT	[148]
		+	38%	NT	NT	[149]
Tropical soda apple mosaic virus (TSAMV)	*Solanaceae*	+	>40%	+	1–16%	[127]
Turnip vein-clearing virus (TVCV)	*Brassicaceae*	+	NT	+	0%	[89]
Zucchini green mottle mosaic virus (ZGMMV)	*Cucurbitaceae*	+	6%	+	1.1%	[141]
		+	NT	+	NT	[144]

NT—not tested

Table 1. Selected seed-borne tobamoviruses.

6. Regulation of virus invasion

Virus invasion does not uniformly occur in plant cultivars. Invasion efficiency varies between virus strains and between various cultivars [15]. Introducing dominant resistance by introgression allows the production of cultivars resistant to virus infection. The R genes (nucleotide binding-site leucine-rich repeat, NB-LRR) so introduced confer the resistance [22]. The *N* gene in *Nicotiana glutinosa*, *Tm* gene in tomatoes, and *L* gene in pepper plants are the known R genes for *Tobamovirus* infection. However, virus strains that overcame these resistance genes have evolved. The R genes induce hypersensitive response (HR) that limits the virus movement to the inoculation area [22]. Metabolic accumulation of salicylic acid, reactive oxygen species, and Ca^{2+} is characteristic to the HR response. Proteolysis through the ubiquitin pathway occurs,

and induced caspase-like proteinases cause local cell death [22, 111]. In the case of *N* NB-LRR gene, translational arrest is involved [112]. The helicase domain of the replicase protein, MP, and CP is the avr proteins that elicit the HR of *N*, *Tm*, and *L* genes, respectively [22].

A new approach that introduces resistance may circumvent the resistance braking tobamoviruses. The clustered regularly interspaced short palindromic repeats (CRISPR-Cas) system is mutagenesis targeted by guide RNAs [113, 114]. The specific guide RNA has sequence homology to a desired DNA site, thus enabling the use of the CRISPR-Cas system to edit specific genome sites. Editing occurs by the introduction of double-stranded DNA breaks by the endonuclease Cas9 at a locus of interest while exploiting cellular repair mechanisms to cause high fidelity heritable genome sequence changes [113, 115]. Successful application of the Cas9 methodology was reported for the virus genera: *Geminivirus* [116], *Potyvirus*, and *Ipomovirus* [117].

The viral MP is a target for various regulatory events. During viral passage through PD, the MP is transiently synthesized, and it is degraded by 26S proteasome [118]. MP binding to viral RNA is associated with repression of viral RNA translation [119]. Phosphorylation of MP apparently has diverse regulatory consequences for MP. Phosphorylated MP no longer represses RNA translation [120], which may indicate temporal regulation of viral movement and synthesis. Kinases that phosphorylate the MP carboxy terminus stabilize the protein and promote its activity on PD dilation [73]. Interestingly the carboxy terminus of MP is susceptible to phosphorylation by the host cell wall-associated protein kinase that may be sequestering the MP to the cell wall [121]. Phosphorylation of ToMV MP at serine 37 apparently confers stabilization to the protein and is essential for its intracellular localization [122].

Suppression of posttranscriptional gene silencing (PTGS) is apparently an elementary mechanism for viral spread. Viruses are initiators of the endogenous PTGS, which degrades RNA. The viral RNA is processed into small interfering RNA (siRNA) by a drosophila Dicer-like enzyme, which is then incorporated into RNA-induced silencing complex (RISK) that degrades RNAs with sequence similarity to the siRNAs. A silencing signal, which may be the siRNAs, spreads between cells via PD and phloem [123]. This signal spread can be prevented by TMV [124]. Study of ToMV suppression of PTGS showed accumulation of siRNAs, suggested to indicate a block in PTGS downstream of siRNA production [125].

As mentioned above, indirect pathway to seed invasion is limited by meristematic exclusion. In addition, seeds apparently have an intrinsic inhibitory effect on virus infection [126].

7. Virus preservation in seeds

RNA viruses have high mutation rate [127] that contributes to rapid evolution dynamics which may ensure adaptation to new host plants or to stressful and fluctuating environments [128, 129]. Seed transmission may comprise a genetic bottleneck, which may reduce population size and induce the immergence of new virus strains [130]. Analysis of the effect of the vertical transmission bottleneck on *Zucchini yellow mosaic virus* (ZYMV), for example, showed that although high variability was observed in the 5' untranslated region, the regions

necessary for vector transmission were unchanged, indicating their contribution to virus spread [131]. Genetic bottlenecks may also cause reduction in virus virulence as suggested to occur in PSbMV seed transmission [132]. Interestingly, experiments on virus seed transmission carried out with CMV infecting Arabidopsis thaliana, under controlled conditions, showed that there is a reduction in virus virulence after several vertical passages [133]. Virus accumulation was reduced as well although seed transmission rate increased. These changes of the virus were concomitant to adaptation of the host plant to the evolving virus, showing an increase in vertical transmission. However, this host adaptation which favors a theory of coevolution of plants and viruses in vertical transmission exposes the cells to high virulence of non-evolved horizontally transmitted viruses [133].

8. Seed-transmitted tobamoviruses

Selected tobamoviruses reported to be transmitted via seeds are listed in **Table 1**. Very low transmission ratios or no transmission at all occurs in most *Tobamovirus* species although seeds are infected. Nevertheless, even a low transmission percentage may be critical for new growing areas. Seed transmission primarily occurs via transplantation procedure, commonly practiced by growers. Most of the tobamoviruses contaminate the seed coat. Study of PMMoV-contaminated pepper seeds using fluorescence probe revealed that the seed coat epidermis and parenchyma cells and the endothelium that surrounds the endosperm all are invaded by PMMoV, leaving the endosperm and the embryo clear of the virus [134, 135]. These cells are of maternal origin. This observation is unlike the reports on ToMV and CGMMV that invade the seed coat as well as the endosperm or the perisperm-endosperm envelope (PEE), respectively. Indeed the seed coat originates from maternal tissues, but the endosperm and its envelope are the result of fertilization process [136]. In most cases, washes of externally attached viruses are not sufficient to prevent seed transmission [4, 34].

CGMMV-contaminated seeds are morphologically modified, as observed by optical coherence tomography. Infected melon seeds show irregularities in the aleurone layer outside the endosperm, and cucumber seeds showed a narrower gap between the seed coat and the endosperm [137]. Interestingly, hair-like structures were observed on the infected seed surface of cucumber and muskmelon [138].

High seed transmission ratio of up to 16% was observed in transmission of *Tropical soda apple mosaic virus* (TSAMV) in tropical soda apple weeds (*Solanum viarum*) [139]. Importantly, the virus can cause leaf deformation and even plant death to the *Solanaceae* plants *Capsicum annuum* cv. Capistrano and *C. annuum* cv. Enterprise, respectively [139]. Weed preservation of tobamoviruses [140, 141] and weed seed transmission may comprise a hurdle difficult to handle by growers [10]. Interestingly, seeds treated with trisodium phosphate (TSP) did not transmit the virus, indicating that TSAMV infection occurred at the outer layers of the seed coat.

Most conspicuous are the results regarding seed transmission of *Sunn-hemp mosaic virus* (SHMV) in sunn-hemp and cowpea plants. While little viral transmission occurred through sunn-hemp seeds, up to 20% transmission ratio occurred when cowpea chlorotic spot isolate infected cowpea seeds. The virus was present in all seed parts including the embryo [142].

9. Seed disinfection treatments

Methods that are used in large-scale commercial seed production are mostly based on various chemical treatments—1–9% hydrochloric acid HCl, 1–5% calcium hypochlorite Ca(OCl)$_2$, 1–3% sodium hypochlorite NaOCl, tetramethylthiuram disulfide (TMTD) (CH$_3$)$_2$NCSS$_2$CSN(CH$_3$)$_2$, and the most commonly used in commercial seed production 10% trisodium phosphate (TSP) Na$_3$PO$_4$ mentioned above [5, 143]—which have been reported to provide satisfactory control of tobamoviruses in cucurbit [144] and solanaceous seeds (e.g., pepper [5, 145, 146] and tomato [147]). In addition to the chemical treatments, several heat treatment protocols at various temperature conditions ranging from 72 to 76°C for a minimum of 12 h up to 72 h [144] are also applied in large-scale production.

However, the recent global outbreaks of *Tobamovirus* diseases emphasize the incompetence of conventional seed disinfection treatments, which could be explained by the preservation of the virus in the inner tissues of the seeds [5]. Analyzing the efficacy of sequential treatments of 10% TSP followed by 72 h heat treatment at 72°C showed that CGMMV infectious particles are preserved in the seeds (**Figure 3(d–e)**) [34].

10. Diagnosis of tobamoviruses in seeds

A technique for the detection of plant viruses that relies on the serological method enzyme-linked immunosorbent assay (ELISA) [148–150] was adapted successfully for the detection of tobamoviruses in seeds. ELISA is considered to be a robust technique [151], and it enables the detection of viral CP subunits [152–159]. However, ELISA suffers from two main limitations: false-negative and false-positive results. The specificity and avidity of the antibodies used for the analysis can vary and may lead to false results. The International Seed Federation (ISF) http://www.worldseed.org/, the International Seed Testing Association (ISTA) https://www.seedtest.org/en/seed-health-methods-_content---1--1452.html, and International Seed Health Initiative for Vegetable Crops (ISHI-Veg) http://www.worldseed.org/our-work/phytosanitary-matters/seed-health/ishi-veg/#protocols adapted, developed, and validated protocols for pathogen diagnosis in seed stock materials (**Table 2**). For the tobamoviruses such as TMV, ToMV, and PMMoV or the *Potexvirus Pepino mosaic virus* (PepMV) that infect *Solanaceae*, proper testing requires 12 batches of 250 seeds each (3000 seeds in total), with detection threshold of 1:249 (1 infected seed/249 healthy seeds). For detection of cucurbit-infecting viruses such as the *Tobamovirus* CGMMV, the *Melon necrotic spot virus* (MNSV, *Carmovirus*), and *Squash mosaic virus* (SqMV, *Comovirus*), and for the detection of the PSbMV (*Potyvirus*) and PEBV (*Chlorovirus*) infecting pea seeds, 20 batches of 100 seeds each (2000 seeds in total) are required. For the latter virus species that belong to five genera (CGMMV, MNSV, SqMV, PSbMV, and PEBV), ELISA detection threshold of 1:99 (1 infected seed/99 healthy seeds) was reported and according to ISTA: "The theoretical detection limit is one seed in 100. To ensure a 95% probability that infestations of 0.15% or higher are detected, it is necessary to test 20 subsamples of 100 seeds." The efficacy of a steadfast seed diagnosis relies on several parameters: (i) random collection of seed samples from the homogenous seed lot and (ii) seed disinfection treatments that are calibrated and examined in parallel to germination assays. In commercial seed production,

Tobamovirus species (acronym) *primary hosts:	Sample size Subsample size X number of subsamples	Assay
Tobacco mosaic virus (TMV) *Pepper/tomato	**3000** 250X12	****Bioassay:** local lesions; indicator plants **Serological: ELISA** **Molecular: RT-PCR/qRT-PCR**
Tomato mosaic virus (ToMV) * Pepper/tomato	**3000** 250X12	**ELISA** ****Bioassay:** local lesions; indicator plants **RT-PCR/qRT-PCR**
Pepper mild mottle virus (PMMoV) *Pepper/tomato	**3000** 250X12	**ELISA** ****Bioassay:** local lesions; indicator plants **Optional:** RT-PCR/qRT-PCR
Cucumber green mottle mosaic virus) CGMMV(*Cucurbit (cucumber, melon, watermelon)	**2000** 100X20	**ELISA** **Optional:** RT-PCR/qRT-PCR
Other virus genera		
Virus genus *Virus* species (acronym) ***Host:**		
Potexvirus *Pepino mosaic virus* (PepMV) * tomato	**3000** 250X12	ELISA ****Bioassay:** indicator plant systemic infection in *Nicotiana benthamiana* **Optional:** RT-PCR/qRT-PCR
Comovirus *Squash mosaic virus* (SqMV) *Cucurbit	**2000** 100X20	**ELISA** **Optional:** RT-PCR/qRT-PCR
Carmovirus *Melon necrotic spot virus* (MNSV) *Cucurbit	**2000** 100X20	**ELISA** **Optional:** RT-PCR/qRT-PCR
Potyvirus *Pea seed-borne mosaic virus* (PSbMV) *Pea	**2000** 100X20	**ELISA**
Chlorovirus *Pea early-browning virus* (PEBV) *Pea	**2000** 100X20	**ELISA**

*Primary host; ****Bioassay**, e.g., *Nicotiana tabacum* "Xanthi NN"; *N. benthamiana*

Table 2. Recommended diagnostic ratios for seed-contamination tests.

applying conventional chemical disinfection treatments followed by extensive washes, which wash out the virus from the seed coat and decrease considerably the viral titer in the tested samples [145, 146], may end up in reducing the virus titer below the detection threshold. Seed analysis under these conditions will show false-negative results because viable viral particles still exist in the internal seed tissues, e.g., the perisperm-endosperm envelope in cucurbit seeds

[34] and the endosperm in *Solanaceae* seeds [4, 35]. The second limitation of the ELISA method, which is false-positive results, may occur in instances that show lack of correlation between the ELISA results and biological significance. The possibility of obtaining false-positive or false-negative ELISA results may lead to situations in which growers purchase treated seed lots that are contaminated. Since the ELISA assay detects only the CP subunit, it is not possible to draw a direct link between ELISA results and the status of the viral particles or the degree of infectivity de facto. It is a major obstacle even when more sensitive molecular-based methods such as reverse transcription polymerase chain reaction (RT-PCR) [3, 160, 161] or quantitative real-time polymerase chain reaction (qRT-PCR) [162–164] are applied, which detect amplified partial genome fragments. Therefore, especially when dealing with treated/disinfected commercial seed lots, the preferable scenario is to validate the ELISA-positive seed subsamples in biological assays on susceptible indicator plants in order to ensure the infectivity status of the tested seed lot before marketing or sowing.

11. Conclusions

The contribution of seed transmission to viral spread may be significant even under conditions of low seed-to-seedling transmission rate. The rapid spread of tobamoviruses by any mechanical contacts is reflected in the spread of the disease especially in plants that are grown trellised in protected structures. This mechanical virus spread may occur when handling seeds and transplanting seedlings. Furthermore, the modern monoculture agriculture contributes to virus buildup, preservation, and spread to new susceptible host plants which increase viral copy number leading to higher viral load in the growing area. Tobamoviruses contaminate both the seed coat and the PEE or the endosperm. The mechanism of *Tobamovirus* transmission in the seed is not quite clear, but it may follow the path suggested for the *Potyvirus* PSbMV in pea seeds. Seeds have regulatory mechanisms that may limit virus transmission, and dominant resistance genes block virus transmission. However, $Tm-2^2$ and L^4 resistance-breaking tobamoviruses have recently been spread, and new approaches for conferring resistance to cultivars against tobamoviruses are in demand. The CRISPR-Cas9 methodology offers exciting prospects and provides an alternative approach to conventional breeding for the acquisition of resistance to viruses. Hopefully, in the near future, it will be applied to *Tobamovirus* species in imported crops.

Acknowledgements

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