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Dept. Biologia Animal,
Biologia Vegetal i Ecologia

Study of *Cucumber mosaic virus* infection in the resistant melon accession PI 161375

Cèlia Guiu Aragonés

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Universitat Autònoma de Barcelona
Facultat de Biociències
Dept. Biologia Animal, Biologia Vegetal i Ecologia
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PhD thesis

Study of *Cucumber mosaic virus* infection in the resistant melon accession PI 161375

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Thesis director	Tutor	PhD candidate
Dra. Ana Montserrat Martín Hernández	Dr. Juan Barceló Coll	Cèlia Guiu Aragonés

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Summaries

The exotic melon accession PI 161375 shows a complex mixture of qualitative and quantitative resistance to *Cucumber mosaic virus* (CMV) infection, depending on the strain. Previously, the presence of a recessive gene (*cmv1*) in the linkage group XII conferring total resistance to a set of CMV strains was reported in our laboratory (Essafi et al., 2009). In this thesis we have extended the knowledge about the *cmv1*-mediated resistance present in melon and have obtained the sequence of the strain CMV-M6 and its infectious clones. This thesis is divided in three chapters.

In the first chapter, we have analysed the *cmv1*-mediated resistance in 11 strains of CMV from subgroup I and II and have established that *cmv1* confers total resistance only to strains of subgroup II. Using infectious clones of strains CMV-LS (subgroup II) and CMV-FNY (subgroup I) we have made combinations between RNAs of both strains showing that the determinant of the virulence is located in RNA3. Chimaeras between CMV-FNY and CMV-LS showed that the determinant of virulence is in the N-terminal 209 amino acids of the movement protein (MP). By directed mutagenesis, we identified a combination of four specific positions that confer to LS the ability to overcome *cmv1*-mediated resistance when exchanged for the corresponding FNY residues.

In the second chapter, we have characterized the resistance mediated by *cmv1.* The strain CMV-LS is able to replicate and move cell to cell in the inoculated leaf of the resistant line. However, it is not able to invade the sieve elements since it has not been detected in the phloem of the resistant line. By immunogold labelling of CMV particles we have identified that the boundary between bundle sheath cells (BS) and vascular parenchyma (VP) or intermediary cells (IC) impedes the systemic infection in the resistant line. Altogether, our results demonstrate that the resistance determined by *cmv1* involves interruption of the virus entry into the vascular system and therefore, inability to develop a systemic infection.

In the third chapter, we have obtained the sequence of CMV-M6 strain and generated infectious clones able to infect systemically *N. benthamiana* and melon.

La accesión exótica de melón PI 161375 presenta una mezcla de resistencia cualitativa y cuantitativa frente a la infección por CMV, dependiendo de la cepa. Anteriormente se describió en nuestro laboratorio la presencia del gen recesivo de resistencia *cmv1* situado en el grupo de ligamiento XII, y que confería resistencia total sólo a algunas cepas de CMV (Essafi et al., 2009). En esta tesis hemos ampliado los conocimientos sobre la resistencia mediada por el gen *cmv1* presente en melón y hemos obtenido la secuencia y los clones infectivos de la cepa M6. La tesis ha sido estructurada en tres capítulos.

En el primer capítulo analizamos la resistencia conferida por el gen *cmv1* en 11 cepas de CMV del subgrupo I y II. Los resultados indicaron que *cmv1* confería resistencia total a las cepas del subgrupo II pero no a las del subgrupo I. Mediante el uso de los clones infecciosos de las cepas CMV-LS (subgrupo II) y CMV-FNY (subgrupo I) hicimos combinaciones entre los RNAs de ambas cepas, pudiendo localizar el determinante de virulencia en el RNA3. Quimeras entre FNY y LS indicaron que el determinante de virulencia estaba en los 209 aminoácidos del extremo N-terminal de la proteína de movimiento (MP). Mediante mutagénesis dirigida identificamos una combinación de 4 posiciones específicas que confieren a LS la habilidad de sobrepasar la resistencia mediada por *cmv1* cuando las sustituimos por los residuos correspondientes de la cepa FNY.

El segundo capítulo trata de la caracterización de la resistencia conferida por el gen *cmv1*. La cepa CMV-LS es capaz de replicarse y moverse célula a célula en la hoja inoculada de la línea resistente. No obstante, LS es incapaz de invadir el floema ya que no hemos podido detectar virus en el floema de la línea resistente. Mediante inmunomarcaje de CMV con oro coloidal hemos identificado el límite entre células de la vaina (BS) y parénquima vascular (VP) o células acompañantes (IC) como barrera que impide la infección sistémica en la línea portadora del gen *cmv1.* Con los resultados obtenidos hemos demostrado que la resistencia determinada por el gen *cmv1* interrumpe la entrada del virus al sistema vascular, impidiendo así una infección sistémica.

En el tercer capítulo hemos obtenido la secuencia de la cepa CMV-M6 y generado clones moleculares capaces de infectar sistémicamente *N. benthamiana* y melón.

L'accessió exòtica de meló PI 161375 presenta una barreja de resistència qualitativa i quantitativa front a la infecció per CMV depenent de la soca. Anteriorment s'ha descrit la presencia del gen recessiu de resistència *cmv1* situat en el grup de lligament XII, i que conferia resistència total només a algunes soques de CMV (Essafi et al., 2009). En aquesta tesi hem ampliat els coneixements sobre la resistència determinada pel gen *cmv1* present en meló i hem obtingut la seqüència i els clons infectius de la soca M6. Aquesta tesi ha estat estructurada en tres capítols.

En el primer capítol vam analitzar la resistència conferida pel gen *cmv1* en 11 soques de CMV del subgrup I i II. Els resultats van indicar que *cmv1* conferia resistència total a les soques del subgrup II però no a les del subgrup I. Mitjançant l'ús dels clons infectius de les soques CMV-LS (subgrup II) i CMV-FNY (subgrup I) vam fer combinacions entre els RNAs d'ambdues soques podent localitzar el determinant de virulència en el RNA3. Virus quimèrics entre FNY i LS van indicar-nos que el determinant de virulència estava en els 209 aminoàcids de l'extrem N-terminal de la proteïna de moviment. Mitjançant mutagènesi dirigida vàrem identificar una combinació de 4 posicions específiques que confereixen a LS l'habilitat de sobrepassar la resistència conferida pel gen *cmv1* quan els substituïm pels residus corresponents de la soca FNY.

El segon capítol tracta de la caracterització de la resistència conferida pel gen *cmv1*. La soca CMV-LS és capaç de replicar-se i de moure's cèl·lula a cèl·lula en la fulla inoculada de la línia resistent. No obstant, LS és incapaç d'envair el floema ja que no hem pogut detectar virus en el floema de la línia resistent. Mitjançant immunomarcatge de CMV amb or col·loïdal hem identificat el límit entre cèl·lules de la beina (BS) i parènquima vascular (VP) o cèl·lules acompanyants (IC) com a barrera que impedeix la infecció sistèmica en la línia portadora del gen *cmv1.* Amb els resultats obtinguts hem demostrat que la resistència determinada pel gen *cmv1* interromp l'entrada del virus al sistema vascular, impedint així una infecció sistèmica.

En el tercer capítol vam obtenir la seqüència de la soca CMV-M6 i vam generar clons moleculars capaços d'infectar sistèmicament *N. benthamiana* i meló.

Abbreviations

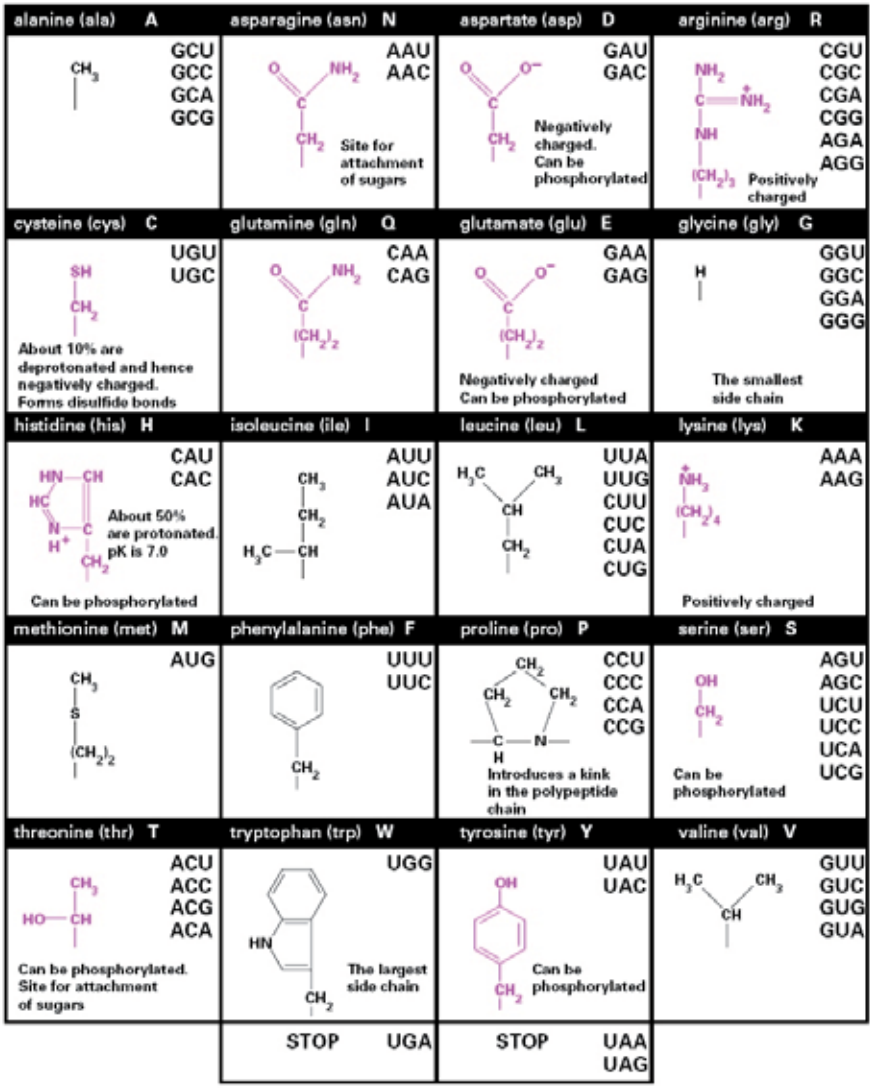
Abbreviations of viruses

AMV	*Alfalfa mosaic virus*
BMV	*Brome mosaic virus*
BPYV	*Beet pseudo-yellows virus*
BYMV	*Bean yellow mosaic virus*
CABYV	*Cucurbit aphid borne yellows virus*
CaMV	*Cauliflower mosaic virus*
CCMV	*Cowpea chlorotic mottle virus*
CGGMV	*Cucumber green mottle mosaic virus*
CMV	*Cucumber mosaic virus*
CuLCrV	*Cucurbit leaf crumple virus*
CVYV	*Cucumber vein yellowing virus*
CYSDV	*Cucurbit yellow stunting disorder virus*
GMMV	*Gayfeather mild mottle virus*
LIYV	*Lettuce infectious yellows virus*
LMV	*Lettuce mosaic virus*
MNSV	*Melon necrotic spot virus*
OLV-2	*Olive latent virus 2*
PepMoV	*Pepper mottle virus*
PRSV	*Papaya ringspot virus*
PSbMV	*Pea seed borne mosaic virus*
PSV	*Peanut stunt virus*
PVMV	*Pepper veinal mottle virus*
PVY	*Potato virus Y*
PZSV	*Pelargonium zonale spot virus*
RTSV	*Rice tungro spherical virus*
RYMV	*Rice yellow mottle virus*
SqMV	*Squash mosaic virus*
TAV	*Tomato aspermy virus*
TBSV	*Tomato bushy stunt virus*
TCV	*Turnip crinkle virus*
TEV	*Tobacco etch virus*
TSV	*Tobacco streak virus*
WMV	*Watermelon mosaic virus*
ZYMV	*Zucchini yellow mosaic virus*

Other abbreviations

BS: Bundle sheath
CC: Companion cell
cDNA: Complementary DNA
CP: Coat protein
DHL: Doubled haploid line
Dpi: Days post-inoculation
eIF: Eukaryotic translation initiation factor
GFP: Green fluorescent protein
HR: Hypersensitive response
IC: Intermediary cell
IGL: Immunogold labelling
ME: Mesophyll cell
MP: Movement protein
NIL: Near isogenic line
ORF: Open reading frames
PCR: Polymerase chain reaction
PD: Plasmodesmata
PPU: Pore-plasmodesma unit
PS: Piel de Sapo
PTGS: Post-transcriptional gene silencing
QTL: Quantitative trait loci
RFP: Red fluorescent protein
RT: Reverse transcriptase
SC: Songwhan Charmi
SE: Sieve element
SEL: Size exclusion limit
TC: Transfer cells
TEM: Transmission electron microscope
UTR: Untranslated region
VP: Vascular parenchyma
VPS: Vacuolar sorting protein

Genetic code



General introduction

1. Melon (*Cucumis melo* L.)

1.1. Origin and economic importance

Melon (*Cucumis melo* L.) is a eudicot diploid plant species (2n = 2x = 24) described and classified by Carolus Linnaeus in 1753 in his compendium "Species planetarum". Melon belongs to, and is the model species of the *Cucurbitaceae* family, represented by 118 genera and 825 species (Jeffrey, 1990), which include agronomical important species such as cucumber (*Cucumis sativus*), pumpkin (*Cucurbita maxima*), squash (*Cucurbita pepo*) and watermelon (*Citrullus lanatus*). The *Cucumis* genus contains 32 species, some of them cultivable, being the most important being, in economical terms, melon and cucumber. The rest correspond mainly to wild African and Asian species. In this genera, 30 species, including melon, have a basic number of chromosome of n=12 and only two of them, *C. sativus* and *C. hystrix* Chakravarty, present a basic number of n=7 (Kerje and Grum, 2000).

Although it was thought that the origin of melon was in Africa, recent data suggest that melon and cucumber may have an Australian and Southeast Asian origin. Sebastian *et al.* (2010) proposed that 10 Ma the Australian ancestor of *C. melo* diverged from its Asiatic relatives. *C. sativus* and probably *C. melo* could have originated 3 Ma in a biogeographic region called Wallacea, where the Southeast Asian and Australian continents converged. Afterwards, melon suffered events of diversification, away from existing primary and secondary diversification areas from the Mediterranean region to Southeast Asia including Japan, China, India, Iran, Iraq or Turkey (Yi *et al.*, 2009; Tanaka *et al.*, 2013). The Indic region is considered a primary centre of diversification, while the Mediterranean region and Southeast Asia are secondary centres of diversification showing genetic erosion (Monforte *et al.*, 2003). Domestication of melon produced a fast

dispersion and diversification of the species in different cultivars with a high morphological variability of the leaf and plant, and especially the fruit. Pitrat (2008) described 16 botanical groups included in two subspecies: *agrestis* (var. *conomon*, var. *makuwa*, var. *chinensis*, var. *momordica* and var *acidulous*) and subespecies *melo* (var. *cantalupensis*, var. *reticulatus*, var. *adana*, var. *chandalak*, var. *ameri*, var. *inodorus*, var. *flexuosus*, var. *chate*, var. *tibish*, var. *dudaim* and var. *chito*). Both subspecies include different cultivars that are consumed for their fruits worldwide.

Melon is an economically important species grown in temperate and tropical regions worldwide with a total production of 32 million tons in 2012 (http://www.faostat3.fao.org). Spain is the seventh leading world producer after China, Iran, Turkey, Egypt, India and United States, producing 870,900 tons in a harvested area of 27,500 hectares. According to the Spanish Agricultural, Food and Environment Ministry (http://www.magrama.gob.es), melon is the third leading horticultural species in economic importance, after tomato and onion.

1.2. Genetic and genomic tools in melon

Melon is an attractive model for studying valuable biological characters, such as diversity of the fruits (Nunez-Palenius *et al.*, 2008), fruit ripening (Pech *et al.*, 2008) and sex determination (Martin *et al.*, 2009; Foucart *et al.*, 2012). In line with the scientific and economic interest of the species, a number of genetic and molecular tools have been developed in recent years, including a collection of Near isogenic lines NILs (Eduardo *et al.*, 2005), genetic maps (Diaz *et al.*, 2011), ESTs (http://www.icugi.org), microarrays (Mascarell-Creus *et al.*, 2009), a BAC library and a physical map (Gonzalez *et al.*, 2010), and reverse genetic tools (Dahmani-Mardas *et al.*, 2010; Gonzalez *et al.*, 2011). To

complete the collection of genomic tools, de novo sequencing of the melon genome was recently published determining 450-Mbases for the whole genome, assembling 375 Mb and predicting 27.427 genes (Garcia-Mas *et al*., 2012).

1.3. Collection of NILs in melon

NILs contain single homozygous chromosome segments from a donor parent, in the genetic background of an elite cultivar, which together provide a complete coverage of the donor parent genome. These lines normally have a high percentage (mostly higher than 95%) of the recurrent parent genome. NIL populations are very useful for the study of complex traits by mendelizing them, verify QTL effects, fine mapping of interesting loci, and generation of breeding lines (Peleman and Van der Voort, 2003). They have also been used to introduce new genetic variability from wild species into elite germplasm (Zamir, 2001). These populations are the basis for understanding complex genetic traits and NIL populations have been developed in tomato (Eshed and Zamir, 1995; Monforte and Tanksley, 2000), cabbage (Ramsay *et al*., 1996), barley (von Korff *et al*., 2004), lettuce (Jeuken and Lindhout, 2004), *Arabidopsis thaliana* (Koumproglou *et al*., 2002) and melon (Eduardo *et al*., 2005).

The melon NILs collection (Eduardo *et al*., 2005) was generated from an intraspecific cross between a Spanish "Piel de Sapo" cultivar (PS) belonging to the horticultural group *inodorus,* chosen as recipient genotype, and the Korean cultivar "Songwhan Charmi" accession PI 161375 (SC), included in the horticultural group *conomon,* chosen as donor genotype. PS and SC present one of the highest distances described between two cultivars within melon germplasm (Garcia-Mas *et al*., 2000; Monforte *et al*., 2003). Both cultivars also present

differences in the fruits, while the recipient cultivar "Piel de Sapo" is a commercial line whose fruits are very sweet, oval, white-flesh coloured and non-climateric, SC fruits are pearshaped green-flesh-coloured and with low-sugar content (Monforte *et al.*, 2004). Additionally, SC is a very interesting cultivar since it shows resistance to some diseases and plagues such as *Cucumber mosaic virus* (Lecoq *et al.*, 1998), *Melon necrotic spot virus* (Coudriet *et al.*, 1981), *Fusarium oxysporum* (Risser *et al.*, 1977) and to virus transmission by *Aphis gossypii* (Pitrat and Lecoq, 1980). The NIL collection developed from these two melon genotypes has 57 lines covering 85% of the genome of the exotic accession in the PS genetic background (Figure I.1).

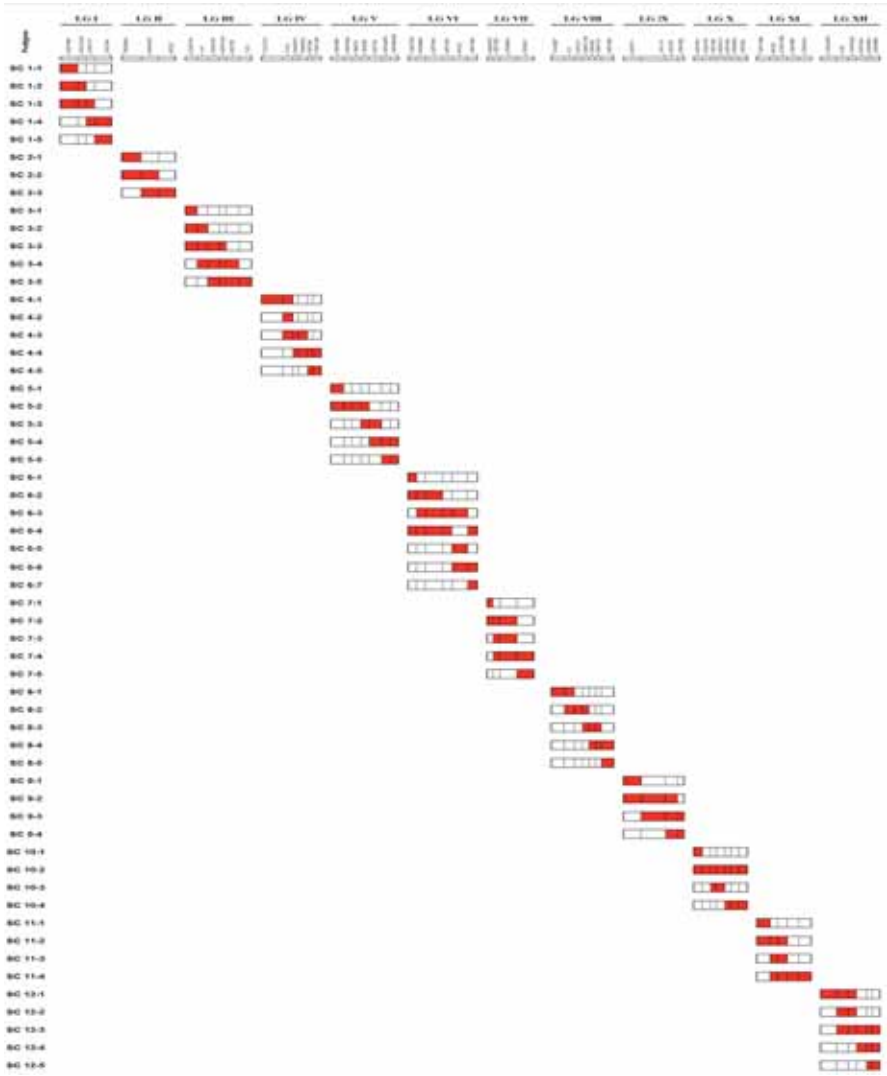


Figure I.1. Graphic genotypes of the melon NILs. Introgressions from SC are represented in red in the PS background. Linkage groups (LG) and molecular markers are represented above. Names of the lines are on the left. For each line, SC indicates the donor parental "Songwhan Charmi", the first number indicates the LG where the introgression maps and the second number indicates the order of the introgression within LG. (Adapted from Eduardo et al, 2005)

2. The plant cell

2.1. Basic organization in a plant cell

The cell is the basic unit of life. Plant cells are eukaryotic cells that present several different aspects from those of other eukaryotic organisms (Figure I.2).

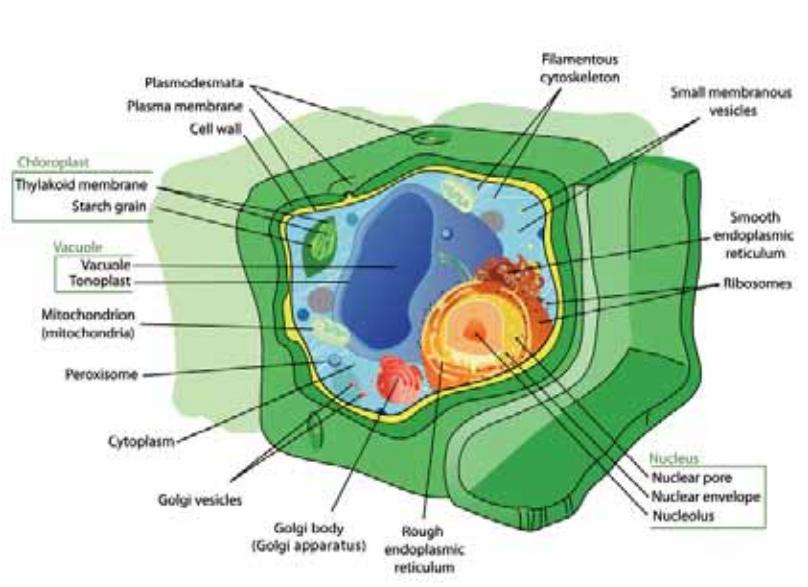


Figure I.2. Plant cell. Schematic representation of the main organelles that constitute a plant cell, including the ones that are different between animal and plant cells: chloroplast, cell wall, vacuole and plasmodesmata, that are indicated in boxes. Adapted from "Plant cell structure" by Mariana Ruiz (Wikipedia).

One of the differential organelles of the plant cell are plastids, the most important being the chloroplasts, which are the responsible for the photosynthesis. Other plastids are amyloplasts, specialized in starch storage, elaioplasts for lipid storage, and chromoplasts for synthesis and storage of pigments.

The vacuole is responsible for the storage of water, ions, salts and other products including toxic products. Apart from the storage

function, the main role of the vacuole is to maintain turgor pressure against the cell wall. The vacuole typically occupies more than 30-80% of the plant cell's volume and is surrounded by a membrane called the tonoplast which controls the movement of molecules between the vacuolar contents and the cell's cytoplasm. Vacuoles are also involved in maintaining the pH and in storage of pigments present in flowers and fruits.

The cell wall is an elaborate extracellular matrix that encloses each cell in a plant. It provides a structural support and protection. The composition of the cell wall depends on the cell type, but in higher plants is mainly composed of polysaccharides. The most abundant is cellulose, a linear polysaccharide consisting on β(1-4) linked D-glucose units. The structure and function of the cell wall changes while the plant cell grows. The primary plant cell wall is flexible and thin, permitting the expansion of growing plant cells. Once the cell has finished its expansion, it synthesises a secondary cell wall between the plasma membrane and the primary cell wall. This secondary cell wall is thicker and more rigid than the primary. The composition of the cell wall also varies; the primary contains approximately the same amount of cellulose, hemicellulose and pectin, and the secondary cell wall does not contain pectin and the main components are xylan (hemicellulose), lignin and higher proportion of cellulose than the primary.

Plasmodesmata are the fourth specialized organelle of the plant cell. Due to their importance in this thesis, they deserve a special section in this introduction.

2.2. Plasmodesmata

Communication through the plant cell wall between two adjacent plant cells is produced through plasmodesmata (PD). PD provide the potential for the exchange of molecules acting as channels for a range of non-cell-autonomous transcription factors (Kurata *et al.*, 2005), pathogenic and non-pathogenic RNAs (Kobayashi and Kobayashi, 2008; Vogler *et al.*, 2008), nutrients and water. Plasmodesmata are key players in the coordination of plant growth, development of the plant and defence against pathogen attack.

Plasmodesmata are pores in the cell wall covered by the plasma membrane (PM) that bridge the cell wall and a central axial element of endoplasmic reticulum (ER), called desmotubule (Figure I.3A), which provides membrane and symplastic continuity between adjacent cells. In the cytoplasmic sleeve inside the PD, between plasma membrane and desmotubule, there are structural proteins that create micro channels that permit the diffusion of small soluble molecules. Plasmodesmata also allow the pass of viral genomes since viruses modify the aperture size of the PD pores (size exclusion limit (SEL)) facilitating the spread of viral infections. Plasmodesmatal trafficking is either passive (e.g., diffusion) or active and responds both to developmental and environmental stimuli. One mechanism proposed for the modulation of PDs flux is based on the dilation/constriction of the neck aperture mediated by a physical collar located in the near-wall, its key component being proposed to be callose (β-1,3-glucan) (Levy *et al.*, 2007). Recently the importance of callose metabolism in PD SEL control has been described. Deposition of callose in the PD neck decreases the SEL and limits the permeability between neighbouring cells. Conversely, removal of callose enlarges PD SEL, enabling large molecules to pass, either via active or passive trafficking. This accumulation is controlled by the antagonistic action

of two types of enzymes: callose synthases and β-1,3-glucanases, which synthesise and degradate β-1,3-glucan polymer respectively (De Storme and Geelen, 2014).

Biogenesis of PD occurs via two distinct pathways. Primary PDs, originated during the cytokinesis, are simple and linear intercellular channels. Secondary PDs on the other hand, are originated independently of cell division and are actively incorporated into pre-existing cell walls by a process requiring cell wall thinning and membrane insertion (Ehlers and Kollmann, 2001). These secondary PDs are more complex showing simple, twinned or branched (X-, Y-, and H-shaped) configurations, respectively (Lee and Sieburth, 2010). Generally, the type of PD structure is temporally and spatially regulated, with young tissues commonly generating simple PDs, whereas complex PD structures are formed during differentiation and cell expansion (Figure I.3B). Special plasmodesmata between companion cell (CC) and sieve element (SE) (see below) are named pore-plasmodesma unit (PPU) with a single pore on the SE cell wall and branching towards the adjacent companion cell (CC) (Kempers *et al.*, 1998; van Bel and Kempers, 1997).

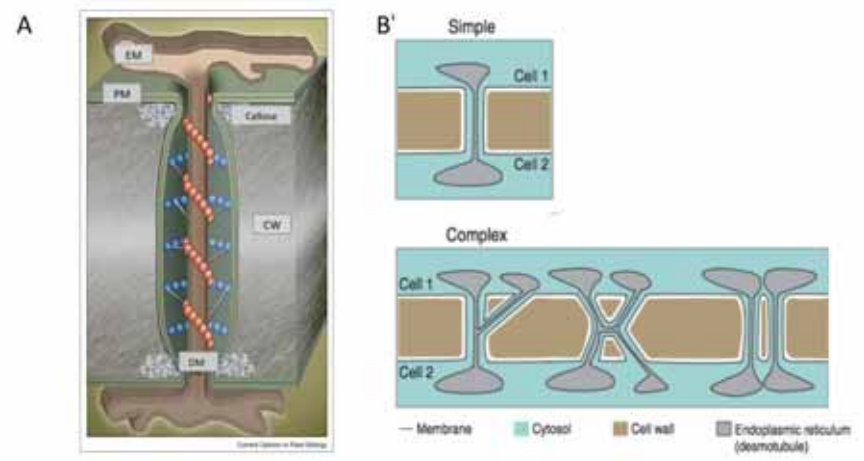


Figure I.3. Plasmodesmata. A. Basic structure of a simple primary plasmodesma. Different parts of PD are represented and indicated. CW: cell wall; EM: Endoplasmic reticulum; PM: plasma membrane; DM: desmotubule (Maule, 2008). B. Plasmodesmata structure ranges from simple to several different complex forms, including, branched, H-shaped and twinned (adapted from Lee and Sieburth (2010)).

3. The transport system

3.1. Transport pathways

Plants have specific mechanisms to exchange information between cells. Long-distance transport occurs via the two conductive tissues, xylem and phloem, whereas for short-range movement of molecules and water, plants use apoplastic and symplastic pathways.

The apoplast consists of free spaces in cell walls and spaces between cells. The apoplastic pathway involves the secretion of water and solutes to the apoplastic space where they can move freely and diffuse to the adjacent cells. In contrast, the symplast is formed by the cytoplasm of individual cells connected by PD. Therefore, in the symplastic pathway, solutes move cell-to-cell through the continuum cytoplasm diffusing through PD. This non-selective, passive cell-to-

cell movement of molecules is driven by diffusion based on a concentration gradient and only applies for molecules that do not exceed the PD size exclusion limit (Mathews, 1991; Hull, 2002) (Figure I.4).

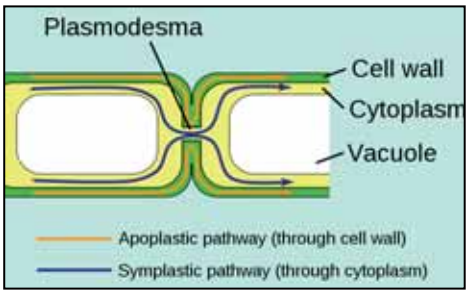


Figure I.4. The apoplastic and symplastic pathways. Apoplastic pathway occurs through the cell wall while in symplastic pathway is through cytoplasm. (Adapted from Apoplastic and symplastic pathways by Jackacon)

3.2. Vein Anatomy

The veins are the vascular tissue of the leaf and are located in the spongy layer of the mesophyll. Veins are subdivided in classes: class I consisting on the primary vein (mid-rib), and classes V and VI representing the finest veins—referred to as minor veins—. The outer layer of the vein is made of mesophyll cells called bundle sheath (BS) cells and they create a circle around the minor veins with no intercellular spaces. The veins consist of sieve elements (SEs), companion cells (CCs) and vascular parenchyma cells (VP). Vascular parenchyma (VP) cells are smaller than BS, highly vacuolated, containing mitochondria and very few chloroplasts. In minor veins VP cells might form a ring around the more internal SEs and CCs. This ring may be irregular, allowing direct contact of some CCs with

bundle sheath (BS) cells (Turgeon *et al.*, 1975; Reidel *et al.*, 2009; Esau *et al.*, 1967; Lucas and Franceschi, 1982).

CC were studied in detail for Gamalei (1989), who grouped plants into two main types: type 1 (open type) has numerous plasmodesmata between CC and other cell types, and type 2 (closed type) has very few. On the basis of ultrastructural features, CC can be classified into three general classes: i) ordinary companion cells, ii) transfer cells (TC) and iii) intermediary cells (IC). All classes are characterized by dense cytoplasm, indicative of high metabolic rate, with numerous mitochondria and ribosomes. ICs are characteristic because they contain many small vacuoles, while the two other types contain only one or two vacuoles. Ordinary CC and TC have few interconnections with the SE and BS, while ICs have numerous plasmodesmata with SE and PD highly branched in IC/VP and IC/BS or IC/IC interfaces (Turgeon *et al.*, 1993; Lalonde *et al.*, 2001).

Both CC and SE originate from a single mother cell by asymmetric cell division. After the division both cells undergo their own developmental programmes and differentiation. The result of SE differentiation is an enucleate cell, that presents the organelles anchored to the plasma membrane and leave the central volume of the cell available for flow of nutrients in solution (Lalonde *et al.*, 2001; Turgeon *et al.*, 1975). CC-SE are connected by specific branched PD – pore plasmodesma unit (PPU)-, whose SEL allows the passage of macromolecules between 10 and 40kDa depending on the species, larger than ordinary PD whose SEL only permit the traffic of 1kDa molecules (Sauer, 1997; Kempers and van Bel, 1997; Kempers *et al.*, 1993).

Plasmodesmata not only provide a direct route for short-range movement, but also integrate local movement of molecules with long distance transport through their role in the loading and unloading of phloem. Solutes and larger molecules including proteins and RNAs move via PD, also viruses and some other pathogens have evolved to use them as their pathway to be spread. The potential of the plant to use a symplastic and/or apoplastic route for loading the polysaccharides into the phloem is indicated by the abundance of plasmodesmal connections between phloem CC and BS cells. In the species that have specialized CC as intermediary cells (IC) with their corresponding highly developed plasmodesmal connections to BS cells, the sucrose diffuse into the IC to be converted into raffinose and stachyose and then be symplastically loaded into the SE, therefore sucrose stays within the symplast all the way from mesophyll cells to the SEs (Zhang and Turgeon, 2009). However, in the species that have as specialized CC transfer cells or ordinary CC, with few PD connecting CC to the surrounding cells, they have symplastic loading of the sugar within the mesophyll cells but in the passage between BS or VP and CC the transport is apoplastic, and sucrose is loaded to the SE without conversion to other compounds (Rennie and Turgeon, 2009; Slewinski *et al.*, 2013; Reidel *et al.*, 2009; Turgeon and Medville, 2004) (Figure I.5).

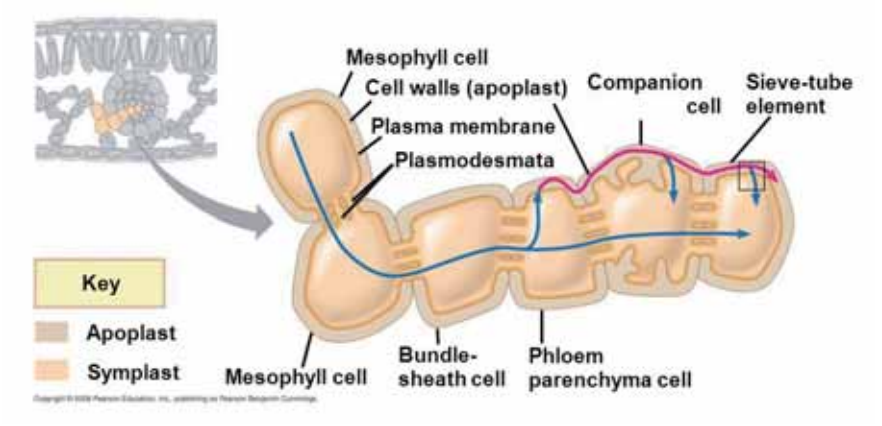


Figure I.5. Model for the apoplastic and the symplastic transport of the sugars in the leaf. In plants that present IC as companion cells the sugar transport is completely symplastic. Contrarily, the plants that have TC and ordinary CC have symplastic transport of the sugar until the VP in which its transport is apoplastic (Modified from Madison Ballard, Pearson Education Inc.).

3.3. Phloem of cucurbits

Cucurbits are models for the study of phloem physiology. They produce a high amount of phloem sap which is easy to sample, and the study of the phloem anatomy is facilitated by easy visualization (Haritatos *et al.*, 1996). Also special is the presence of double phloem (Zhang *et al.*, 2010). The fascicular phloem (FP) is within the main vascular bundles, and is composed of internal phloem (iP) and external phloem (eP), which flank the xylem on two sides. The extra-fascicular phloem (EFP) is an extensive system of sieve tubes distributed throughout the cortex tissues in stems and petioles. It consists of peripheral sieve tubes at the margins of the fascicular phloem and entocyclic sieve tubes that are inside the sclerenchyma ring, both of which are connected by commissural sieve tubes. Extrafascicular phloem also includes ectocyclic sieve tubes that are

found outside the sclerenchyma ring (figure I.6) (Golecki *et al.*, 1999; Crafts, 1932; Zhang *et al.*, 2012).

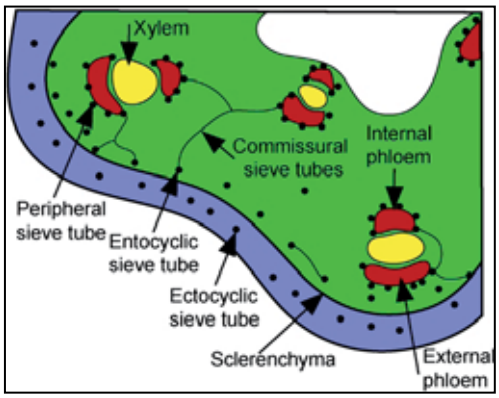


Figure I.6. Diagram of a transverse section of cucurbit stem. The fascicular phloem is bicollateral, with both internal and external phloem. The extrafascicular phloem is composed by the entocyclic and peripheral sieve tubes, connected by commissural sieve tubes, and ectocyclic sieve tubes that are found outside the sclerenchyma ring. (Adapted from Zhang *et al.* (2012)).

4. Plant viruses

Viruses are sub microscopic infectious agents potentially pathogenic in plants, animals, fungi and bacteria that are unable to reproduce outside a living host cell. Virus particles consist of the genetic material, one or more nucleic acid molecules and a coat protein that can be surrounded by an envelope of lipids.

The studies of viral diseases started in 1886 when Adolf Mayer demonstrated that the mosaic disease ("Mosaikkrankheit") that his tobacco plants presented could be transmitted by using the sap from the infected tobacco as inoculum to the healthy plants. Mayer concluded that the infectious agent was some sort of bacteria. Years later, Dimitri Ivanovsky in 1892 and Martinus Beijerinck in 1898

performed filtration experiments with paper and finest porcelain and concluded that the agent of the tobacco mosaic disease was a contagious living fluid, denominated *contagium vivum fluidum,* which was in fact filterable, since the filtered sap infected tobacco. The invention of the electron microscope in 1931 led to the first plant virus images in 1935 by Wendell Meredith Stanley. The second half of the 1900s resulted in the discovery of more than 2000 virus species infecting animals, plants and bacteria.

Viruses cause many important diseases being the second plant pathogens in importance after fungi. Viruses are responsible for huge losses in crop production and quality worldwide. Typical symptomatology shows usually chlorotic, necrotic or yellowing leaves, leaf distortion and/or other growth distortions like abnormalities in flower or fruit formation.

Although viruses are simple in nature, they exhibit great diversity in morphology of the capsid and mode of replication (Lucas, 1998). Based on Baltimore's classification system and International Committee on Taxonomy of Virus (ICTV), viruses are classified based on the mechanism of mRNA synthesis. Viruses must generate mRNAs from their genomes to produce proteins and replicate themselves, but different mechanisms are used to achieve this in each virus family. Viral genomes can be single-stranded (ss) or double-stranded (ds) RNA or DNA and may need the use of reverse transcriptase (RT). In addition, genomic viral ssRNA may be either sense (+) or antisense(-). This classification places viruses into seven groups: i) dsDNA, ii)ssDNA, iii) dsRNA, iv)(+)ssRNA, v) (-)ssRNA, vi) ssRNA-RT: RNA with a DNA intermediate in life-cycle and vii) dsDNA-RT viruses, which replicate through an RNA intermediate. Of these, the majority of plant viruses identified to date are in class iv, the single-stranded

RNA of positive polarity group. Plant viruses can be classified in 83 genera which are grouped in 20 families (King *et al.*, 2012).

Plant viruses differ greatly from other plant pathogens in a number of key aspects. Diseases caused by viruses do not consume host cells or produce toxins, but alter the normal host cell metabolism as a result of the viral invasion. This change in metabolism leads to an imbalance in the metabolic pathways resulting in conditions deleterious to the function of the cell. As a "parasite" within plant cells, viruses in their simplest form consist only of a nucleic acid core enclosed in a protein capsid. Also, unlike many other pathogens, viruses do not produce reproductive structures but need to use the host cell machinery to synthesize their viral components (genome and capsid) (Hull, 2002; Agrios, 2005). Because viruses present a reduced viral genome, they use multiple strategies to translate several proteins from the same nucleic acid sequence: shifting and overlapping the open reading frames (ORF), using weak stop codons, organizing multipartite genome, synthesising proteins from subgenomic RNAs or synthesizing a polyprotein that will later be processed into individual proteins.

4.1. A general virus life cycle

Plant virus transmission must be mediated in nature by biological vectors such as insects, fungi and nematodes (Campbell, 1996; Brown and MacFarlane, 2001; de Vos and Jander, 2010). Once in the cytoplasm, the virion must be disassembled to make the viral genome available for the translation and replication machineries (Maia and Haenni, 1994). Replication requires the viral encoded polymerase in conjunction with host factors (Hull, 2002). Local movement involves transport of the virus through various cell types, from the epidermis,

which is usually the first tissue to be mechanically infected, through mesophyll tissues until reaching the minor veins where the virus will gain access to the SE. From mesophyll cells to the SE, the virus has to pass through the BS, VP and CC, the last cell type before being loaded into the SE. The virus spreads between all cell types through plasmodesmata (Carrington *et al.*, 1996; Roberts and Oparka, 2003). In their normal state , PD only allow passage of molecules smaller than 1 kDa, meaning that free passage of the much larger viral nucleoprotein complexes or virions is excluded. To achieve movement, plant viruses encode movement proteins (MPs), whose function is to modify and gate the PD (Wolf *et al.*, 1989; Lazarowitz and Beachy, 1999a). There are two main strategies described for cell-to-cell movement (Figure I.7.): the first concerns cell-to-cell movement of a viral RNA-MP complex in a non virion form following the symplastic continuity provided by the desmotubule provided by the adjacent cells (Rhee *et al.*, 2000); the second is based on tubule-guided movement of mature virions or non-enveloped nucleocapsids through tubules built-up from the MPs. Although some viruses may travel long distance through the xylem, (Moreno *et al.*, 2004; Verchot *et al.*, 2001), most viruses move systemically through phloem, which follows the non-selective, pressure-driven stream of photosynthetic assimilates from source to sink organs (Leisner and Turgeon, 1993; Santa Cruz, 1999).

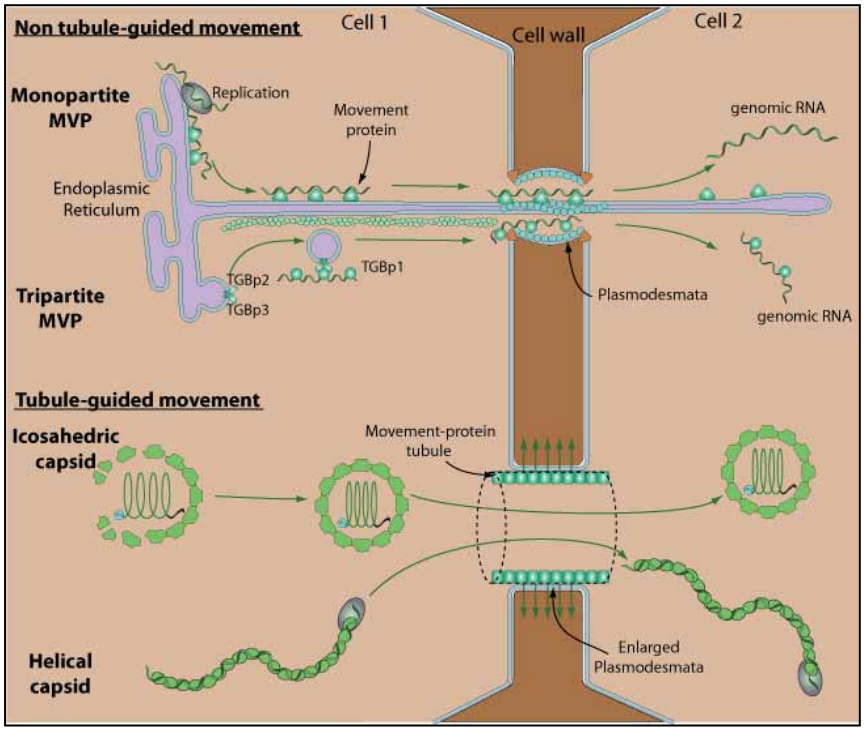


Figure I.7. Cell-to-cell movement of viruses. Representation of the two main strategies for cell-to-cell movement: in the first, cell-to-cell movement is achieved through a viral RNA-MP complex in a non virion form; the second is based on tubule-guided movement of mature virions or non-enveloped nucleocapsids. Movement proteins (MP or MVP) are proteins dedicated to enlarge the pore size of plasmodesmata and actively transport the viral nucleic acid into the adjacent cell, thereby allowing local and systemic spread of viruses in plants (scheme obtained from ExPASy).

5. Resistances to viruses

During a viral infection different interaction processes are established between virus and the host plant. Although viruses are relatively simple genetic entities, interactions between plant and virus are very complex and some of the mechanisms of resistance are still unknown even though they have been studied in detail for the last years.

Successful infection of a plant by a virus requires a series of compatible interactions between the host and a limited number of viral gene products (Hull, 2002) (Figure I.8.). Recessive resistance in plants, also termed "passive resistance" (Fraser, 1990) might be the result of the lack or inactivation of a specific host factor required by the virus to complete its cycle.

In contrast, dominant resistance is the result of an active and specific recognition event that occurs between host and viral factors leading to the induction of host defence responses. This resistance is known as the gene-for-gene response (Dangl and Jones, 2001; Flor, 1971). Genes that contribute to this response are likely to be dominant or incompletely dominant, unless the resistant response occurs as a result of a de-repression of a defense pathway (Buschges *et al.*, 1997). This type of resistance depends on the ability of a plant to recognize a specific "avirulence" (*Avr*) gene product from the pathogen and to quickly mount a range of defensive measures that is often associated with the hypersensitive response (HR) (Flor, 1971). HR, induced by specific recognition of the virus, limits virus spread by rapid programmed cell death surrounding the infection site, which results in visible necrotic local lesions (Hammond-Kosack and Jones, 1997). The recognition process of the pathogen is conferred by single dominant resistance genes (R-genes). Several viral R-genes have been characterized to date, and all belong to the nucleotide binding site leucine-rich repeat (NBS-LRR) superfamily of R-genes (Hammond-Kosack and Parke, 2003; De Ronde *et al.*, 2014). In contrast to dominant R genes, many recessive resistance genes appear to function at the single cell level or affect cell-to-cell movement.

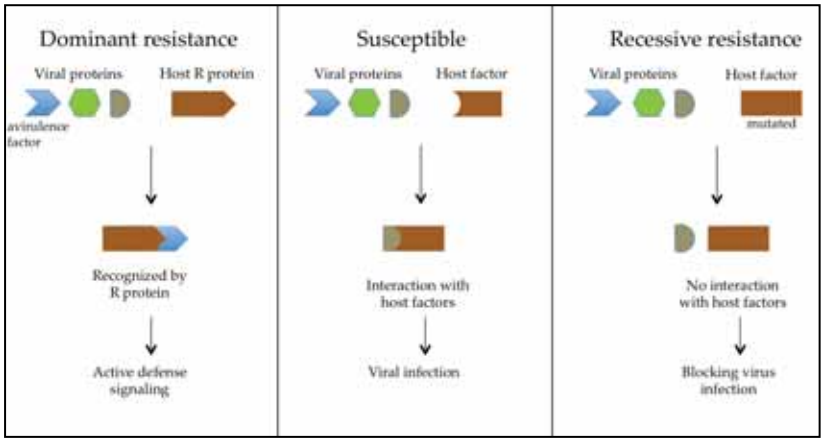


Figure I.8. Resistances to viruses. Possible virus resistance mechanisms showing dominant or recessive inheritance contrasted with a susceptible interaction. (Adapted from Kang *et al.* (2005b))

On the decade of 1990s, a defence mechanism that differs from the classic resistance responses was described, consisting of the specific homology-dependent degradation of viral RNA in the cytoplasm of the infected cell through post-transcriptional gene silencing (PTGS) (generally known as RNA silencing) (Carrington, 2000; Baulcombe, 2000). Specifically, when RNA silencing is triggered by a virus that shares some homology with a plant gene, it is called virus induced gene silencing (VIGS). Nowadays, it is known that most plant viruses have adopted strategies to avoid this defensive response of the plant by expressing suppressors of silencing, viral proteins that suppress RNA silencing at different levels (Roth *et al.*, 2004). Examples of these viral suppressors are the protein 2b from *Cucumber mosaic virus* (CMV) (Brigneti *et al.*, 1998), p25 from *Potato virus X* (PVX) (Voinnet *et al.*, 2000), P1-HcPro from *Tobacco etch potyvirus* (TEV) (Pruss *et al.*, 1997) or p19 from *Tomato bushy stunt virus* (TBSV) (Voinnet *et al.*, 1999). These examples of suppressors belong to viruses that are not related phylogenetically, which suggests that the capacity to suppress RNA

silencing is a general characteristic of viruses (Qu and Morris, 2005; Brodersen and Voinnet, 2006).

6. Resistance to viruses in melon (*Cucumis melo* L.)

One of the main objectives in breeding new crop cultivars is achieving disease resistance. The relative importance of these diseases varies according to the geographic locations, the type of culture (open-field or protected) and the climatic conditions of the growing seasons. Control of the disease includes preventive measures such as the use of healthy seeds, soil disinfection for soil-borne pathogens, removing weeds or spraying chemicals. However, the use of resistant cultivars is for the producer one or the easiest ways to control diseases. The use of marker-assisted selection, the molecular knowledge on resistance genes and the use of transgenic plants permit the introduction of improved cultivars with novel genetic resistances to viruses, bacteria or fungi that have been found in the genetic resources (Olczak-Woltman *et al.*, 2011; Orzaez *et al.*, 2010).

More than 50 viruses have been described in melon but resistance has been described for only 13 of them. Resistance under dominant and under recessive genetic control has been observed for the viruses detailed in Table I.1. (Pitrat, personal communication).

Table I.1. Viruses controlled under dominant or recessive resistances in melon.

Dominant resistances	Resistance gene
Beet pseudo-yellows virus (BPYV)	*My*
Cucumber mosaic virus (CMV)	*Creb-2*
Cucumber vein yellowing virus (CVYV)	*Cvy-1*
Cucurbit yellow stunting disorder virus (CYSDV)	*Cys*
Lettuce infectious yellows virus (LIYV)	*Liy*
Melon necrotic spot virus (MNSV)	*Mnr-1, Mnr-2*
Papaya ringspot virus (PRSV)	*Prv, Prv-2*
Watermelon mosaic virus (WMV)	*Wmr*
Zucchini yellow mosaic virus (ZYMV)	*Zym, Zym-1, Zym-2, Zym-3*
Recessive resistances	**Resistance gene**
Beet pseudo-yellows virus (BPYV)	-
Cucumber green mottle mosaic virus (CGGMV)	*cgmmv-1, cgmmv-2*
Cucumber mosaic virus (CMV)	*cmv1*
Cucumber vein yellowing virus (CVYV)	*cvy-2*
Cucurbit aphid borne yellows virus (CABYV),	*cab-1, cab-2*
Cucurbit leaf crumple virus (CuLCrV),	*culcrv*
Cucurbit yellow stunting disorder virus (CYSDV)	*cys-2*
Melon necrotic spot virus (MNSV)	*nsv*
Squash mosaic virus (SqMV)	*sqmv*
Watermelon mosaic virus (WMV)	*wmr-2*

Two genes involved in virus resistance have been cloned in melon by a chromosome walking strategy. One is *nsv* for MNSV recessive resistance, that was identified as an eukaryotic translation initiation factor 4E (*Cm-eIF4E*) and is located in linkage group XII (Nieto *et al.*,

2006). The *Prv* gene for PRSV resistance is dominant, located on LGIX and belongs to the TIR-NB-LRR family (Brotman *et al.*, 2012).

One approach to describing new resistance genes is by studying exotic melon cultivars. Most of the sources of resistance belong to the *momordica* and *acidulus* groups from India and the *conomon, makuwa* and *chinensis* groups from Eastern Asia (China, Japan and Korea). One exception is the resistance to MNSV which has been found in several accessions from Eastern Asia and USA.

In melon, resistances to CMV are almost absent. After a screening of 253 melon accessions, Diaz *et al.* (2003) determined that there are three exotic accessions that are resistant to some strains of CMV: "Freeman's Cucumber" and the Korean accession "Songwhan Charmi PI 161375" (SC) already described by Karchi *et al.* (1975) and the Japanese accession C-189, all of them show strain specific resistance (Diaz *et al.*, 2003). SC is resistant to almost all CMV isolates with the exception of the "Song" isolates. Recently, three indian accessions resistant to CMV were described, although their resistance has not been characterized (Fergany *et al.*, 2011; Dhillon *et al.*, 2007). Three Momordica accessions and one Cantalupensis accession were described as resistant to strains of subgroup IB of CMV (Malik *et al.*, 2014). Additionally, a single dominant gene, *Creb-2*, was recently described in the accession "Yamatouri", although its identity and function remain unknown (Daryono *et al.*, 2010). Some reports indicated that the resistance present in SC had a major QTL in linkage group XII (Dogimont *et al.*, 2000; Pitrat, 2002). Using line SC12-1 from the melon NIL collection, *cmv1*, a recessive gene conferring resistance to some strains of CMV, was reported to map in LGXII, (Essafi *et al.*, 2009). Recently, three QTLs from SC were reported to be necessary for the resistance to the strain M6. One of these QTLs, *cmvqw12.1,* co-located with *cmv1* that, in

this case was necessary, but not sufficient to confer resistance to CMV-M6 (Guiu-Aragonés et al., 2014).

7. The *Bromoviridae* family

The *Bromoviridae* is one of the most important families of plant RNA viruses, with members distributed worldwide. The natural host range of the members ranges from very narrow (genus *Bromovirus*) to extremely broad (genus *Cucumovirus*). They are predominantly transmitted by insects, in a non-persistent manner, or mechanically. Virions from this family are non enveloped, and the capsid is icosahedral or bacilliform of 26-35 nm in diameter. Its symmetry is icosahedral (T=3, or T=1).

The *Bromoviridae* family consists of six genera (Table I.2)(King *et al.*, 2012). All viruses in these genera have a segmented, tripartite linear ssRNA (+) genome composed or RNA1, RNA2 and RNA3. Each of the RNAs has a 5'-cap structure (Symons, 1975) and a 3' terminus that can adopt a pseudoknot structure similar to that of a tRNA (Ahlquist *et al.*, 1981; Rietveld *et al.*, 1983) Genomic RNAs serve as messenger RNAs. RNA1 and RNA2 encode respectively proteins 1a and 2a, both involved in genome replication. RNA3 encodes two non-overlapping ORFs for the 3a, the movement protein and 3b, the capsid protein separated by a non coding region. The CP is expressed via a subgenomic RNA (sgRNA4). *Cucumovirus* and *Ilarvirus* have and additional overlapping ORF, the 2b encoded by RNA2, which encodes a viral suppressor of RNA silencing and is expressed as a subgenomic RNA (sgRNA4A).

Table I.2: Genera of the *Bromoviridae* family with each type species.

***Bromoviridae* family**	
Genus	**Type species**
Alfamovirus	*Alfalfa mosaic virus* (AMV)
Anulavirus	*Pelargonium zonale spot virus* (PZSV)
Bromovirus	*Brome mosaic virus* (BMV)
Cucumovirus	*Cucumber mosaic virus* (CMV)
Ilarvirus	*Tobacco streak virus* (TSV)
Oleavirus	*Olive latent virus 2* (OLV-2)

Recombination and segment reassortment between genera are key contributors to the important genetic variation of RNA viruses. Codoner and Elena (2008) identified several cases of these events between members of the genera *Bromovirus*, *Cucumovirus* and *Ilarvirus*.

8. *Cucumovirus* genus

The genus *Cucumovirus* contains four described species: *Cucumber mosaic virus* (CMV) (Doolittle, 1916), *Peanut stunt virus* (PSV, described Mink in 1972), *Tomato aspermy virus* (TAV, described by Hollings and Stone in 1971) and the recently described *Gayfeather mild mottle virus* (GMMV)(Adams *et al.*, 2009). Virions from these species are non-enveloped, spherical, about 29nm in diameter with T=3 icosahedral symmetry and composed of 180 coat proteins. The type member of the genus is CMV.

CMV has an extremely broad host range. The other *cucumovirus* species have narrower host ranges: PSV is largely limited to legumes

and solanaceous hosts, TAV predominantly infects composites and solanaceous plants and GMMV infects a series of indicator plants (*Nicotiana sp.* and *Chenopodium sp.)* but it still has to be tested in more hosts. All species are transmitted by aphids in a non-persistent manner.

The genome of the cucumoviruses is represented in Figure I.9. In this section only the differences with the other members of the *Bromoviridae* family are mentioned. Genomic RNA1 ranges from 3350 to 3412 nts. RNA2 ranges from 2935 to 3074 nts encoding 2a and 2b proteins in two different reading frames, with 2b overlapping the 3'-terminal part of ORF 2a (+1 frame shift) (Ding *et al.*, 1994). RNA3 ranges from 2170 to 2386 nts. The proteins encoded by each RNA were already mentioned in the *Bromoviridae* section. The 2b and the capsid proteins are translated from subgenomic messenger RNA4A and RNA4 respectively (Habili and Francki, 1974). RNA4 is encapsidated by all cucumoviruses, whereas RNA4A is encapsidated by some strains of CMV and TAV. RNA5 is a heterogeneous mixture of the 3'UTR of RNAs 2 and 3, and is encapsidated by some strains of CMV. RNA5 of TAV is identical to the 3'end of TAV RNA3. RNA3B is generated from the 3'end of TAV RNA3, with a duplicated region in the 5' end of 163 nucleotides. Minor RNAs of PSV and GMMV have not been characterized yet.

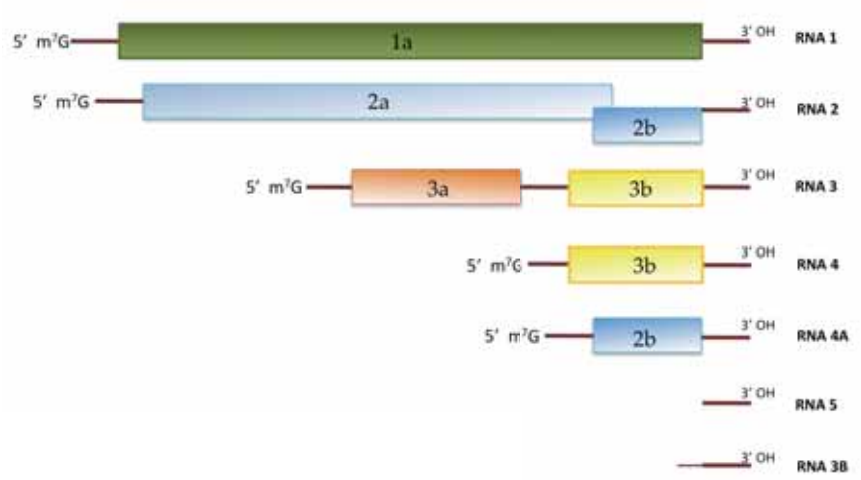


Figure I.9. Genome organization of cucumoviruses. The genomes of CMV, PSV, TAV and GMMV, each consist of three genomic RNAs (1-3s) and two major subgenomic RNAs (RNA4 and RNA4A). TAV and some CMV contain a minor RNA (RNA5), and TAV contains a second minor RNA (RNA3B).

Nucleotide sequence similarity among these four cucumoviruses is about 55-65% (table I.3.) and species can be differentiated serologically and by sequence similarity (Adams *et al.*, 2009). All the genomic RNA of isolates of the same species could be re-assorted into new functional isolates; however, only RNA3 could be exchanged between species (Rao and Francki, 1981). On the other hand, it has been shown that a mixture of RNA2 of both CMV and TAV can be maintained stably in infected plants by TAV RNA1 and CMV RNA3 (Masuta *et al.*, 1998).

Table I.3. Percentage of nucleotide identity between the complete genomes of cucumoviruses (*Cucumber mosaic virus* (CMV), *Peanut stunt virus* (PSV), *Tomato aspermy virus* (TAV) and *Gayfeather mild mottle virus* (GMMV).

	CMV	**PSV**	**TAV**
PSV	65,03%	-	
TAV	64,84%	64,12	-
GMMV	58,81%	56,81	59,94

9. *Cucumber mosaic virus* (CMV)

Cucumber mosaic virus is the type member of the genus *Cucumovirus*, belonging to *Bromoviridae* family. CMV was described for the first time in 1916 infecting cucumber and melon fields of the USA (Doolittle, 1916; Jagger, 1916). Since then, CMV has been detected worldwide, infecting more than 1200 species belonging to more than 100 families (Edwardson and Christie, 1991) and being one of the most important pathogens affecting crops worldwide.

9.1. The viral genome

As a member of the *Bromoviridae* family, CMV is a single stranded, positive sense RNA viruses that shares the above mentioned characteristics of this family (Figure I.10.). Out of its three genomic RNAs, RNA1, has a length of 3357-3391 nucleotides depending on the isolate, with one open reading frame (ORF), encoding protein 1a of 111kDa. RNA2, has 3036-3060 nucleotides and gives rise to proteins 2a

(97kDa) and 2b (11kDa). RNA3, has 2197-2220nts, encoding a movement protein of 30kDa (Boccard and Baulcombe, 1993) and a coat protein of 24,5kDa (Palukaitis *et al.*, 1992). Protein 1a and 2a are both involved in replication (Hayes and Buck, 1990a). Protein 1a possesses a putative methyltransferase domain in its N-terminal part and a helicase motif in the C-terminal, which is essential for viral replication. Also in RNA1 there is a determinant for the hypersensitive response (Salanki *et al.*, 2007) and for seed transmission of the virus (Hampton and Francki, 1992). Protein 2a possesses the GDD motif typical for an RNA-dependent RNA polymerase (RdRp) (Ishihama and Barbier, 1994). It is involved in replication of the virus and also in short and long distance movement interacting with protein 3a (Hwang *et al.*, 2005). Although the MP has no affinity for the 1a protein, it interacts indirectly with the 1a protein via the 2a polymerase protein (Hwang *et al.*, 2005). Protein 2b has been described as a suppressor of post-transcriptional gene silencing (PTGS) (Brigneti *et al.*, 1998) and involved in plant defence response inhibiting salicylic acid-mediated virus resistance (Ji and Ding, 2001). It is also involved in the long distance movement of CMV within the plant (Soards *et al.*, 2002). Protein 3a or movement protein is essential for cell-to-cell and long distance movement (Boccard and Baulcombe, 1993; Li *et al.*, 2001). Finally, CP (3b) is required for functions involving systemic and cell-to-cell movement (Boccard and Baulcombe, 1993). There are determinants associated with this protein affecting long-distance movement in several hosts (Wong *et al.*, 1999; Takeshita *et al.*, 2001; Taliansky and Garcia-Arenal, 1995; Saitoh *et al.*, 1999; Ryu *et al.*, 1998) and it has been described as the only determinant for viral transmission by aphids (Liu *et al.*, 2002).

In addition to RNAs 1, 2, 3, 4, and 4A, cucumber mosaic virus also encapsidates other small RNAs: RNA5 and satellite RNA. RNA5 is

only present in subgroup II of CMV. It is 300 to 400nt long, non-capped and non-polypeptide associated, consists of a mixture of 3'termini of RNA2 and RNA3 and is independent of virus multiplication. CMV satellite RNAs are small noncoding RNAs with no sequence similarity to the viral genome, but dependent on functions encoded by the CMV genome (the helper virus). The presence of a satellite RNA modifies the pathogenesis, accumulation and transmission of CMV depending on the strain of helper virus and satellite RNA and on the species of host plant (Palukaitis and Garcia-Arenal, 2003; Garcia-Arenal and Palukaitis, 1999).

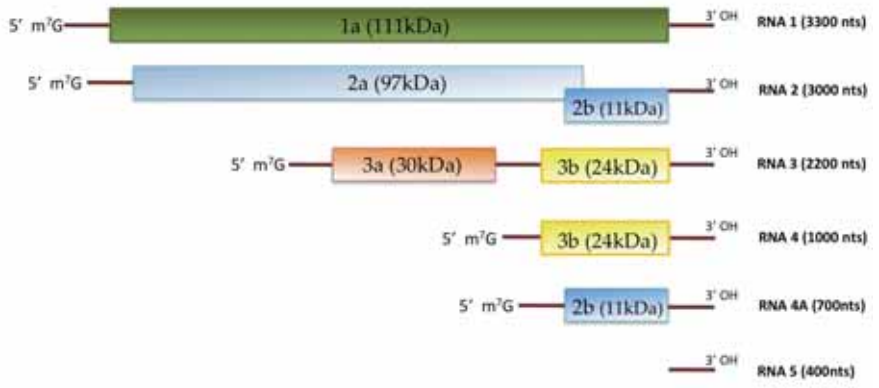


Figure I.10. Genomic organization of CMV. The number of nucleotides (nts) is approximate. ORF are indicated in boxes and named according to the proteins they encode indicating the mass of the protein in kilodaltons (kDa). Schematic representation is not drawn to scale.

RNAs and subgenomic RNAs of CMV are encapsidated in icosahedral particles with a T=3 quasi-symmetry (Figure I.11.). Particles are 29nm in diameter, composed of 180 capsid protein subunits (Finch *et al.*, 1967) and contain about 18% RNA. Viral particles are stabilised by protein-RNA unions (Kaper and Geelen, 1971). RNA1 and RNA2 are

encapsidated in separate particles while RNA3 and RNA4 are in the same (Habili and Francki, 1974; Lot and Kaper, 1976), the three of them show similar morphology and sedimentation. Viral particles can also encapsidate some copies of RNA4A, RNA5 and satellite RNA depending on the isolate (Palukaitis *et al*., 1992).

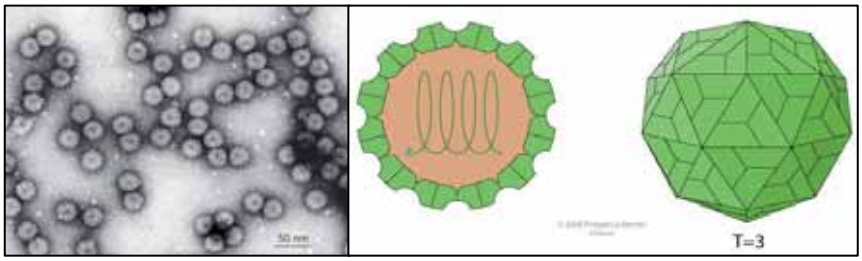


Figure I.11. CMV virion. Electron microscopy image of viral particles of CMV (image from ICTVdB) and schematic representation of a virion and its symmetry (image from ExPASy).

9.2. Epidemiology, transmission and control

CMV has one of the widest geographical distribution of any plant virus, having been detected world-wide, both in tropical and temperate climates (Palukaitis *et al*., 1992). The most common symptoms of a CMV infection are mosaic and deformation of the leaves, dwarf plants and, in some cases, necrosis. However, in general symptoms depend on the viral isolate, host plant, presence or absence of satellite RNA, co-infections with other virus and climate conditions (Palukaitis *et al*., 1992).

CMV, in natural conditions, is transmitted by a number of aphid species in a nonpersistent manner; the most important aphids transmitting virus in vegetable crops are *Aphis gossypii* and *Myzus persicae* that have been extensively used in laboratory experiments.

Vector transmission takes place in a nonpersistent manner, meaning that vectors can only transmit the virus at short distance because viruses remain viable on aphid's mouthparts only for a few hours; the virus is not retained in aphid's moult as happens in persistent transmission. Efficiency of aphid transmission varies depending on the accumulation of viral particles in the source leaf, presence of the satellite RNAs (Escriu *et al.*, 2000), and on the viral strain and aphid species (Carmo-Sousa *et al.*, 2013; Ng *et al.*, 2000; Ziebell *et al.*, 2011). The determinants of virulence for aphid transmission are localised on amino acid positions 161 (Ng *et al.*, 2005) and 162 (Pierrugues *et al.*, 2007) of the CP. CMV can also be transmitted by seeds, its efficiency varies from 1-50% depending on the viral strain and the plant species (Palukaitis *et al.*, 1992). The mechanisms of transmission of CMV have an important role in its epidemiology. For this reason, weeds play an important role as inoculum reservoir for annual crops.

9.3. Variability and evolution

Studies on this virus demonstrate that isolates of CMV are extremely heterogeneous. Initially, on the basis of serological typing, peptide mapping of the coat protein and physical criteria, strains were classified in two subgroups, I and II (Owen and Palukaitis, 1988). Strains belonging to subgroup I were called heat-resistant, while strains belonging to subgroup II were called heat-sensitive. Subgroup I and II can also be named DTL or ToRS referring to the serotype specificity of the antibodies (Porta *et al.*, 1989). Overall, nucleotide sequence similarity between isolates from subgroup I is 90-98% and about 98% within subgroup II. Similarities between groups are around 70-75%, which constitutes a uniquely high degree of sequence diversity within a plant virus species. Analysis of 5' non coding region

of RNA3 divided subgroup I into subgroup IA and IB (Roossinck *et al.*, 1999) which are 92-94% identical. Phylogenetic analysis of the CP ORF confirmed this new grouping, and suggested three distinct major events in CMV evolution. A first radiation would have given rise to subgroup II strains; a second would have led to subgroup IB, from which subgroup IA would have derived in a third event (Roossinck *et al.*, 1999). Phylogenetic analysis of RNA1 and RNA2 ORFs showed different results, which indicate that, each of the three genomic RNAs had a different evolutionary history, including differential re-assortments and recombinations that account for different lineages for different parts of the viral genomes (Roossinck, 2002).

Point mutations are one of the main sources of genetic variability in RNA viruses. RNA-dependent RNA polymerases (RdRp) do not have proofreading activity, so this permits the introduction of mutations during the replication process. Another very important mechanism of variability on RNA viruses is recombination, both within and between isolates, existing in CMV hot spots of recombination in the 3' UTR regions (Canto *et al.*, 2001; Roossinck, 2002). Natural reassortment and recombination events between strains are highly frequent in natural populations of CMV (Bonnet *et al.*, 2005; Chen *et al.*, 2007).

9.4. Virus cycle

Viruses use the cellular components and machinery of their hosts to drive their own amplification and spread through the plant. There are three main steps in a virus infection cycle:

9.4.1. Virus replication

Since the genome of CMV is positive-stranded RNA, when the virus enters the cell, virus particles are uncoated and genomic RNAs are

translated for production of viral proteins. The replication occurs on the tonoplast, in which 1a and 2a proteins, involved in the replication complex, are localized (Cillo *et al.*, 2002). CMV 1a protein, either is associated directly with the tonoplast or anchors to a tonoplast membrane protein and recruits 2a protein that then also becomes associated with the tonoplast (O'Reilly *et al.*, 1998). In CMV it is suggested that the C-terminal part of the 1a protein interacts with the N-terminal region of protein 2a. In association with one or more cellular components and viral RNA, the 1a-2a protein complex forms the active CMV replicase, which binds to the positive sense strands of the viral RNA and begins the replication cycle (Kim *et al.*, 2002). Also, Kim *et al.* (2002) demonstrated that phosphorylation in the N-terminal region of 2a protein prevents the interaction with the 1a protein and therefore inhibits the formation of new replicase complexes (Figure I.12). The N-terminal phosphorylated 2a protein is then available to interact with other host factors involved in host-specific disease responses either hindering or promoting virus movement (Kim and Palukaitis, 1997).

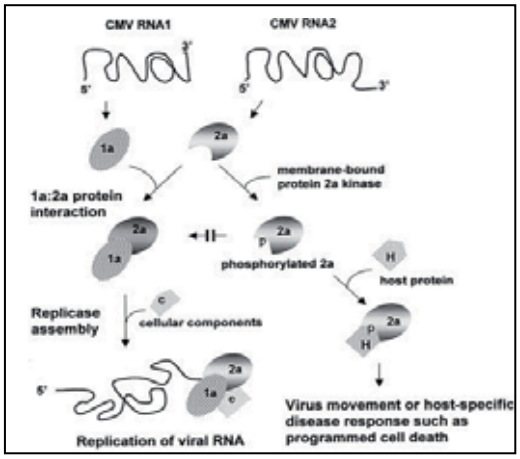


Figure I.12. Model for the effect of phosphorylation of 2a protein. The 1a and 2a proteins are translated from RNA1 and RNA2. Proteins 1a and 2a can directly interact and form with other cellular components, the CMV replication complex. When 2a protein is phosphorylated, 1a and 2a protein cannot interact and 2a would interact with other host factors. (Adapted from Kim *et al.* (2002)).

There are some host proteins described as necessary for the regulation of virus replication. Tsi1-interacting protein 1 (Tsip1) described in tobacco, has a zinc-finger-like domain, which is necessary for the interaction with the viral proteins 1a and 2a. The role of this protein is addressing and/or anchoring proteins 1a and 2a to the tonoplast in the replication complex (Huh *et al.*, 2011). Two tonoplast intrinsic proteins (TICs) from *Arabidopsis thaliana*, TIP1 and TIP2 interact only with protein 1a and they can have a role in anchoring and/or virus replication (Kim *et al.*, 2006).

The replication process consists on the synthesis of minus (-) strand RNA, which is in turn used for production of plus (+) strands. These (+) strands then have three functions: mRNA for translation, template for further transcription and production of virions (Jacquemond, 2012).

9.4.2. Cell to cell movement

Viruses spread from the initially infected cells into adjacent ones through plasmodesmata (PD) and need the MP for this function. The 3a protein is considered the major movement protein of CMV even though all CMV-encoded proteins have been shown to have some role in viral movement. CMV MP belongs to the "30K superfamily" of MPs, which also includes MPs of other members of *Bromoviridae*, *Como* and *Tobamovirus* genus. The most studied "30K superfamily" protein is the MP of *Tobacco mosaic virus* (TMV). Even though there is a low similarity in the sequence between some MP proteins, all of them have the same functions and also a similar structure of the domains described for TMV-MP. Within this superfamily, there are MPs with and without the capacity to form tubules. The 3a movement protein of CMV possesses the main characteristics of MPs: localization to plasmodesmata (PD) (Blackman *et al.*, 1998); ability to increase the plasmodesmal size exclusion limit (SEL) (Vaquero *et al.*, 1994); promotion of the trafficking of RNAs and itself through the PD; binding to single-stranded RNAs cooperatively (Li and Palukaitis, 1996); and the ability to act *in trans* and to complement a CMV isolate deficient for virus movement when expressed in transgenic plants (Sanz *et al.*, 2000; Kaplan *et al.*, 1995).

The MP binds to the viral RNA and forms ribonucleoprotein complexes (Andreev *et al.*, 2004). These complexes interact with host proteins of the PDs, facilitate the movement of RNA and transport the RNA through them. Although CMV does not seem to migrate as virions, the virus needs both the MP and the CP for cell-to-cell movement (Canto *et al.*, 1997). Deletions of either the MP or the CP did not affect the ability of the virus to replicate in protoplasts, but impaired the movement of CMV in plants. Studies combining RNA3 of CMV and TAV RNA3 showed that efficient movement requires

compatibility between the 29 C-terminal amino acids of the MP and the C-terminal two-thirds of the CP (Salanki *et al.*, 2004). Also, the 33 C-terminal amino acids of the MP determine the dependence or independence of the CP to move the viral genome, since the MP with these amino acids deleted is able to move the viral RNA cell-to-cell without the presence of CP (Kim *et al.*, 2004; Nagano *et al.*, 2001). These studies suggest the need for some interaction by both proteins to facilitate movement, however, no direct interactions between the two proteins have been observed in vitro (Palukaitis and Garcia-Arenal, 2003). The general concept is that 3a protein is required for cell-to-cell movement, whereas the capsid protein is not required directly for cell-to-cell movement, but affects the 3a protein in some indirect capacity.

CMV MP also has the ability to sever F-actin filaments and this is required to increase the PD SEL (Su *et al.*, 2010). Two amino acids, forming part of a zinc finger domain in the central part of the MP, are determinant for PD targeting and cell-to-cell movement. A third amino acid plays a role only in cell-to-cell movement, suggesting that factors other than the zinc-binding activity are required for efficient trafficking into and/or out of the cell (Sasaki *et al.*, 2006). Studies from Canto and Palukaitis (1999) described that the MP generates tubules on the surface of protoplasts, which could be involved in virus movement, although there are no reports of the presence of tubules in infected tissues. Furthermore, a 3a mutant that was unable to form such tubules on the surface of the protoplasts was able to support both local and systemic movement of CMV in a number of CMV hosts (Canto and Palukaitis, 1999; Li *et al.*, 2001). This suggests that either such tubules are not required for movement or that they are required for movement between particular tissues or in certain host species.

9.4.3. Long distance movement

The systemic movement of CMV has been shown to occur only through the phloem (Moreno *et al.*, 2004). Long distance movement starts when the virus enters the phloem of the host plant following the pathway BS to VP to CC/SE.

Generally, the ultrastructure of PD that connects BS and VP is similar to that of the PD connecting mesophyll cells. Therefore, PD between BS and ME cells are not a significant barrier for the virus movement (Nelson and van Bel, 1998). However, most of the studies performed in minor veins report a higher percentage of vascular parenchyma infected cells than companion cells, also in the susceptible plants, suggesting that the invasion of the companion cells is a limiting barrier for systemic infection (Nelson and van Bel, 1998; Moreno *et al.*, 2004). It is also known that the host factors that interact with viral proteins and nucleic acids are different from those involved in the cell-to-cell movement though mesophyll cells, meaning that different mechanisms are involved for cell-to-cell movement and long-distance movement (Carrington *et al.*, 1996; Ding *et al.*, 1992). Studies from Kempers *et al.* (1993) revealed that PPU connecting the CC-SE complex have different morphological characteristics from the other PD in that they present a higher SEL which permit the passage of larger molecules. This SEL would permit the direct access of molecules and viruses from the CC to the SE, but Omid *et al.* (2008) suggested that the traffic of proteins from the CCs to the SE is under molecular control in virally infected plants and it does not occur via simple diffusion. In the case of CMV, it is postulated that before the virus is loaded into the SE, virus particles disassemble in the cytoplasm of companion cells (CC), move through the PPU as a ribonucleoprotein complex, with the aid of the MP, and they reassemble in the SE, since CMV virions have been detected in vesicle aggregates in mature SEs but not inside the PD

connecting CC-SE (Blackman *et al.*, 1998) (Figure I.13.). In the SE, viral particles interact with a protein present in the phloem exudate of infected cucumber, which is a homolog of phloem protein 1 (PP1) of pumpkin. PP1 has plasmodesmal-gating ability and translocates with the phloem stream. Its function could be to facilitate the movement of ribonucleic complexes or protect the viral RNAs against the ribonucleases present in the phloem (Requena *et al.*, 2006).

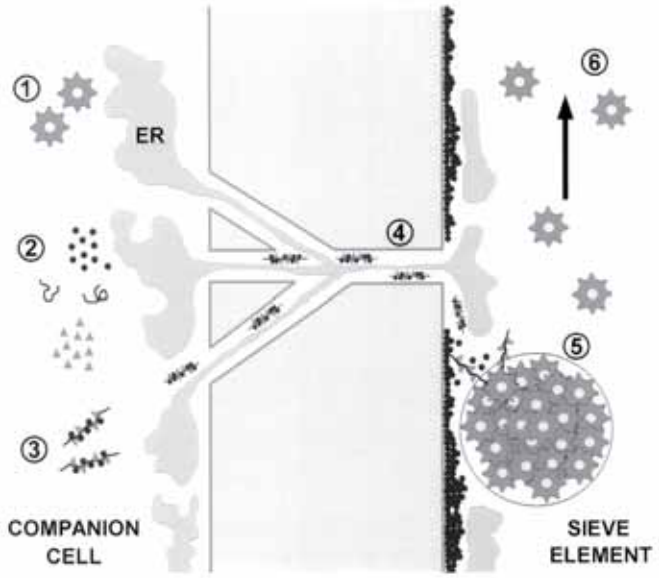


Figure I.13. Schematic model for the movement of CMV into the minor vein sieve elements. The virus particles assemble in the CC (1). To pass through the PPU, virus disassembles (2) and form the ribonucleoprotein complex composed by vRNA (black strands), MP (black circles) and CP subunits (grey triangles)(3). Once the complex has passed to the SE through the PPU (4), virions assemble (5) and virus is spread systemically to sink leaves (6) (Blackman *et al.*, 1998).

Studies on different combinations of strains and host species show that all CMV viral proteins can have effects on long-distance

movement, but CP, MP and 2b proteins seem to be the most important. CP is essential for efficient long-distance movement but the capsid protein expressed transgenically was not sufficient to fulfil this requirement (Palukaitis and Garcia-Arenal, 2003). The CP role in long-distance movement has been observed in a number of hosts, including several cucurbits, maize, *Tetragonia expansa* and *Physalis floridana* (Palukaitis and Garcia-Arenal, 2003; Kobori *et al.*, 2002; Salanki *et al.*, 2011; Taliansky and Garcia-Arenal, 1995). CP is also determinant for the invasion of shoot apical meristem (SAM) in tobacco (Mochizuki and Ohki, 2005). The MP has been associated with the ability of the strain SC to infect wild soybean (Hong *et al.*, 2007). A MP mutant in amino acid position 60 was described as temperature sensitive for long-distance movement but not for cell-to-cell movement, suggesting that both functions reside in different regions of the MP (Li *et al.*, 2001). Protein 2b apart from being a silencing suppressor, is also a pathogenicity determinant in solanaceous hosts and a movement determinant that prevents long-distance migration depending on the host. Deletion of the *2b* gene prevented systemic infection in cucumber, tomato, pepper and squash but not in *Nicotiana* sp. or *A.thaliana* (Ding *et al.*, 1995; Wang *et al.*, 2004; Lewsey *et al.*, 2009). The 2b was also shown to determine SAM invasion in tobacco (Sunpapao *et al.*, 2009), but in this case the role of the 2b protein in virus movement is associated to its silencing suppressor activity rather than to a direct role of the protein itself.

Once the virus has gained access to the conducting elements (SE) through the symplastic pathway, virus particles move very fast to sink tissues. In plant families such as *Solanaceae* and *Cucurbitaceae,* which present two different fascicular phloem types, movement towards the root occurs through the external (eP), while the internal phloem (iP) is

used for translocation toward the shoot. This differential distribution reflects phloem specialization for the downward and upward transport of viruses and has been described for MNSV in melon plants (Gosalvez-Bernal *et al.*, 2008).

The mechanism of unloading from SE has not been extensively studied, but it is thought to reach the sink tissues almost entirely from major veins as occurs in other viruses like MNSV (Gosalvez-Bernal *et al.*, 2008), TMV (Cheng *et al.*, 2000) or CPMV (Silva *et al.*, 2002).

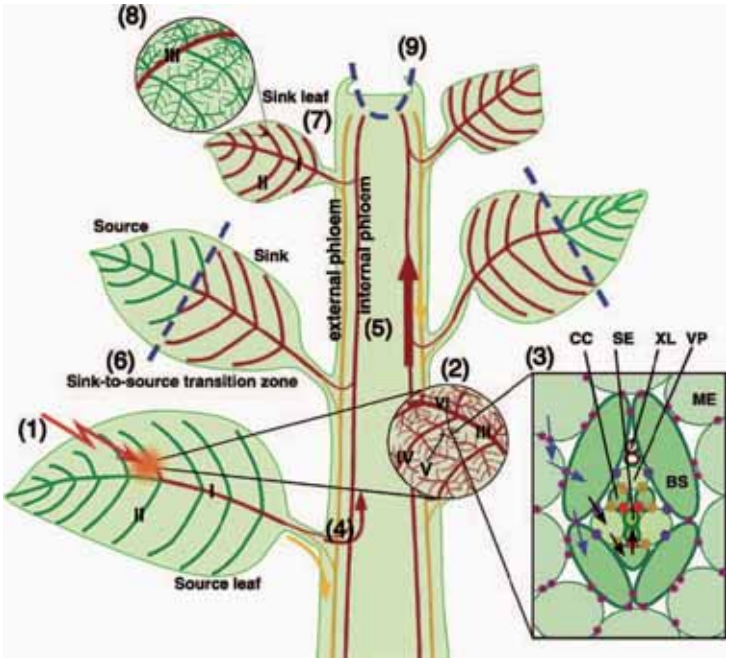


Figure I.14. Cellular routes for cell-to-cell and systemic movement of plant viruses. When a virus starts an infection (1), it replicates in the host cells and then it moves cell-to-cell through epidermal and mesophyll cells (ME) until it reaches the veins (2). Once in a vein, the virus has to pass through BS, VP and CC to enter the SE (3). Once in the SE, the virus moves using internal and external phloem of the stem to infect systemically the sink leaves (4-8). The apical meristem is separated from the rest of the plant by a boundary that does not permit the viral infection (9) (Waigmann *et al.*, 2004).

Objectives

This thesis has as a general objective to study in detail the *Cucumber mosaic virus* infection in the resistant melon NIL SC12-1-99, which carries the resistant gene *cmv1*, and to start addressing the study of CMV resistance in the accession PI 161375 cultivar "Songwhan Charmi". The specific objectives to achieve this main goal are:

1. Identification of the viral factor that determines the virulence against the recessive resistant gene *cmv1.* This factor differentiates strains of subgroup I from strains of subgroup II.

2. Characterization of the *cmv1*-mediated resistance to the progress of CMV infection in melon. This objective will determine which step of the viral cycle is blocked in the resistant line.

3. Sequencing and development of an infectious clone of the strain M6 of CMV. This objective will generate a tool for further studies of characterization of other resistances to CMV in melon.

Chapter I

Four sequence positions of the Movement Protein of *Cucumber mosaic virus* determine the virulence against *cmv1*-mediated resistance in melon.

Four sequence positions of the Movement Protein of *Cucumber mosaic virus* determine the virulence against *cmv1*-mediated resistance in melon.

Cèlia Guiu-Aragonés and Ana Montserrat Martín-Hernández

IRTA, Centre for Research in Agricultural Genomics (CRAG) CSIC-IRTA-UAB-UB. Edifici CRAG, Campus UAB. Cerdanyola del Vallès, 08193, Barcelona, Spain.

Corresponding author: A.M. Martín-Hernández, montse.martin@irta.es

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Abstract

Resistance to a set of strains of *Cucumber mosaic virus* (CMV) in the melon accession PI 161375, cultivar "Songwhan Charmi", depends on one recessive gene, *cmv1*, which confers total resistance, whereas a second set of strains, are able to overcome it. We have tested 11 strains of CMV subgroups I and II in the melon line SC12-1-99, which carries the gene *cmv1*, showing that this gene only confers resistance to strains of subgroup II. Using infectious clones of CMV strain LS (subgroup II) and FNY (subgroup I), we have generated rearrangements and viral chimaeras between both strains and established that the determinant of virulence against the gene *cmv1* resides in the first 209 amino acids of the Movement Protein. A comparison of the sequences of strains of both subgroups in this region shows that there are five main positions shared by all subgroup II strains, which are different from those of subgroup I. Site-directed mutagenesis of the CMV-LS clone to substitute these residues for those of CMV-FNY revealed that a combination of four of these residues (the group 64-68 (SNNLL) to (HGRIA), and the point mutations R81C, G171T and A195I) was required for a complete gain-of-function of the LS MP in the resistant melon plant.

Introduction

To be able to infect a plant, viruses must interact with the host products by means of their own virulence factors. This interaction allows the virus to start or continue its infectious cycle and develop a disease. For genomes as tightly packed as those of viruses, it is important and convenient to be able to use all their gene products, even in multiple functions, to facilitate the invasion of the host. Consequently, virulence factors can be either parts of the viral RNA itself (Díaz et al., 2004; Albiach-Marti et al., 2010) or gene products of the virus (Decroocq et al., 2009; Mansilla et al., 2009; Mochizuki and Ohki, 2011; Choi et al., 2013). *Cucumber mosaic virus* is able to infect more than 1,200 species worldwide from over 100 families (Edwardson and Christie, 1991). To infect such a number of species, CMV has evolved to develop a vast genetic diversity (Palukaitis and Garcia-Arenal, 2003). CMV isolates belong to two subgroups, I and II, defined by their serological and biological properties and showing a 73%-78% sequence similarity among them. The subgroup I is also divided in IA and IB, with 92%-94% sequence similarity between them (Roossinck, 2001). CMV has three genomic and two sub genomic RNAs. The RNA1 encodes for the 1a, a protein with two domains, a methyltransferase and a helicase. RNA 2 encodes for the 2a, an RNA-dependent RNA polymerase, that together with the 1a protein are involved in the replication of the viral RNA. RNA2 also encodes the 2b protein, a suppressor of RNA silencing that is also involved in long distance movement. RNA 3 is bi-cistronic and encodes for the 3a, the Movement Protein (MP), and the 3b, the Coat Protein (CP). The 2b and 3b proteins are translated from two subgenomic RNAs, RNA4A and 4, respectively (Palukaitis and Garcia-Arenal, 2003). CMV has also evolved the capacity to use as determinants of virulence all protein products encoded by its genome, depending on the host. All CMV

proteins have been found to act as determinants of virulence in different systems (for a review, see Mochizuki and Ohki, 2011). The 1a protein is involved in producing necrosis in some species of *N. benthamiana* (Divéki et al., 2004) and can be methylated by a host protein, increasing the spread of CMV (Kim et al., 2008). In pepper, the helicase domain determines systemic infection in *Cmr1*-mediated resistant plants (Kang et al., 2012). The 2a protein is related to the production of necrotic lesions in cowpea and Arabidopsis (Kim and Palukaitis, 1997) and also determines the systemic symptoms in squash by facilitating host-specific viral movement (Choi et al., 2005). The 2b protein is a silencing suppressor that determines long distance movement (Brigneti et al., 1998) and additionally determines symptoms by interfering with the microRNA pathways (Diaz-Pendon et al., 2007; Lewsey et al., 2007). The 3b (CP) is the most frequent virulence determinant reported in CMV in different host systems, both in monocots (Ryu et al., 1998) and dicots (Taliansky and García-Arenal, 1995; Ryabov et al., 1999; Wong et al., 1999; Takahashi et al., 2001; Thompson et al., 2006; Salánki et al., 2011) and often, by affecting cell-to-cell movement and/or systemic spread. In cucumber, a role for the CP as determinant for long distance movement was reported using exchanges between two Cucumoviruses. *Tomato Aspermy virus* (TAV) is not able to infect cucumber systemically, whereas CMV can. Exchanges between TAV and CMV showed that only the CP of CMV could complement the lack of movement of TAV in cucumber (Taliansky and García-Arenal, 1995). The 3a protein has been the CMV product less frequently reported as determinant of virulence in different systems. It has been related with the appearance of cyclic symptoms in tobacco (Gal-On et al, 1996) but also with systemic infection in squash (Choi et al., 2005) and other cucurbits (Kaplan et al., 1997) and determines the infection in soybean (Hong et al, 2007). Mutants generated in several domains of the MP affect the

pathogenicity in different hosts, enabling the virus to invade systemically species or cultivars previously not allowed, or presenting altered symptoms in normal hosts (Kaplan et al., 1997; Li et al., 2001), indicating that different domains of the protein were involved in determining the infection in different hosts.

In the melon accession "Songwhan Charmi" PI 161375 (SC) the resistance to CMV is oligogenic and recessive (Karchi et al., 1975). One of the genes involved, *cmv1*, confers total resistance against CMV strains P9 and P104.82 (Essafi et al., 2009), but not to the strains TL and M6. For these strains, the contribution of other two QTLs, *cmqw3.1* and *cmqw10.1*, which must act together with *cmv1*, is necessary to abort the infection (Guiu-Aragonés et al., 2014). In this report, we show that strains of subgroup I are able to overcome *cmv1*-mediated resistance and that this gene is only effective against strains of subgroup II. We have used the infectious clones of CMV-FNY (subgroup I) and CMV-LS (subgroup II) (Rizzo and Palukaitis, 1990; Zhang et al., 1994), to analyze the difference between both subgroups and identify the determinant that confers virulence to FNY in the resistant line, demonstrating that the MP is the sole part of FNY needed to confer virulence to the strain LS in the *cmv1*-carrying melon. Moreover, we have demonstrated that a combination of four amino acids or groups of amino acids of the MP, common to all tested strains of subgroup I, is sufficient to overcome *cmv1*-mediated resistance.

Results

The gene *cmv1* only confers resistance to strains of subgroup II

As *cmv1* confers total resistance to some strains, but not to others, we tested 11 CMV strains of both, subgroup I and II, for their ability to overcome *cmv1*-mediated resistance to see if there was a correlation of the virulent phenotype with any of the subgroups. Plants of the resistant line SC12-1-99, carrying *cmv1* were inoculated with sap from the strains FNY, I17F, Ri-8, M6 and Y (belonging to subgroup IA), Pl2 and Co1 (belonging to subgroup IB) and p104.82, WL, LS and NG (belonging to subgroup II). All subgroup I (IA and IB) strains were infectious in SC12-1-99, starting to produce symptoms at 7 dpi and developing a full systemic infection, whereas type II strains were unable to infect systemically this resistant line. The results are shown only for CMV-LS and FNY, the two most experimentally used CMV strains (figure CI.1). The plants inoculated with FNY showed the systemic infection typical from CMV, whereas the resistant line inoculated with LS was completely asymptomatic. Accordingly, RT-PCR analysis was able to detect CMV-FNY in the SC12-1-99 plants, but failed to detect CMV-LS in systemic leaves of this resistant line (fig 1B). Therefore, there was a correlation between virulence in the *cmv1* plant and subgroup I, indicating that the gene *cmv1* only confers resistance to strains of subgroup II. Both subgroups must, therefore, be different in the viral factor that determines the virulence in the line carrying *cmv1*.

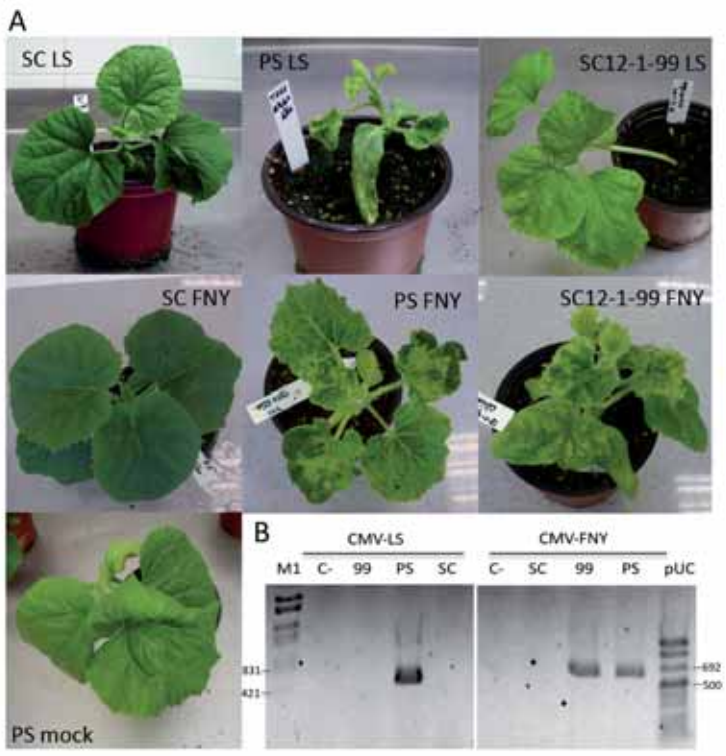


Figure CI.1. Symptoms and virus detection in melon genotypes inoculated with CMV-LS and CMV-FNY. A. Symptoms in melon SC (Songwhan Charmi), PS (Piel de Sapo) and in SC12-1-99 line (*cmv1*) inoculated with CMV-LS and CMV-FNY. Pictures were taken at 20dpi. **B.** RT-PCR for virus detection in melon genotypes Songwhan Charmi (SC), Piel de Sapo (PS) and SC12-1-99 (99); C-, mock- inoculated plant; M1, Lambda Marker (*EcoRI*, *HindIII*); pUC, pUC Mix Marker 8 (Fermentas).

The MP is the determinant of virulence in *cmv1*-mediated resistance.

The molecular clones of the strains LS and FNY are available for inoculation and manipulation (Rizzo and Palukaitis, 1990; Zhang et al., 1994) and were used to produce combinations between the three independent RNAs of each strain. "In vitro" transcribed RNAs were combined to produce all possible pseudo recombinants (CI.2) and

inoculated into *N. benthamiana* plants to produce high viral yield for ulterior inoculation into melon plants. As shown in figure CI.2, the only combination that resulted virulent in the plant SC12-1-99 carried RNA3 from FNY and RNA1 and 2 from LS. The corresponding complementary combination (RNA3 from LS and RNA1 and 2 from FNY) was unable to infect the resistant plant SC12-1-99. All combinations shown in figure CI.2 were infectious in PS and none was in SC, therefore, the fitness of the resulting combinations was not compromised. RT-PCR analysis using primers specific of the unique RNA confirmed the presence of the correct combination in systemic leaves. Therefore, the determinant of virulence that confers virulence to CMV-LS in the presence of the gene *cmv1* was encoded in RNA3.

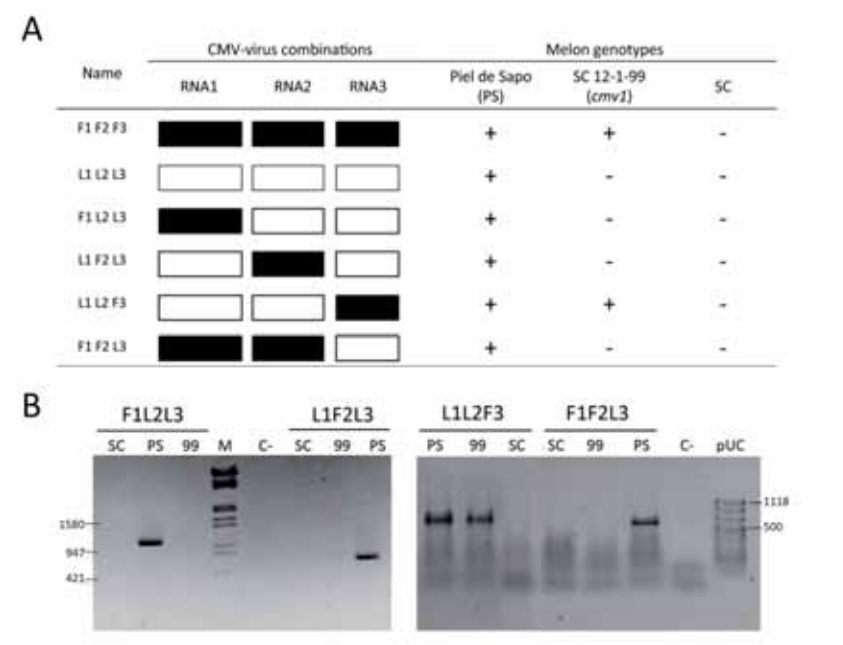


Figure CI.2. The determinant of virulence is in RNA3. A. Schematic representation of the genome structure of pseudo recombinants between CMV-FNY, CMV-LS. CMV-FNY (F1 F2 F3) is represented in black and CMV-LS (L1 L2 L3) is represented in white. Results from both RT-PCR and visual symptoms of virus are indicated as infected (+) or non infected (-). **B.** Infectivity of pseudo recombinants tested by RT-PCR in melon genotypes Songwhan Charmi (SC), Piel de Sapo (PS) and SC12-1-99 (99). M, Lambda marker (*EcoRI, HindIII*); pUC, pUC Mix Marker 8 (Fermentas); C-, mock- inoculated plant.

To further determine which element of RNA3 was responsible for the phenotype, we generated chimaeras exchanging either the three untranslated regions (5'UTR, Middle UTR and 3'UTR) independently or the two open reading frames, MP or CP genes. To assign a clear role to each region, the chimaeras were designed to exchange only the exact nucleotides corresponding to each region. As shown in figure CI.3A, a virulent phenotype was only produced when the MP of FNY was present. Even when only the MP of FNY was present in the

background of LS (clone L3(MPFNY)), the virus was able to overcome the resistance. It produced the typical mosaic symptoms of CMV and was detected by RT-PCR in systemic leaves using primers specific for the recombinant chimaera.

Using the program SCRATCH protein predictor (http://scratch.proteomics.ics.uci.edu/), we have observed that it predicts two well differentiated domains in the MPs of the two strains, domain 1 from residue 1 to 225 and domain 2 from 226 to 279. A close look at the sequence of both MPs showed that there was a *Bpu1102I* restriction site shared by both strains at nucleotide position 628 (209 amino acids). Therefore, the *Bpu1102I* restriction site separates most of the domain 1 (209 out of 225 amino acids, being the remaining 16 residues identical or much conserved (see figure CI.4A)) from domain 2. Thus the *Bpu1102I* site was used to exchange the domain 1 of the gene to obtain clones with only the 628 5'-terminal nucleotides of FNY MP in the background of LS (L3(MP1FNY)) and, likewise, the complementary clone with the 628 first nucleotides of LS MP in the background of FNY (F3(MP1LS)). After testing the virulence of the resulting clones in the resistant SC12-1-99 line, the only one that produced infection was the clone carrying the domain 1 of FNY in the background of LS (fig 3, clone L3(MP1FNY)). Therefore, this indicates that the virulence was conferred by one or several residues present in the N-terminal 209 amino acids of the domain 1 of the MP of FNY or by the structure generated when these residues are present.

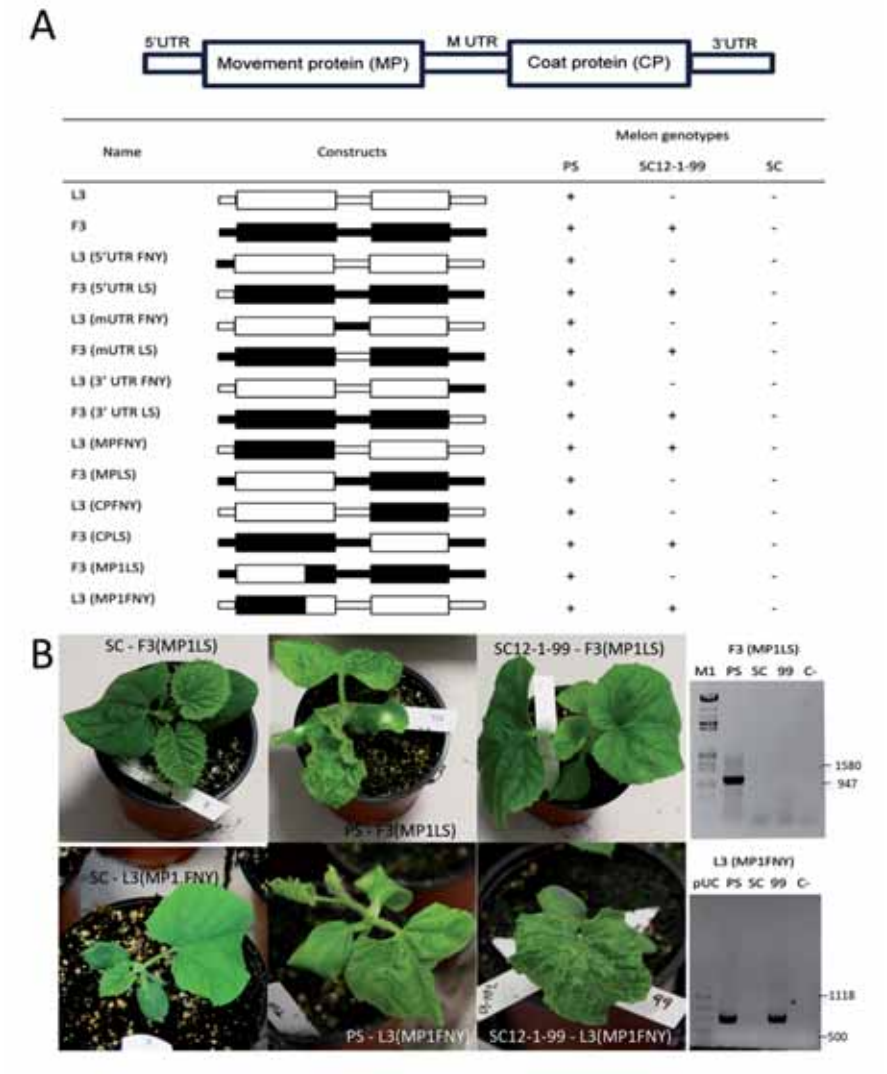


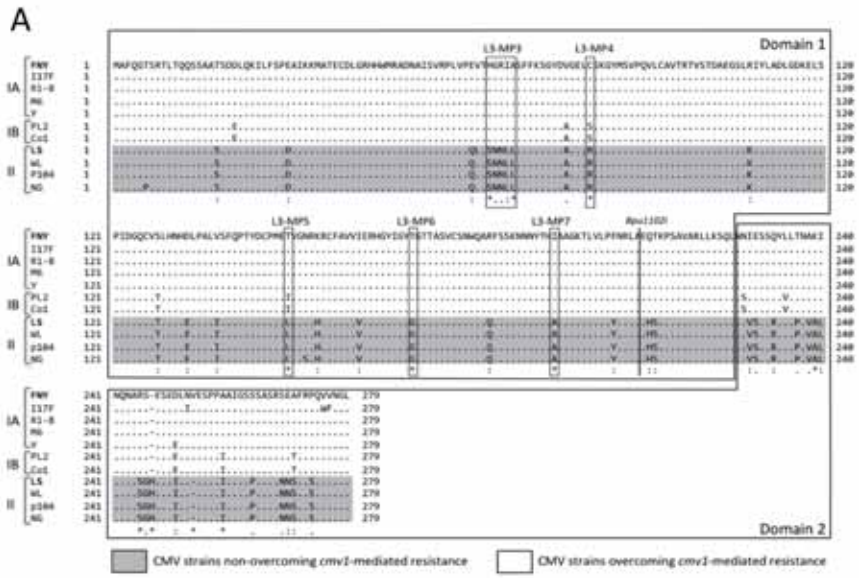
Figure CI.3. The determinant of virulence is in the MP. A. Chimeric viruses between CMV-FNY (represented in black) and CMV-LS (white) exchanging untranslated regions (UTR) and open reading frames (ORF) in RNA3. Results from both RT-PCR and visual symptoms of virus are indicated as infected (+) or non infected (-). **B.** Pictures of virus symptoms at 20 dpi and RT-PCR in melon genotypes Songwhan Charmi (SC), Piel de Sapo (PS) and SC12-1-99 (99) inoculated with F3(MP1LS) or L3(MP1FNY). C-: mock-inoculated plants; M1, Lambda marker (*EcoRI, HindIII*); M2, pUC Mix Marker 8 (Fermentas).

Identification of the residues relevant for virulence.

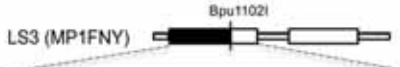
A comparison of the MPs of the two strains showed that they were 83.5% identical in residues and 93.1% similar. The comparison of the corresponding N-terminal 209 amino acids of the two strains showed that they were 89.5% identical and differ in 22 amino acids, and that only in five positions there were amino acid changes with very different chemical properties, more likely to produce important changes in the final structure. There was a group of 5 amino acids that changed as a block at positions 64-68 (SNNLL in LS) to (HGRIA in FNY), and four unique changes: R81C, L150T, G171T and A195I. A comparison between the MPs of the 11 CMV strains, belonging to subgroup I and II, used above, showed that they were also different in these same five positions, although inside subgroup I there were different residues at positions MP4 and MP5 in the strains of subgroup IB than in those of subgroup IA (CI. 4A). Therefore, the presence of these 5 residues or groups of residues in all subgroup I strains was a general feature for all the strains that overcome the resistance provided by *cmv1*.

To assign virulence to a particular residue or group of residues, we performed site-directed mutagenesis in the MP gene of LS to introduce the corresponding FNY residue. First we introduced the mutations in independent clones to test the effect of each change on the virulence, but the inoculation of these mutants into the resistant line 12-1-99 did not produce infection (figure CI.4B). Therefore, none of the five residues individually can produce a gain-of-function mutant able to overcome the resistance provided by *cmv1*. Then combinations of two, three, four and the five changes were generated. Although all of them were infectious in the susceptible plant PS, inoculation of these clones into SC12-1-99 resistant plants revealed that the infectivity of the strain LS was restored when the changes 64-

68 (SNNLL) to (HGRIA), R81C, G171T and A195I were present in the same clone (L3-MP3/4/6/7, see figure CI.4B), producing infection after 10 days pi. Consistently, the clone L3-MP3/4/5/6/7, carrying the five mutations was also infectious. However, the clone with the mutations 64-68 (SNNLL) to (HGRIA), R81C and G171T (clone L3-MP3/4/6) produced also a late infection, starting after 3 weeks pi, suggesting that some additional compensatory mutation in other viral gene(s) had been necessary to restore the virulence. The clones L3-MP3/4 and L3-MP6/7 were not infectious in the resistant plant, indicating that the changes 64-68 (SNNLL) to (HGRIA) and R81C are required amino acid changes for overcoming *cmv1* resistance, but they need the contribution of G171T and A195I to completely restore the virulence and that these are the only four mutations required. The mutation L150T was, therefore, dispensable for this phenotype. In all cases, the relevant fragment of the out coming viruses was sequenced confirming that the only mutations present were the ones introduced for this experiment.



B



Combinations	Amino acid substitutions					Melon genotypes		
	64-68 (MP3)	81 (MP4)	150 (MP5)	171 (MP6)	195 (MP7)	PS	SC 12-1-99	SC
FNY	**HGRIA**	**C**	**T**	**T**	**I**	+	+	-
LS	SNNLL	R	L	G	A	+	-	-
L3-MP3	**HGRIA**	R	L	G	A	+	-	-
L3-MP4	SNNLL	**C**	L	G	A	+	-	-
L3-MP5	SNNLL	R	**T**	G	A	+	-	-
L3-MP6	SNNLL	R	L	**T**	A	+	-	-
L3-MP7	SNNLL	R	L	G	**I**	+	-	-
L3-MP3/4	**HGRIA**	**C**	L	G	A	+	-	-
L3-MP6/7	SNNLL	R	L	**T**	**I**	+	-	-
L3-MP3/4/5	**HGRIA**	**C**	**T**	G	A	+	-	-
L3-MP3/4/6	**HGRIA**	**C**	L	**T**	A	+	+/-	-
L3-MP5/6/7	SNNLL	R	**T**	**T**	**I**	+	-	-
L3-MP3/4/6/7	**HGRIA**	**C**	L	**T**	**I**	+	+	-
L3-MP4/5/6/7	SNNLL	**C**	**T**	**T**	**I**	+	-	-
L3-MP3/4/5/6/7	**HGRIA**	**C**	**T**	**T**	**I**	+	+	-

Figure CI.4. Comparison of the Movement Protein (MP) of subgroup I and II strains and amino acid substitutions. A. Alignment between MPs of strains of subgroups I and II showing the two domains, *Bpu1102I* restriction site and location of amino acid substitutions. CMV-FNY and CMV-LS strains are indicated in bold. Non conserved amino acids are indicated as"*"; semi-conserved amino acids are indicated as "."; and conserved amino acid substitutions are indicated as ":" below the

sequences. **B.** Representation and results of single and multiple amino acid substitutions. Amino acids of FNY and LS are indicated in bold and plain lettering, respectively. Presence of virus was observed by RT-PCR, at 21 dpi in the melon genotypes Piel de Sapo (PS), Songwhan Charmi (SC) and SC12-1-99: +, infected plant; -, non-infected plant.

Discussion

We have analyzed 11 CMV strains for their ability to overcome *cmv1*-mediated resistance in the melon accession SC and established that this resistance was only efficient against strains of subgroup II, whereas strains of subgroup I can infect systemically the resistant line SC12-1-99. Subgroups I and II of CMV strains have been defined based mainly on sequences of the CP and RNA3 5′ UTR (Roossinck et al., 1999). Correlation of subgroups with traits has only been observed concerning temperature and symptoms in tobacco plants. Strains of subgroup I are also called heat-resistant since they can infect plants at higher temperature than strains of subgroup II, also called heat-sensitive. To our knowledge, this is the first resistance phenotype where both subgroups have a different response to the resistance gene. Subgroups I and II are worldwide distributed, without a clear prevalence of one of them in a geographical area (Palukaitis and Garcia-Arenal, 2003). Only subgroup IB is more frequent in East Asia. However, in this report, we have observed that the presence of a gene, *cmv1*, in a Korean melon cultivar confer resistance only against strains of subgroup II, suggesting that some positive selection pressure must have happened. This selection pressure involved the selection of a group of mutations and three additional point mutations in the MP which could possibly be necessary to maintain a protein structure able to overcome the resistance conferred by *cmv1*. More work is needed to understand the epidemiological significance of this correlation between *cmv1* and to characterize the resistance against subgroup II strains. Additionally, this observation could allow the development of strategies of breeding to select melon plants resistant to CMV strains of subgroup II.

The ability to invade systemically a plant involves short and long-distance movement and in both, viral and host factors are involved. The viral factors are the elements that enable the virus to overcome the plant barriers. The analysis of the determinant of virulence that enables CMV to overcome *cmv1*-mediated resistance established that the determinant is in the MP coding region. All CMV-encoded proteins have been identified as virulence determinants in different systems (for a review see (Mochizuki and Ohki, 2011)). Although the most frequent determinant of virulence in CMV is the CP (see above), the MP has also been reported to achieve the main role in virulence in different hosts. In soybean, Hong et al, 2007 constructed chimaeras between two strains, CMV-SC, that was able to infect the cultivar Hyuongo, and CMV-SD that was unable and reported the MP as a determinant that enabled CMV-SC strain to infect this cultivar (Hong et al., 2007). In cucurbits, mostly the CP and MP have been implicated in viral spread. The MP has been implicated in slower cell-to-cell movement in the inoculated cotyledons and also in systemic spread in squash (Gal-On et al., 1996; Kaplan et al., 1997; Choi et al., 2005). The CP controls systemic infection in squash (Wong et al., 1999; Thompson et al., 2006) and cucumber (Taliansky and García-Arenal, 1995; Salánki et al., 2011). Sometimes, the determinant is not a single gene, but a combination of viral gene products. For example, a combination of residues in both the MP and CP determines viral spread in bottle gourd (Takeshita et al., 2001). In the system CMV-melon reported here, we have not detected a role for the CP alone or in combination with the MP in overcoming the resistance provided by *cmv1*. However, there are at least other two QTLs that contribute to the resistance to the strains of subgroup I in melon (Guiu-Aragonés et al., 2014). The CP (and other CMV genes) could well be involved in determining the virulence against one or both of these QTLs. Moreover, CMV is thought to move systemically as particles

(Blackman et al., 1998) and therefore, it is likely that the CP has a role in the systemic movement of the strains of subgroup I by interacting with the products of those QTLs.

Although CMV does not move cell-to-cell as virions, CMV CP is needed for cell-to-cell movement together with MP. Both proteins communicate through a region of 33 C-terminal amino acids of the MP that determine the dependence or independence of the CP to move the viral genome, since Δ33CMV MP is able to move CMV RNA without the need of the homologous CP (Nagano et al., 2001; Kim et al., 2004). In the melon accession SC, the CP of CMV-LS is able to move the chimaeric virus LS3(MPFNY) that carries the MP of FNY, since this virus produces a full systemic infection. Additionally, all combinations made are infectious in the susceptible line PS. Therefore, this region of the MP does not affect the crosstalk between both proteins when they belong to different strains despite being the most divergent part of this protein between both strains (figure CI.4A). Moreover, we have shown that the MP fragment involved in overcoming the resistance of *cmv1* is the N-terminal domain, and therefore, interaction with the CP should not be involved in this phenotype. In fact, this region of the MP has been suggested to be involved in host-specific functions both in CMV and in *Brome Mosaic virus* (Fujita et al., 1996; Li et al., 2001). We cannot rule out interactions of MP with other CMV proteins, although for cell-to-cell movement only the CP has been reported to be necessary. The 2a and MP have been involved together, but independently in virus movement (Choi et al., 2005), although an interaction between them has also been reported (Hwang et al., 2007). Nevertheless, in the chimaeric virus L3 (MPFNY) these interactions, if any, would take place between LS proteins and the FNY-MP and don't disturb the possible direct or indirect interaction of this MP with the product of *cmv1*.

We have generated a series of mutant clones in the MP of LS by introducing the corresponding FNY residues and observed that a combination of four mutations (the group 64-68 (SNNLL) to (HGRIA), and the point mutations R81C, G171T and A195I) is sufficient to overcome the resistance provided by *cmv1*. Since the structure of the CMV MP has not been resolved, it is difficult to visualize the impact of these mutations in the folding of the protein. However, some functional domains have been reported (Kaplan et al., 1997; Li et al., 2001). There is a hydrophobic core domain between residues 86 to 108, an RNA binding domain between amino acids 174 and 233 (Vaquero et al., 1997) and a Cys-rich, putative zinc finger domain between amino acids 126 and 194, with two nucleic acid binding domains also included in this region (Sasaki et al., 2006). Mutants in these domains have revealed implication in the MP functions. Li et al, (2001), found that mutants in the putative zinc-finger domain, were impaired in cell-to-cell movement although can replicate in protoplasts. In this report, the four changes that together restore the virulence in the *cmv1* plant are dispersed in different domains: MP6 and MP7 are in the putative zinc finger domain, MP4 is in the hydrophobic core and the group 64-68 (SNNLL) to (HGRIA), located out of putative domains. MP5, the only one not affecting the gain-of-function phenotype, is out of any domain. The mutation G172D, in the zinc-finger domain, produce a MP with no localization to plasmodesmata and the virus does not move to the adjacent cell (Sasaki et al., 2006). It is just beside the mutant MP6 (G171T) in this report, which has a role in overcoming *cmv1* resistance. However, the MP of the strain LS localizes to the PD in epidermal melon cells (data not shown). Therefore, the gain-of-function mutation MP6 studied here does not seem to be related to PD localization, suggesting that the zinc-finger domain is not involved in overcoming the resistance of *cmv1* or that the mutation MP6 does not alter the zinc-finger domain. Mutant M9 from Li et al, (2001), at

position 60, can move cell-to-cell, but is temperature sensitive for long-distance movement and therefore suggests that cell-to-cell movement and long distance movement functions of CMV MP reside in different regions of the protein. This mutation is only four residues far from the residues 64-68 (SNNLL) to (HGRIA), suggesting an implication of this region in long distance movement. The chemical nature of the mutations introduced in LS MP predicts relevant changes in the predicted tertiary structure of the protein. For example, the program SCRATCH protein predictor, mentioned above, predicts three disulfide bonds in FNY MP and two in LS MP, making the structure of LS MP more relaxed. The mutation R81C would recover this disulfide bond, and with it, a tertiary structure more FNY-like of the MP. FNY MP can probably interact directly or indirectly with both, the PS CMV1 protein and the resistant SC cmv1 to continue the infection, whereas LS MP would only interact directly or indirectly with the susceptible PS CMV1 protein to produce a systemic infection. The conformation of the mutant L3-MP3/4/6/7 would keep the interaction FNY-like enabling the crosstalk with both, the PS and the SC alleles of *cmv1*.

Recessive resistances against viruses reported most frequently are related to impairment of translation of the virus, since the resistance genes were eukaryotic translation initiation factors. The implication of the MP as determinant of virulence in the resistance mediated by *cmv1* suggests that it will be related to impairment of the viral movement, rather than with viral translation. This resistance is effective only for strains of subgroup II. However, there are at least other two QTLs in the resistant cultivar SC (Guiu-Aragonés et al., 2014) involved in resistance against strains of subgroup I that might also be involved in impairing viral movement. Further work is needed to characterize the resistance provided by these three genes and to demonstrate that a limitation in viral movement is involved.

Materials and methods

Plant and virus materials

Genotypes of melon (*Cucumis melo* L.) used for the study of the resistance to CMV were: the Korean accession PI 161375 cultivar "Songwhan Charmi" (SC) and the Spanish cultivar Piel de Sapo, line T111 (PS) as resistant and susceptible controls, respectively. The near isogenic line (NIL) SC12-1-99 was derived from the NIL SC12-1 (Essafi et al., 2009), carrying a shorter introgression of SC on the linkage group XII that contains *cmv1* gene. Seeds were pre-germinated by soaking them in water overnight and then kept for 2 to 4 days in continuous light at 28°C. Seedlings were grown in growth chambers SANYO MLR-350H in long-day conditions consisting of 22°C for 16h with 5,000 lux of light and 18°C for 8h in dark for all infections.
Viruses used in this study were the infectious clones of CMV-LS, belonging to subgroup II, and CMV-FNY, belonging to subgroup I (Rizzo and Palukaitis, 1990; Zhang et al., 1994). Other CMV strains used were M6 (Diaz et al., 2003), I17F (Jacquemond and Lauquin, 1998), Ri-8, Co1, PL2 (Aramburu et al., 2007) and Y, from subgroup I and NG (Aramburu et al., 2007), WL (Namba et al., 1991) and P104.82 from subgroup II.

Inoculations and virus detection

Viral inocula were freshly prepared from infected zucchini squash Chapin F1 (Semillas Fito SA, Barcelona, Spain) and the sap obtained, rub-inoculated onto the cotyledons of 7- to 10-days-old melon plants. Infectious RNAs of the pseudo recombinants and chimaeric viruses were inoculated to *Nicotiana benthamiana* leaves. RNAs were generated from 1µg of the linearized infectious cDNA clones by "in vitro" transcription using T7 RNA polymerase (Roche Diagnostics,

Germany) and Cap Analog (Roche Diagnostics, Germany) according to manufacturer's protocols. Infectious clones from CMV-FNY (Rizzo and Palukaitis, 1990), chimeric viruses and amino acid substitution mutant clones were linearized using *PstI*. Infectious clones CMV-LS1, CMV-LS2 and CMV-LS3 were linearized with *NotI*, *HindIII* and *PstI*, respectively (Zhang et al., 1994). The three transcribed RNAs, without further purification, were rub-inoculated together onto *N. benthamiana* leaves. Sap produced from systemically infected leaves was used to inoculate melon cotyledons. Symptoms in melon plants were scored visually 20 days after inoculation. Viral detection was performed by Reverse Transcriptase (RT)-Polymerase Chain Reaction (PCR) from young newly developed leaves. For RT-PCR, RNA was isolated using TriReagent (Sigma-Aldrich, St Louis, MO, USA) following manufacturer's protocol. RT-PCR was made using PrimeScript (Takara Biotechnology, Dalian, China) and Taq polymerase (Promega Corporation, Madison, WI) according to manufacturer's instructions as reported previously (Essafi et al., 2009). Primers used for RT and PCR are listed in supplementary materials S1. Combinations of primers from both strains, CMV-LS and CMV-FNY, were used to confirm viral infection, when necessary. To confirm the amino acid substitution mutants, the PCR products were sequenced using primer LS3-1F (Table CI.1).

Table CI.1. Specific combinations of primers used for virus detection in plants inoculated with pseudo-recombinants, chimaeras with UTR and ORF exchanges and amino acid substitution mutants.

Pseudore-combinants	RT Primer	PCR primers
F1L2L3	F109-3'R (5'TGGTCTCCTTTTAGAGACCC)	F109-2200F (5'CGGGACCATTAGTCAAGTTG) F109-3'R (5'TGGTCTCCTTTTAGAGACCC)

L1F2L3	F209-3'R (5'TGGTCTCCTTTTGGAGGC)	F209-2200F (5'GAATGTCTCAGTCGTGTATC) F209-3'R (5'TGGTCTCCTTTTGGAGGC)
L1L2F3	F309-3′R (5′TGGTCTCCTTTTGGAGGCC)	F309-400F (5'TACTCGAACAGTTTCCACTG) F309-1400R (5'GTTAATAGTTGGACGACCAG)
F1F2L3	LS3-3′R (5′TGGTCTCCTTATGGAGAAC)	LS3-400F (5'GTTTCTACGGATGCTGAGG) LS3-1400R (5'CTTTCACTACCCACGAAGG)
UTR and ORF exchanges	RT Primer	PCR primers
L3 (5′UTRFNY)	LS3-3′R (5′TGGTCTCCTTATGGAGAAC)	F309-1F (5'GTAATCTTACCACTGTGTGTG) LS3-500R: (5′TGGGGCAATCGTAGGTAGG)
F3 (5′UTRLS)	F309-3′R (5′TGGTCTCCTTTTGGAGGCC)	LS3-1F (5'GTAATCTTACCACTTTCTTTTC) F309-500R (5'CTTGCCAATTACTACACACG)
L3 (mUTRFNY)	LS3-3′R (5′TGGTCTCCTTATGGAGAAC)	LS3-400F (5'GTTTCTACGGATGCTGAGG) LS3-1400R (5'CTTTCACTACCCACGAAGG)
F3 (m UTRLS)	F309-3′R (5′TGGTCTCCTTTTGGAGGCC)	F309-900F (5'CGCAATCGGGAGTTCTTCC) F309-1400R (5'GTTAATAGTTGGACGACCAG)
L3 (3′UTRFNY)	F309-3′R (5′TGGTCTCCTTTTGGAGGCC)	LS3-1600F (5'CGCCATCTCTGCTATGTTTG) F309-3′R (5′ TGGTCTCCTTTTGGAGGCC)
F3 (3′UTRLS)	LS3-3′R (5′TGGTCTCCTTATGGAGAAC)	F309-1600F (5'TTCGAGTTAATCCTTTGCCG) LS3-3′R (5′TGGTCTCCTTATGGAGAAC)
F3 (CPLS)	F309-3′R (5′TGGTCTCCTTTTGGAGGCC)	F309-1F (5'GTAATCTTACCACTGTGTGTG) F309-3′R (5′ TGGTCTCCTTTTGGAGGCC)

L3 (CPFNY)	LS3-3′R (5′TGGTCTCCTTATGGAGAAC)	LS3-1F (5'GTAATCTTACCACTTTCTTTTC) LS3-3′R (5′TGGTCTCCTTATGGAGAAC)
F3 (MPLS)	F309-3′R (5′TGGTCTCCTTTTGGAGGCC)	LS3-400F (5'GTTTCTACGGATGCTGAGG) F309-1400R (5'GTTAATAGTTGGACGACCAG)
L3 (MPFNY)	LS3-3′R (5′TGGTCTCCTTATGGAGAAC)	F309-400F (5'TACTCGAACAGTTTCCACTG) LS3-1400R (5'CTTTCACTACCCACGAAGG)
L3 (MP1.FNY)	LS3-1400R (5'CTTTCACTACCCACGAAGG)	LS3-1F (5'GTAATCTTACCACTTTCTTTTC) F309-500R (5'CTTGCCAATTACTACACACG)
F3 (MP1.LS)	F309-1400R (5'GTTAATAGTTGGACGACCAG)	LS3-400F (5'GTTTCTACGGATGCTGAGG) F309-1400R (5'GTTAATAGTTGGACGACCAG)
Amino acid substitution mutants	RT Primer	PCR primers
	LS3-1400R (5'CTTTCACTACCCACGAAGG)	LS3-1F (5'GTAATCTTACCACTTTCTTTTC) LS3-1400R (5'CTTTCACTACCCACGAAGG)

Construction of chimeric viruses and amino acid substitution mutants

Chimeric viruses between CMV-LS and CMV-FNY were generated exchanging all the untranslated regions (UTR) and the open reading frames (ORF) separately. Briefly, overlapping PCR fragments sharing around 20 nucleotides at the ends were amplified. For each pair of fragments, the overlapping sequence contained part of LS and part of FNY. Gel-purified PCR fragments were submitted to chimaeric PCR performed in two steps. The first 8 cycles are based on the temperature

given by the overlapping sequence, usually higher than the temperature of the remaining cycles, given by the flanking primers. These cycles amplify the chimaeric PCR generated during the first cycles. The chimaeric fragments were exchanged in the viral construct using unique restriction sites. In the cases of ORF exchanges and middle UTR exchanges, three PCR fragments were amplified creating two overlapping regions and then, the chimaeric PCR assembled the three fragments. All PCRs were performed in the presence of 0,2mM dNTPs with Pfu DNA polymerase (Promega Corporation, Madison, WI, USA) and specific primers (Table CI.2).

Table CI.2. Primers used to generate UTR and ORF exchanged regions.

Constructs	Primary PCR primers[a]	Chimeric PCR[b]
L3 (5'UTRFNY)	LS3-500R: (5'TGGGGCAATCGTAGGTAGG) LS-MPF: (5'TTCCCGAGGC**ATGGCTTTCCAAGGTACC**) V3F: (5'TTTTCCGAAGGTAACTGGCT) FNY-5'UTR-R: (5'**GGAAAGCCAT**GCCTCGGGAAATCTAACACAC)	V3F LS3-500R
F3 (5'UTRLS)	F309-500R: (5'CTTGCCAATTACTACACACG) FNY-MPF: (5'**ATTACGAAGGTT**ATGGCTTTCCAAGGTACC) V3F: (5'TTTTCCGAAGGTAACTGGCT) LS 5'UTR-R: (5'TGGAAAGCCAT**AACCTTCGTAATCTAGACAC**)	V3F F309-500R
L3 (mUTRFNY)	LS3-400F: (5'GTTTCTACGGATGCTGAGG) LS 5-R: (5'GCACCAAAGTGCTA**AAGACCGTTAACCACCT**) LS3-F: (5'ACTATATAGAGA**GTGACGCGAAGCCGCCT**) LS3-1400R: (5'CTTTCACTACCCACGAAGG) FNY-M1F: (5'**TTAACGGTCTTTAG**CACTTTGGTGCGTATTAG) FNY-M2R: (5'**GCTTCGCGTCAC**TCTCTATATAGTCAGTAG)	LS3-1400R LS3-400F
F3 (mUTRLS)	F309-400F: (5'TACTCGAACAGTTTCCACTG) FNY 5R: (5'**GTAACAAAACACTA**AAGACCGTTAACCACCT) FNY 3F: (5'**GGTAGACATCT**GTGTTTGTGCTGTGTTTTCTC) F309-1400R: (5'GTTAATAGTTGGACGACCAG) LS M1F: (5'GTTAACGGTCTTTAG**TGTTTTGTTACGTTGTACC**) LS M2R: (5'ACAGCACAAACAC**AGATGTCTACCGTTACA**)	F309-1400R F309-400F
L3 (3'UTRFNY)	LS3-900F:(5'AGCTTCAGATCGCAGGTGG) LS3-CPR: (5'GGAACACGGAACTA**AGTCGGGAGCATCC**) V3R: (5'GTGAAATACCGCACAGATGC) F309-3'utr-F: (5'**TCCCGACTTAG**TTCCGTGTTCCCAGAATC)	V3R LS3-900F

F3 (3'UTRLS)	F309-900F: (5'CGCAATCGGGAGTTCTTCC) F309-CPR: (5'**AAACACACGGA**TCAGACTGGGAGCACTC) V3R: (5'GTGAAATACCGCACAGATGC) LS-3'utr-F: (5'TCCCAGTCTGA**TCCGTGTGTTTACCGGC**)	V3R F309-900F
F3 (CPLS)	F309-400F: (5'TACTCGAACAGTTTCCACTG) F309-ORF.CPR2: (5'**AGATTTGTCCAT**GACTCGACTCAATTCTACGA) LS3-ORF.CPF2: (5'TGAGTCGAGTC**ATGGACAAATCTGGATCTCC**) LS3-ORF.CPR: (5'ACACGGAATCA**AGTCGGGAGCATCCG**) F309-ORF.CPF: (5'**GCTCCCGACT**TGATTCCGTGTTCCCAGAAT) V3R: (5'GTGAAATACCGCACAGATGC)	V3R F309-400F
L3 (CPFNY)	LS3-400F: (5'GTTTCTACGGATGCTGAGG) LS3-5CPR2: (5'GATTTGTCCAT**AGGCACACTAAGACGCGAA**) F309-5CPF2: (5'**CTTAGTGTGCCT**ATGGACAAATCTGAATCAACC) F309-3CPR: (5'**CACACGGACTA**GACTGGGAGCACTCC) LS3-3CPF: (5'GCTCCCAGTC**TAGTCCGTGTGTTTACCGG**) V3R: (5'GTGAAATACCGCACAGATGC)	V3R LS3-400F
F3 (MPLS)	F309-ORF.MPR: (5'**TGGAAAGCCAT**GCCTCGGGAAATCTAA) V3F: (5'TTTTCCGAAGGTAACTGGCT) F309-ORF.MPF: (5'**TTAACGGTCTT**TAGCACTTTGGTGCGTATTA) F309-1400R: (5'GTTAATAGTTGGACGACCAG) LS3-5MPF: (5'TTCCCGAGGC**ATGGCTTTCCAAGGTACCAG**) LS3-3MPR: (5'CACCAAAGTGCTA**AAGACCGTTAACCACCT**)	V3F F309-1400R
L3 (MPFNY)	V3F: (5'TTTTCCGAAGGTAACTGGCT) LS3-ORF.MPR: (5'CTTGGAAAGCCAT**AACCTTCGTAATCTAGAC**) F309-5MPF: (5'**TACGAAGGTT**ATGGCTTTCCAAGGTACCA) F309-3MPR: (5'**CGTAACAAAACACTA**AAGACCGTTAACCACC) LS3-ORF.MPF: (5'AACGGTCTT**TAGTGTTTTGTTACGTTGTAC**) LS3-1400R: (5'CTTTCACTACCCACGAAGG)	V3F LS3-1400R

[a]: Primary PCR primers used to amplify the different overlapping fragments. Nucleotides from CMV-LS are represented in bold and CMV-FNY, underlined. [b]: Chimaeric PCR primers used to amplify the whole chimaeric PCR fragment.

Constructs where the 209 N-terminal amino acids of the MP were exchanged, were generated using restriction sites. In the case of the construct F3(MP1LS), the chimaeric construct L3(5'UTRFNY) was digested with *Bpu1102I* and *XmaI*, and the fragment cloned into the

same restriction sites of the construct F309, that carries the whole RNA3 of FNY, to have only the 209 amino acids of LS in the background of FNY. The complementary construction, L3 (MP1FNY) was done using the same approach on the clone F3(5'UTRLS) and cloned using the same restriction sites on LS3. The amino acid substitution mutants introducing specific residues of MP-FNY into MP-LS were built using GENEART® Site-Directed Mutagenesis System (Invitrogen Life Technologies, Carlsbad, CA, USA), following manufacturer's instructions. 20ng of DNA of plasmid LS3 were hybridized to the mutagenic primer and allowed to polymerize in the presence of 0,2mM dNTPs and Pfu DNA polymerase (Promega Corporation, Madison, WI, USA). All mutagenic primers used are listed in Table CI.3. The substitutions were done independently at five positions. Multiple amino acid combinations were done using the same technique over constructs carrying previously introduced mutations. Sequences to confirm all chimaeric constructs were analyzed using Sequencher™ version 4.8 (Gene Codes Corporation; Ann Arbor, MI).

Table CI.3. Primers used for site-directed mutagenesis.

Primer	Sequence[a]
L3-MP3F	CTCTCGTTCCCCAATTAACCCACGGTCGTATTGCTTCTT TCTTTAAATCTGGG
L3-MP4F	ATGATGCCGGTGAATTGTGCTCTAAAGGCTATAT
L3-MP5F	ACGATTGCCCCATGGAAACAGTTGGCAATCGGCA
L3-MP6F	ATGGTTATATTGGTTACACCGGTACCACTGCTAG
L3-MP7F	ATAATAATTACACACACATCGCTGCTGGTAAGAC

[a]: The mutations introduced into the clone CMV-LS are underlined.

Movement Protein sequences and alignment.

Sequences of the movement protein of all the strains used were obtained performing RNA extraction and RT-PCR of infected material as described previously (Essafi et al., 2009). The Movement Proteins of strains of subgroup II were sequenced with primers specific for LS: LS3-1F: (5'GTAATCTTACCACTTTCTTTTC), LS3-400F: (5'GTTTCTACGGATGCTGAGG) and LS3-1400R: (5'CTTTCACTACCCACGAAGG). Primers used for strains of subgroup I were specific for CMV-FNY: F309-1F: (5'GTAATCTTACCACTGTGTGTG), F309-400F: (5'TACTCGAACAGTTTCCACTG) and F309-1400R: (5'GTTAATAGTTGGACGACCAG). Alignment of movement proteins was done using Protein-Protein BLAST 2.2.25. Sequences of the MP of strains FNY, I17F, Ri-8, Y and LS were identical to the corresponding sequences published in GenBank: FNY: BAA01396.1; I17F: CAA77064.1; Ri-8: CAJ65583.1; Y: AAA46419.1 and LS: AAD45246.1.

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Author contributions

C. Guiu-Aragonés performed the inoculations with CMV-FNY, CMV-LS and all mutants, clonings and site-directed mutagenesis, sequencing and sequence comparisons. A.M.Martín-Hernández did the inoculations of CMV strains, designed the experiments and wrote the manuscript.

References

Albiach-Marti, M., Robertson, C., Gowda, S., Tatineni, S., Belliure, B., Garnsey, S., Folimonova, S., Moreno, P., and Dawson, W. 2010. The pathogenicity determinant of *Citrus tristeza virus* causing the seedling yellows syndrome maps at the 3'-terminal region of the viral genome. Molecular plant pathology 11:55-67.

Aramburu, J., Galipienso, L., and López, C. 2007. Reappearance of *Cucumber mosaic virus* Isolates Belonging to Subgroup IB in Tomato Plants in North-eastern Spain. Journal of Phytopathology 155.

Blackman, L.M., Boevink, P., Santa Cruz, S., Palukaitis, P., and Oparka, K.J. 1998. The movement protein of *Cucumber mosaic virus* traffics into sieve elements in minor veins of Nicotiana clevelandii. The Plant Cell 10:525-537.

Brigneti, G., Voinnet, O., Li, W.X., Ji, L.H., Ding, S.W., and Baulcombe, D.C. 1998. Viral pathogenicity determinants are suppressors of transgene silencing in *Nicotiana benthamiana*. Embo J 17:6739-6746.

Choi, S., Palukaitis, P., Min, B., Lee, M., Choi, J., and Ryu, K. 2005. *Cucumber mosaic virus* 2a polymerase and 3a movement proteins independently affect both virus movement and the timing of symptom development in zucchini squash. The Journal of General virology 86:1213-1222.

Choi, S.H., Hagiwara-Komoda, Y., Nakahara, K.S., Atsumi, G., Shimada, R., Hisa, Y., Naito, S., and Uyeda, I. 2013. Quantitative and qualitative involvement of P3N-PIPO in overcoming recessive resistance against *Clover yellow vein virus* in pea carrying the cyv1 gene. Journal of virology 87:7326-7337.

Decroocq, V., Salvador, B., Sicard, O., Glasa, M., Cosson, P., Svanella-Dumas, L., Revers, F., García, J., and Candresse, T. 2009. The determinant of potyvirus ability to overcome the RTM resistance of *Arabidopsis thaliana* maps to the N-terminal region of the coat protein. Molecular plant-microbe interactions : MPMI 22:1302-1311.

Diaz-Pendon, J., Li, F., Li, W.-X., and Ding, S.-W. 2007. Suppression of antiviral silencing by *Cucumber mosaic virus* 2b protein in Arabidopsis is associated with drastically reduced accumulation of three classes of viral small interfering RNAs. The Plant cell 19:2053-2063.

Díaz, J., Nieto, C., Moriones, E., Truniger, V., and Aranda, M. 2004. Molecular characterization of a *Melon necrotic spot virus* strain that overcomes the resistance in melon and nonhost plants. Molecular plant-microbe interactions : MPMI 17:668-675.

Diaz, J.A., Mallor, C., Soria, C., Camero, R., Garzo, E., Fereres, A., Alvarez, J.M., Gómez-Guillamón, M.L., Luis-Arteaga, M., and Moriones, E. 2003. Potential sources of resistance for melon to nonpersistently aphid-borne viruses. Plant Disease 87:960-964.

Divéki, Z., Salánki, K., and Balázs, E. 2004. The necrotic pathotype of the *Cucumber mosaic virus* (CMV) Ns strain is solely determined by amino acid 461 of the 1a protein. Molecular Plant-Microbe Interactions 17:837-845.

Edwardson, J.R., and Christie, R.G. 1991. Cucumoviruses. in: CRC Handbook of Viruses Infecting Legumes, CRC Press, Boca Raton, FL.

Essafi, A., Diaz-Pendon, J.A., Moriones, E., Monforte, A.J., Garcia-Mas, J., and Martin-Hernandez, A.M. 2009. Dissection of the oligogenic resistance to *Cucumber mosaic virus* in the melon accession PI 161375. Theor Appl Genet 118:275-284.

Fujita, Y., Mise, K., Okuno, T., Ahlquist, P., and Furusawa, I. 1996. A single codon change in a conserved motif of a bromovirus movement protein gene confers compatibility with a new host. Virology 223:283-291.

Gal-On, A., Kaplan, I.B., and Palukaitis, P. 1996. Characterization of *Cucumber mosaic virus*: II. Identification of movement protein sequences that influence its accumulation and systemic infection in tobacco. Virology 226:354-361.

Guiu-Aragonés, C., Monforte, A.J., Saladié, M., Corrêa, R.X., Garcia-Mas, J., and Martín-Hernández, A.M. 2014. The complex resistance to cucumber mosaic cucumovirus (CMV) in the melon accession PI161375 is governed by one gene and at least two quantitative trait loci. Molecular Breeding DOI 10.1007/s11032-014-0038-y:1-12.

Hong, J.S., Ohnishi, S., Masuta, C., Choi, J.K., and Ryu, K.H. 2007. Infection of soybean by *Cucumber mosaic virus* as determined by viral movement protein. Arch Virol 152:321-328.

Hwang, M., Kim, K., Lee, J., and Park, Y. 2007. Identification of amino acid sequences determining interaction between the *Cucumber mosaic virus*-encoded 2a polymerase and 3a movement proteins. The Journal of general virology 88:3445-3451.

Jacquemond, M., and Lauquin, G. 1998. The cDNA of *Cucumber mosaic virus*-associated satellite RNA has in vivo biological properties. Biochem Biophys Res Commun 151.

Kang, W.-H., Seo, J.-K., Chung, B., Kim, K.-H., and Kang, B.-C. 2012. Helicase domain encoded by *Cucumber mosaic virus* RNA1 determines systemic infection of Cmr1 in pepper. PLoS ONE 7.

Kaplan, I., Gal-On, A., and Palukaitis, P. 1997. Characterization of *Cucumber mosaic virus*. III. Localization of sequences in the movement protein controlling systemic infection in cucurbits. Virology 230:343-349.

Karchi, Z., Cohen, S., and Govers, A. 1975. Inheritance of resistance to *Cucumber mosaic virus* in melons. Phytopathology 65:479-481.

Kim, C., and Palukaitis, P. 1997. The plant defense response to *Cucumber mosaic virus* in cowpea is elicited by the viral polymerase gene and affects virus accumulation in single cells. The EMBO journal 16:4060-4068.

Kim, M.J., Huh, S.U., Ham, B.-K., and Paek, K.-H. 2008. A novel methyltransferase methylates *Cucumber mosaic virus* 1a protein and promotes systemic spread. Journal of virology 82:4823-4833.

Kim, S.H., Kalinina, N.O., Andreev, I., Ryabov, E.V., Fitzgerald, A.G., Taliansky, M.E., and Palukaitis, P. 2004. The C-terminal 33 amino acids of the *Cucumber mosaic virus* 3a protein affect virus movement, RNA binding and inhibition of infection and translation. Journal of General Virology 85:221-230.

Lewsey, M., Robertson, F.C., Canto, T., Palukaitis, P., and Carr, J.P. 2007. Selective targeting of miRNA-regulated plant development by a viral counter-silencing protein. Plant J 50:240-252.

Li, Q., Ryu, K., and Palukaitis, P. 2001. *Cucumber mosaic virus*-plant interactions: identification of 3a protein sequences affecting infectivity, cell-to-cell movement, and long-distance movement. Mol plant-microbe interactions.

Mansilla, C., Sánchez, F., Padgett, H., Pogue, G., and Ponz, F. 2009. Chimeras between oilseed rape mosaic virus and tobacco mosaic virus highlight the relevant role of the tobamoviral RdRp as pathogenicity determinant in several hosts. Molecular plant pathology 10:59-68.

Mochizuki, T., and Ohki, S. 2011. *Cucumber mosaic virus*: viral genes as virulence determinants. Molecular plant pathology 13:217-225.

Nagano, H., Mise, K., Furusawa, I., and Okuno, T. 2001. Conversion in the requirement of coat protein in cell-to-cell movement mediated by the *Cucumber mosaic virus* movement protein. Journal of virology 75:8045-8053.

Namba, S., Ling, K.S., Gonsalves, C., Gonsalves, D., and Slightom, J.L. 1991. Expression of the gene encoding the coat protein of *Cucumber mosaic virus* (CMV) strain WL appears to provide protection to tobacco plants against infection by several different CMV strains. Gene 107:181-188.

Palukaitis, P., and Garcia-Arenal, F. 2003. Cucumoviruses. Adv Virus Res 62:241-323.

Rizzo, T.M., and Palukaitis, P. 1990. Construction of full-length cDNA clones of *Cucumber mosaic virus* RNAs 1, 2 and 3: generation of infectious RNA transcripts. Mol Gen Genet 222:249-256.

Roossinck, M.J. 2001. *Cucumber mosaic virus*, a model for RNA virus evolution. Molecular plant pathology 2:59-63.

Roossinck, M.J., Zhang, L., and Hellwald, K.-H. 1999. Rearrangements in the 5' nontranslated region and phylogenetic analyses of *Cucumber mosaic virus* RNA3 indicate radial evolution of three subgroups. Journal of Virology 73:6752-6758.

Ryabov, E.V., Roberts, I.M., Palukaitis, P., and Taliansky, M. 1999. Host-specific cell-to-cell and long-distance movements of *Cucumber mosaic virus* are facilitated by the movement protein of groundnut rosette virus. Virology 260:98-108.

Ryu, K.H., Kim, C.-H., and Palukaitis, P. 1998. The coat protein of *Cucumber mosaic virus* is a host range determinant for infection of maize. Molecular Plant-Microbe Interactions 11:351-357.

Salánki, K., Kiss, L., Gellért, A., and Balázs, E. 2011. Identification a coat protein region of *Cucumber mosaic virus* (CMV) essential for long-distance movement in cucumber. Archives of virology 156:2279-2283.

Sasaki, N., Park, J.-W., Maule, A., and Nelson, R. 2006. The cysteine-histidine-rich region of the movement protein of *Cucumber mosaic virus* contributes to plasmodesmal targeting, zinc binding and pathogenesis. Virology 349:396-408.

Takahashi, H., Suzuki, M., Natsuaki, K., Shigyo, T., Hino, K., Teraoka, T., Hosokawa, D., and Ehara, Y. 2001. Mapping the virus and host genes involved in the resistance response in *Cucumber mosaic virus*-Infected Arabidopsis thaliana. Plant Cell Physiol 42:340-347.

Takeshita, M., Suzuki, M., and Takahami, Y. 2001. Combination of amino acids in the 3a protein and the coat protein of *Cucumber mosaic virus* determines symptom expression and viral spread in bottle gourd. Archives of virology 146:697-711.

Taliansky, M., and García-Arenal, F. 1995. Role of cucumovirus capsid protein in long-distance movement within the infected plant. Journal of virology 69:916-922.

Thompson, J.R., Doun, S., and Perry, K.L. 2006. Compensatory capsid protein mutations in *Cucumber mosaic virus* confer systemic infectivity in squash (Cucurbita pepo). J Virol 80:7740-7743.

Vaquero, C., Liao, Y., and Fischer, R. 1997. Mapping of the RNA-binding domain of the *Cucumber mosaic virus* movement protein. Journal of General Virology 78:2095-2099.

Wong, S.-M., Thio, S.S.-C., Shintaku, M.H., and Palukaitis, P. 1999. The rate of cell-to-cell movement in squash of *Cucumber mosaic virus* is affected by sequences of the capsid protein. Molecular Plant-Microbe Interactions 12:628-632.

Zhang, L., Handa, K., and Palukaitis, P. 1994. Mapping local and systemic symptom determinants of cucumber mosaic cucumovirus in tobacco. The Journal of General Virology 75:3185-3191.

Chapter II

Characterization of the resistance mediated by *cmv1*

Introduction

Plants that are susceptible to viruses must provide a permissive environment for the viral infection cycle. To complete their life cycle, viruses undergo a multistep process that implicate the entrance into the plant cells, uncoating the nucleic acid, translation of the viral proteins, replication of viral nucleic acid, assembly of the virions, cell-to-cell movement through plasmodesmata, systemic movement to colonize the whole plant and interaction with vectors to spread the virus in other plants (Carrington *et al.*, 1996; Hipper *et al.*, 2013). Successful infection of a plant by a virus therefore depends on the presence of host factors necessary to complete different steps of the viral life cycle. The absence of a necessary host factor (or mutation to impede its function) has been postulated for recessively inherited disease resistance in plants, and it is termed passive resistance (Fraser, 1990). About half of the 200 known virus resistance genes in plants are recessively inherited, suggesting that this form of resistance is the most common for viruses (Kang *et al.*, 2005b).

Resistance at the single cell level is characterized by a state where virus replication either does not occur, or occurs at essentially undetectable levels in inoculated cells. When virus particles enter a susceptible plant cell, the viral genome is liberated from the capsid, and the genome becomes available for translation, in which the virus requires the involvement of many host proteins, mainly eukaryotic translation initiation factors (eIFs). Most of the recessive resistance genes cloned from model and crop species encode for eIF4E and eIF(iso)4E, affecting at the single cell level or cell-to-cell movement (Truniger and Aranda, 2009; Diaz-Pendon *et al.*, 2004; Robaglia and Caranta, 2006). *Pvr2* encoding for eIF4E confers resistance to *Potato virus Y* (PVY) and *Tobacco etch virus* (TEV) in pepper (Ruffel *et al.*, 2002; Deom *et al.*, 1997), *pvr2* and *pvr6* coding for eIF4E and its isoform

respectively, confer resistance to *Pepper veinal mottle virus* (PVMV) (Ruffel *et al.*, 2006), *pvr1*, encoding for eIF4E confers resistance in chilli pepper to three viruses: *Pepper mottle virus* (PepMoV), PVY and TEV (Murphy *et al.*, 1998; Kang *et al.*, 2005a; Murphy and Kyle, 1995; Kyle and Palloix, 1997), *nsv*, encoding for eIF4E provides resistance in melon to *Melon necrotic spot virus* (Nieto *et al.*, 2006; Nieto *et al.*, 2007; Truniger *et al.*, 2008), and *pot-1* , encoding for eIF4E to PVY and TEV in *Solanum hirsutum* (Parella *et al.*, 2002; Ruffel *et al.*, 2002). All of them inhibit the accumulation of the virus via impairing the translation of the genomic or replicated viral RNA. Other resistance genes like *mo1* conferring resistance to *Lettuce mosaic virus* (LMV) in lettuce (Nicaise *et al.*, 2003) or *sbm1* to *Pea seed borne mosaic virus* (PSbMV)(Keller *et al.*, 1998; Gao *et al.*, 2004) and *Bean yellow mosaic virus* (BYMV)(Bruun-Rasmussen *et al.*, 2007) in pea are also eIF4E but they reduce the accumulation and the cell-to-cell movement instead of inhibiting the replication. It is difficult to speculate on a plausible role for eIF4E in virus movement, because processes that account for cell-to-cell movement are not well understood (Kang *et al.*, 2005b). Even though the majority of the genes are coding for eIF4E, eIF4G and its isoform are also involved in resistance: *rymv-1* encoding for eIF(iso)4G confers resistance to *Rice yellow mottle virus* (RYMV)(Albar *et al.*, 2006), *cum2* (an eIF4G) provides resistance to *Turnip crinkle virus* (TCV) in Arabidopsis (Yoshii *et al.*, 2004), or *tsv1*, encoding for eIF4G, confers resistance to *Rice tungro spherical virus* (RTSV) in rice (Lee *et al.*, 2010).

In the absence of any resistance preventing the multiplication, plant viruses move from the site of replication into surrounding cells to mount a successful infection. Resistance at this level can result in the failure of interactions between plant and viral factors necessary for cell-to-cell movement. Host factors may be involved in intracellular transport to plasmodesmata (PD) or may be proteins specifically

associated with PD that can block viral entrance into the next cell if they are non functional. The cytoskeleton and associated motor proteins have a crucial role in virus movement, and viruses may also use the host endomembrane system for intracellular movement (Lazarowitz and Beachy, 1999b; Harries and Ding, 2011; Harries *et al.*, 2010).

There are at least two cases in which genes involved in translation are not candidate for recessive resistances. A vacuolar protein sorting 4 (VPS4) gene, has recently been proposed as candidate for the *zym* gene, that confers resistance to *Zucchini yellow mosaic virus* (ZYMV) in cucumber (Amano *et al.*, 2013) but its function has not been characterized. For *cmv1,* that confers the resistance characterized in the present study to some strains of *Cucumber mosaic virus* (CMV) in melon (Essafi *et al.*, 2009), there are three putative candidate genes in the current mapping interval, none of which is a eIF. In fact, the most plausible candidate gene is also a VPS (A. Giner, personal communication). The fact that the candidate genes are not eukaryotic translation initiation factors hint that different mechanisms of resistance, such as impairing of movement, exist and reveals that much remains to be learned about the nature of recessive resistance.

In case that the susceptible host does not show restrictions to cell-to-cell movement, plant viruses move from the mesophyll, to bundle sheath (BS) cells, vascular parenchyma (VP), companion cells (CC) and from here, into phloem sieve elements (SE), where they are translocated and then unloaded in new leaves (Carrington *et al.*, 1996; Santa Cruz, 1999). This pathway up to the SE occurs though plasmodesmata, which establish a symplastic network for the virus movement. Due to the lack of transcription/translation capacity, the SEs are dependent of the neighbouring CCs for their maintenance and

function, and their obligatory association is considered as the CC-SE complex (Lough and Lucas, 2006). The entry of the virus into the SE-CC complex is currently the most significant barrier to long-distance movement. The interface between the BS and CC-SE complex was found to be a boundary for the systemic movement of *Cowpea chlorotic mottle virus* infecting soybean plants (Goodrick *et al.*, 1991), for a TMV mutant infecting tobacco plants (Ding *et al.*, 1996b), and for systemic movement of CMV in transgenic tobacco plants expressing the replicase gene (Wintermantel *et al.*, 1997). Soybean and tobacco, as apoplastic-loading plants, have CC with few PD in the BS interface. This can be a boundary in some cases, but generally viruses are able to pass through these PDs to complete the systemic infection. On the contrary, cucurbits, that are symplastic loaders, present specialized CC, named intermediary cells (IC) that have numerous plasmodesmata with BS, VP and SE interfaces. Interestingly, even though in cucurbits there is a high amount of PD connecting the minor vein cells, BS/CC was found to be a barrier for viral movement of *Tomato aspermy virus* in cucumber (Thompson and García-Arenal, 1998) and for *Cucumber mosaic virus* (CMV) in *Cucumis figarei* (Kobori *et al.*, 2000). In both cases, the virus was accumulating in the BS and was not detected in the CC.

Few natural genes for resistance to CMV are known. In many cases the resistances are multigenic and recessive (Caranta *et al.*, 2002; Ben-Chaim *et al.*, 2001; Pitrat, 2002; Guiu-Aragonés *et al.*, 2014; Ohnishi *et al.*, 2011). In other cases, such as studies in potato (Celebi-Toprak *et al.*, 2003) or in bottle gourd (Takeshita *et al.*, 2001), the phenotype is well characterised but little is known about the genetics of resistance. Although many QTLs conferring resistance to CMV have been described (Jacquemond, 2012; Palukaitis and Garcia-Arenal, 2003; Guiu-Aragonés *et al.*, 2014; Pitrat, 2002), there are some single genes that have been characterized. Regarding the dominant genes, *Cry* in

cowpea (Nasu *et al*., 1996), *RT4-4* in bean (Seo *et al*., 2006) and *RCY1* in *A.thaliana* (Sekine *et al*., 2006; Takahashi *et al*., 2001) are NBS-LRR proteins and act producing a hypersensitive response; *Cmr1* in pepper (Kang *et al*., 2010), whose identity is still unknown, permits the replication and cell-to-cell movement of the virus but impedes the systemic infection. The described recessive resistance genes to CMV are all related to inhibition of virus movement, both cell-to-cell or systemic movement. Additionally, some resistance genes confer resistance only to some strains of CMV: two resistance genes have been described in cucumber (Boulton *et al*., 1985), two in pumpkin (Pink, 1987), one gene in *Lactuca saligna*, accession PI 26153 (Edwards *et al*., 1983). In Arabidopsis, *ssi2* involved in lipid synthesis produces constitutive accumulation of the pathogenesis-related-1 (PR-1) gene transcript and salicylic acid (SA) and restricts CMV to the inoculated leaf (Sekine *et al*., 2004). Mutations of *cum1*and *cum2* inactivate eIF4E and eIF4G respectively, affecting the efficiency of translation of protein 3a in a manner dependent on the 5'noncoding sequence, altering CMV movement (Yoshii *et al*., 2004).

In cucurbits CMV resistance has been described as partial, polygenic and recessive (Risser *et al*., 1977). In melon, there are identified several QTLs conferring resistance to some strains of CMV (Pitrat, 2002; Guiu-Aragonés *et al*., 2014), one dominant gene Creb-2 (Daryono *et al*., 2010) and one recessive gene, *cmv1,* that confers resistance to strains from subgroup II of the virus (Essafi *et al*., 2009) (Guiu-Aragonés et al. submitted).

In this chapter, we have used a near isogenic line (NIL), SC12-1-99 to characterize the resistance mediated by *cmv1*. We have followed the progress of CMV infection leading the limitation of systemic infection of subgroup II strains of CMV.

Material and methods

Plants, virus, bacteria and inoculations

The genotypes of *C.melo* used for the study were: the Korean accession PI 161375 cultivar "Songwhan Charmi" (SC) and the Spanish type PS (Piel de Sapo) as resistant and susceptible controls, respectively. The NIL SC12-1-99 was derived from SC12-1 (Essafi *et al.*, 2009) and has an introgression of SC on the linkage group XII that contains the *cmv1* gene.
Seeds were pre-germinated by soaking them in water overnight and then maintained for 2-4 days in light at 28°C. Seedlings were grown in growth chambers SANYO MLR-350H in long-day conditions consisting of 22°C for 16 h with 5000 lux of light and 18°C for 8 h in the dark for all infections.

Virus strains used in this study were CMV-LS, belonging to subgroup II, and CMV-FNY, belonging to subgroup I, both provided by Prof. P. Palukaitis as infectious clones (Rizzo and Palukaitis, 1990; Zhang *et al.*, 1994).

Viral inocula were freshly prepared from infected *Nicothiana benthamiana* or zucchini (*Cucurbita pepo* L.) and rub-inoculated onto either the cotyledons of 7- to 10-day-old melon plants, or onto the first true leaf.

The bacteria used were *E.coli* strain DH5α (F-, endA1, glnV44, thi-1, recA1, relA1, gyrA96, deoR, nupG, Φ80dlacZΔM15 Δ(lacZYA-argF)U169, hsdR17(r_{K^-} m_{K^+}), λ–), *E.coli* strain BL21(DE3) pLysS (F-, ompT, gal, dcm, lon, hsdS_B(r_{B^-} m_{B^-}) λ(DE3) pLysS(cm^R)) and *A. tumefaciens* GV3101 and C58C1 both Tet^R and Rif^R.

Plasmid constructs

For transient expression in plant tissues, the complete MP of both strains was amplified with primers that generate *BamHI* (5'ACTGGATCCATGGCTTTCCAAG) and *XhoI* (5'TCTCGAGTGAAGACCGTTAAC) sites (underlined) at 5' and 3' end of gene respectively. Stop codon of the MP is missing from reverse primer because the fusion of fluorescent protein is at C-terminal. PCR products were cloned into GATEWAY® pENTR™ 3C (InVitrogen Corporations, Carlsbad, CA, USA) at *BamHI-XhoI* sites. To express C-terminally tagged fluorescent protein fusions of MP:RFP and MP:GFP, both pENTR™3C MP-LS and pENTR™3C MP-FNY were recombined with destination vectors pB7WGF2 and pH7WGR2 (Karimi *et al.*, 2002) for C-terminal fusions to enhanced green fluorescent protein (GFP) or mCherry red fluorescent protein (RFP) respectively, using LR clonase mix (Invitrogen Corporations, Carlsbad, CA, USA) according to the instructions of the manufacturer. Plasmids were transferred to *A. tumefaciens* GV3101 by electroporation. For all constructs the correct cloning and insert orientations were confirmed by sequencing.

For purification of the MP protein, MP genes of CMV-LS and CMV-FNY were cloned. CMV MP genes were amplified from the full-length RNA3 cDNA clones of CMV-LS and CMV-FNY by PCR using two primers that generated two restriction sites: *NdeI* (5'GAGCATATGGCTTTCCAAGGTACCAG) and *XhoI* (5'GAACTCGAGAAGACCGTTAACCAC) sites (underlined) at the 5' and 3' end of the gene respectively. PCR product was then cloned into the expression vector pET23 (Novagen) at the *NdeI-XhoI* site, obtaining the expression plasmids pET23-MPFNY and pET23-MPLS. These constructs were transformed into *E.coli* strain BL21(DE3) pLysS cells.

Virus detection

Detection of virus in cotyledons, tissue printing and dot blot were done by molecular hybridization.

Accumulation in cotyledons

Fully expanded cotyledons of 7 days old melon plants were rub-inoculated and were sequentially collected at 2 dpi and 5 dpi from the same plant. Total RNA extractions were performed using TriReagent (SIGMA-ALDRICH, St Louis, MO, USA) following manufacturer's protocol.

Northern analysis

Northern blot analysis was performed following García-Cano *et al.* (2006). Briefly, 6 μg of total RNA obtained from inoculated cotyledons were denatured, electrophoresed in 1% agarose gel and vacuum-transferred (VacuGene XL, HealthCare) onto a positively charged nylon membrane (Roche Diagnostics, Barcelona, Spain). Samples were crosslinked under UV light (Crosslinker RPN 2500, Amersham Life Science).

For hybridizations, a digoxygenin-11-UTP-labelled RNA probe was synthesized from the construct (p73, kindly provided by J.A. Díaz Pendón) containing partial sequences of CMV-LS and CMV-FNY coat protein (CP) genes using a digoxigenin-labelling and detection kit (Roche Diagnostics, Barcelona, Spain). For transcription, the plasmid was linearized with ApaI, and cRNA was synthesized using SP6 RNA polymerase (Amersham Pharmacia Biotech, little Chalfont, U.K). Hybridization was performed at 65ºC. Washing steps and detection with the alkaline phosphatase chemiluminiscent substrate CDP-Star

(Roche Diagnostics, Barcelona, Spain) was performed as recommended by the manufacturer.

Tissue printing and dot blot

Distribution of CMV in the leaves was studied by tissue printing hybridization of the inoculated leaf. The first true leave was rub-inoculated and 3 dpi foliar discs were printed onto a positively charged nylon membrane (Roche Diagnostics, Barcelona, Spain) according to (Más and Pallás, 1995). Leaves were printed on the membrane by using a roller and applying a uniform pressure. To avoid an excess of sap during pressing, leaf discs were frozen at -80ºC for one minute.

Dot blot was performed using the same plants. Freshly made cross-sections of the petiole of inoculated leaf, sections of the stem and from other petioles were squashed on nylon positively charged membrane at 12 dpi. As positive control, we used plants inoculated with CMV-FNY, which produce systemic infection.

To detect the distribution of CMV, the membranes were processed as described previously for the Northern hybridization.

Microscopy

In situ hybridization

In situ hybridization (ISH) was performed as previously described (Javelle *et al.*, 2011; Gosalvez-Bernal *et al.*, 2008). Samples from inoculated leaves were collected at 6 dpi, fixed by vacuum infiltration in a FAA solution (4% formaldehyde, 5% acetic acid and 50% ethanol, in water) and stored overnight at 4ºC. The fixed tissues were then dehydrated in a series of alcohol baths, embedded in paraplast,

sectioned in 8 μm thickness slices and mounted on poly-L-lysine-coated slides. Sections were rehydrated and incubated 15 min at 37ºC with 1 μg/ml proteinase K. Hybridisations with the riboprobe p73, (see above) were performed overnight at 42ºC with 200 ng/ml of the riboprobe diluted in hybridisation buffer (10% dextran sulfate, 50% formamide, 4xSSC, 1% Denhardt, 100 μg/ml tRNA). After a 0.2xSSC wash at 42ºC, slides were submitted during 30 min to RNAse A treatment (10 μg/ml at 37ºC). Then, the sections were washed at 42ºC in 0.2xSSC and incubated 30 min in blocking solution (100 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% BSA, 0.1% Triton). Sections were incubated with anti-digoxigenin sheep antibody coupled to alkaline phosphatase (Roche Molecular Biochemicals), diluted 1:1000 in blocking solution. After washing to remove the excess of antibody, sections were rinsed in staining buffer (100 mM Tris-HCl pH 9.5, 150 mM NaCl, 2.5 mM $MgCl_2$) and incubated for 30 minutes in the staining buffer supplemented with nitroblue tetrazolium and 5-bromo-4-cloro-3-indolyl phosphate (NBT/BCIP) (Roche Molecular Biochemicals) The hybridization signal is seen as blue areas in the sections. Negative controls on sections of non-inoculated plants were carried for each experiment.

Sections were examined using a DMRB LEITZ microscope (Leica Microsystems, Wetzlar, Germany) and photographed with a Leica DFC420C digital colour camera.

Fluorescent microscopy

A. tumefaciens cultures carrying CMV MPs fused to GFP or RFP were harvested by centrifugation, resuspended in water to a final OD_{600} of 0.2 and infiltrated into the abaxial side of the leaf of *N.benthamiana* and melon tissues using a syringe without needle. Expression of GFP or RFP was observed at 48-72 h after agroinfiltration.

Confocal Laser Scanning Microscopy (CLSM) was performed using Zeiss LSM510 microscope with a C-Apo-chromat (63×/1.2-W Korr) water objective lens in multitrack mode. Leaf samples were mounted on a microscope slide and vacuum infiltrated with water to avoid tissue desiccation. Excitation/emission wavelengths were 488 nm/505 to 545 nm for GFP and 543 nm/585 to 615 nm for RFP. Images were acquired using LSM510 version 2.8 software (Zeiss) and processed with Image J software.

Transmission electron microscopy (TEM)

Leaf-tissue samples (8 x 3 mm^2) from Piel de Sapo and SC12-1-99 were taken from the inoculated leaf at 7 dpi and from a non inoculated leaf as negative control. Samples were vacuum-infiltrated for 2 min in a freshly made mixture of 1% (v/v) glutaraldehyde and 3% (v/v) paraformaldehyde (for LR-White embedding) or 3% (v/v) glutaraldehyde (for Embed 812 embedding) in 0,1 M (pH 7.2) sodium phosphate buffer. Samples were treated for 12 h at 4°C. Tissue was stained for 2 h in 0,1% (v/v) osmium tetroxide and 24 hours in 2% (w/v) uranyl acetate in 150 mM sodium phosphate buffer (pH 7.2). Samples were then dehydrated through an ethanol series and infiltrated with London Resin White (Sigma-Aldrich, St Louis, MO, USA) or Embed812 resin (Electron Microscopy Sciences, Hatfield, PA, USA). Polymerization was performed at 60ºC for 72 h.

For morphological analysis by light microscopy, semi thin sections were mounted on slides and stained with toluidine blue. For the ultra-structural study, ultrathin sections of samples embedded with Embed 812 were collected on formvar-coated EM grids and stained with uranyl acetate and lead citrate.

Samples embedded with LR-White were used for

immunocytochemistry. Ultrathin sections were collected on nickel grids formvar-coated. Samples were incubated with anti-CP (Loewe ® Biochemica GmbH, Sauerlach, Germany) diluted 1:5. After that, sections were incubated with commercial secondary antibody antirabbit IgG conjugated with 10 nm colloidal gold (Electron Microscopy Sciences, Hatfield, PA, USA) for one hour using a dilution of 1:100. Finally, after washes with PBS, samples were stained for 15 minutes with uranyl acetate.

Controls were made on infected samples incubated without the primary antibody and on sections of non inoculated plants. Ultrastructural samples observed with a Zeiss EM10 transmission electron microscope and other samples were visualised in a Philips Tecnai 12 and also in the Zeiss EM10 transmission electron microscope.

Quantification and statistical analysis of gold particles

Gold labelling was quantified in the entire cell of vascular parenchyma and intermediary cells of the susceptible (PS) and resistant (SC12-1-99) plants. Quantification in the same cell types of non inoculated plants was also performed to determine nonspecific labelling.

Morphometric measurements to determine the surface of the cells (μm^2) were performed using the free software Image J. The data were analysed using T-student test with $P \leq 0.05$. Results are represented in box plot.

Graftings

Piel de Sapo (PS) plants were rub-inoculated to produce the stocks needed for graftings. Ten days after inoculation, infected plants were used as virus sources by cutting them either below or above the cotyledons depending on the thickness of the stem.

The scions were either 3 weeks old subNIL SC12-1-99 (*cmv1*) plants or PS as susceptible control. Apical parts were collected, given a V shape at the cut end, and grafted onto the stock. The grafted region was wrapped with Parafilm M (American National Can, Chicago, IL) and a high humidity (60 to 80%) was maintained during the following 2 days to avoid dehydration of the scion. The survivors were grown at usual conditions.

Expression and purification of MP

Antibody against MP of CMV was produced by immunization of rabbits with the protein expressed in *E. coli* BL21 (DE3) pLysS. Expression of the CMV movement protein was induced for 4 hours at 37ºC by the addition of isopropylthio-b-galactoside (IPTG)(0,4 mM) prior to bacterial cell harvest. Inclusion bodies containing CMV MP were isolated from lysed cells by sonication and lysozyme incubation; following steps to purify CMV MP were performed as described in Citovsky *et al.* (1990). Protein amounts were determined by Bradford assays or by SDS-PAGE using 10% polyacrylamide gels followed by Coomassie blue staining. An equal mix of both purified movement proteins was sent to Custom Antibody Service, a scientific platform of CIBER-BBN (Biomedical Research Networking center in Bioengineering, Biomaterials and Nanomedicine, Barcelona) to generate the polyclonal antibody.

Results

CMV-LS accumulates in cotyledons of the resistant line

The NIL SC12-1-99, carrying the *cmv1* gene, is resistant to CMV strains of subgroup II, but susceptible to strains of subgroup I (Guiu-Aragonés et al. submitted). To determine if *cmv1*-mediated resistance was acting at the replication level, we used CMV-LS and CMV-FNY as type members of each subgroup to study the viral accumulation in inoculated cotyledons.

Cotyledons of SC12-1-99 and PS (susceptible parental) were rub-inoculated with sap of CMV-LS or CMV-FNY-infected squash plants. Two cotyledons from different plants of each line were collected 2 days post inoculation (dpi). The other cotyledon from the same plants was sampled 5 dpi. Then RNA was extracted and viral RNA was detected by northern blot. The results shown in figure CII.1 demonstrated that, independently of the CMV strain or the melon line, there was more viral accumulation at 5 days post inoculation (dpi) than at 2 dpi, indicating that both strains, CMV-LS and CMV-FNY, were able to replicate in both melon lines, SC12-1-99 and PS. In mock-inoculated, negative controls plants, no viral RNA accumulation was detected, as expected. The results were reproduced in two independent experiments. Although the northern blot hybridization data showed comparable levels of viral accumulation in the melon cotyledons of both resistant and susceptible lines, it is interesting to note that the rate of accumulation of both CMV-LS and CMV-FNY was always higher in PS than in SC12-1-99.

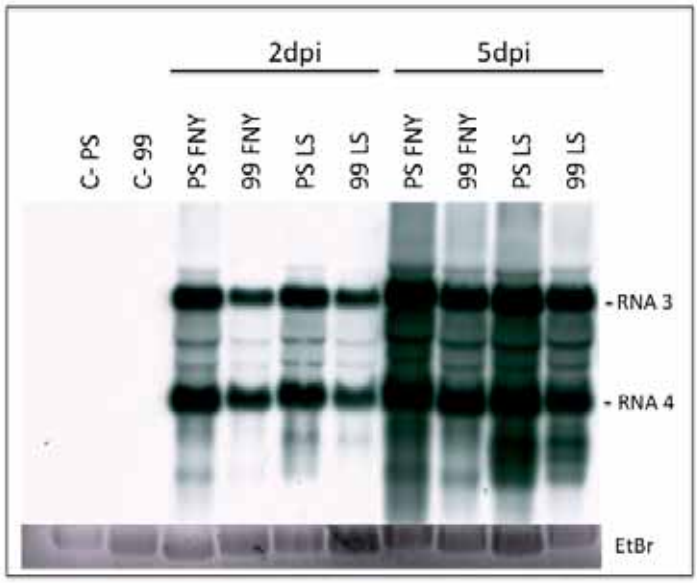


Figure CII.1.Viral accumulation of CMV-LS or CMV-FNY in inoculated cotyledons of PS or SC12-1-99 at 2 and 5 days post inoculation (dpi). Viral RNA was detected by northern blot hybridization with a probe from the CP gene. Loading control was detected with ethidium bromide staining. C-: negative control; PS: Piel de Sapo, line susceptible to CMV-LS; 99: SC12-1-99, line resistant to CMV-LS.

Localization of CMV in inoculated leaves

Once established that CMV-LS was able to replicate in the resistant plant, it was still possible that the resistance was due to inability of the virus to move cell-to-cell. To investigate this possibility, the first melon leaves of resistant and susceptible plants were inoculated with sap of either CMV-LS or FNY and 3 dpi were inspected for local spread of the virus by tissue printing of leaf discs. As shown in figure CII.2, whereas no signal was detected in the mock-inoculated controls, there was no difference in the detection of CMV-LS in PS and in SC12-1-99. In both cases the virus had moved all over the leaf blade and localized mainly in the veins of the inoculated leaf. CMV-FNY showed the same

pattern of accumulation in the veins. Therefore, both viruses spread through the inoculated leaf and reached the veins although only CMV-FNY produced a systemic infection in SC12-1-99 and PS, whereas CMV-LS only infected systemically PS plants. This indicates that the resistance mediated by *cmv1* is not acting at the level of cell-to-cell movement. Interestingly, both viruses could also be localized in the veins of the resistant parental SC, which carries at least three genes involved in the resistance to CMV (Guiu-Aragonés et al, 2014). This indicates that these new genes present in SC are not preventing neither replication nor cell-to-cell movement of the virus and therefore, they must be acting at another level to provide resistance against CMV.

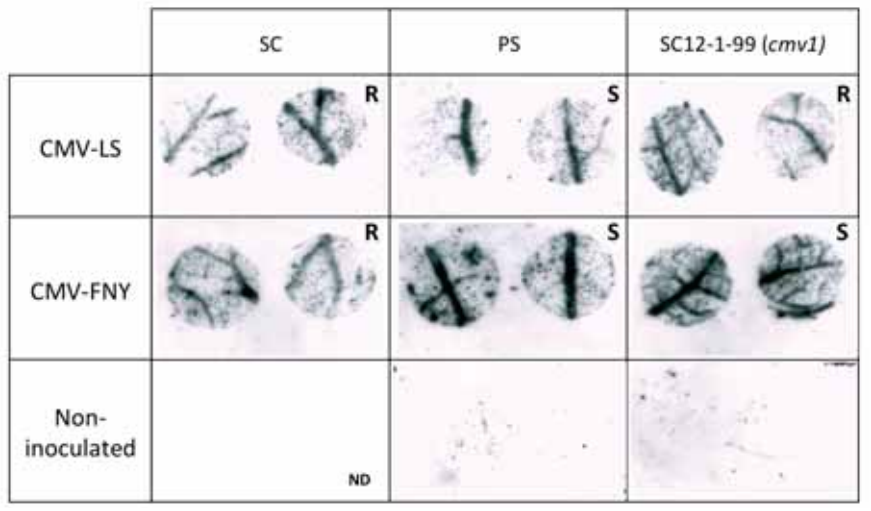


Figure CII.2. Tissue printing analysis of the localization of CMV in leaf discs of inoculated leaf. First true leaves were inoculated with CMV-LS or CMV-FNY in Piel de Sapo (PS), PI 161375 (SC) and SC12-1-99. Samples were taken at 3 dpi. ND: No-data. R: resistant to systemic infection. S: susceptible to systemic infection.

CMV-LS is absent from the phloem of the resistant line

Once we determined that CMV-LS is able to replicate and move cell to cell in the resistant line we performed two experiments to test whether the virus could enter or not the phloem. One consisted of detecting the virus in the phloem by tissue printing hybridization, and the other of *in situ* hybridization to observe microscopically the virus in the phloem cells. For the first experiment, we used the same plants that were processed in the tissue printing of the inoculated leaf in the previous section that were allowed to grow and develop a systemic infection if it was present. In this case, at 12 dpi, plant stems and petioles were cut and the cross-sections were blotted onto the membrane to detect the virus. The results showed in figure CII.3 show the distribution of CMV in the different sections of the plant. In PS, CMV-LS developed a systemic infection, with the virus detected in all the sections, whereas in the resistant line SC12-1-99, CMV-LS could not be detected in any of the sections. This indicated that the virus was not able to exit from the inoculated leaf and suggests that CMV-LS might not enter the phloem, since we didn't detect virus even in the petiole of the inoculated leaf. CMV-FNY, which infects systemically both melon lines, was detected in all the petiole and stem sections.

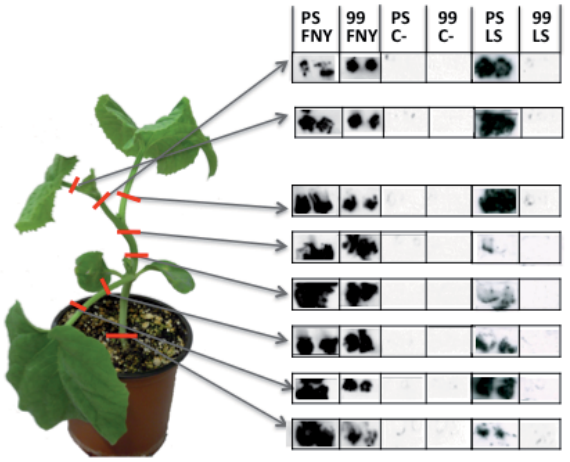


Figure CII.3. Tissue printing analysis of the localization of CMV in the phloem of melon plants. Representation of an inoculated plant with CMV-FNY or CMV-LS and detection of the virus in Piel de Sapo (PS) and SC12-1-99 (99) in the petiole of the inoculated leaf, in main stem and upper petioles of the plant 12 days post inoculation.

On the second experiment, we performed *in situ* hybridization of the inoculated leaf to microscopically determine whether the virus is present in the phloem cells. First true leaves were rub-inoculated with sap of CMV-LS and at 6 dpi leaves from both susceptible and resistant plants showed some foci, more chlorotic than background (see Figure CII.4), which were collected to fix the tissue for ulterior *in situ* hybridization. Results of the hybridized sections of both lines showed a specific signal of viral RNA (blue signal) only located in the foci area. As shown in figure CII.5b and c, viral RNA was detected in epidermal, palisade and mesophyll cells in both lines and regularly distributed along the tissues. Focusing in detail on the vascular system of the minor veins, the virus was detected in the phloem tissues of PS (Figure CII.5d) whereas no viral RNA was detected in the phloem tissues of SC12-1-99 (Figure CII.5e), which indicates that the virus is not capable of invading the phloem. The movement of CMV-LS is interrupted in

certain cell types surrounding the phloem of the resistant line. However, light microscopy of longitudinal and transversal leaf sections didn't allow us to identify in detail the anatomy of the vascular system and the distribution of CMV-LS in the vascular tissues of PS and SC12-1-99 lines (Figure CII.5f, g and h). To obtain a better resolution to differentiate and identify all the cell types, samples were studied under the transmission electron microscope.

Figure CII.4. Foci present in the rub-inoculated melon leaf.

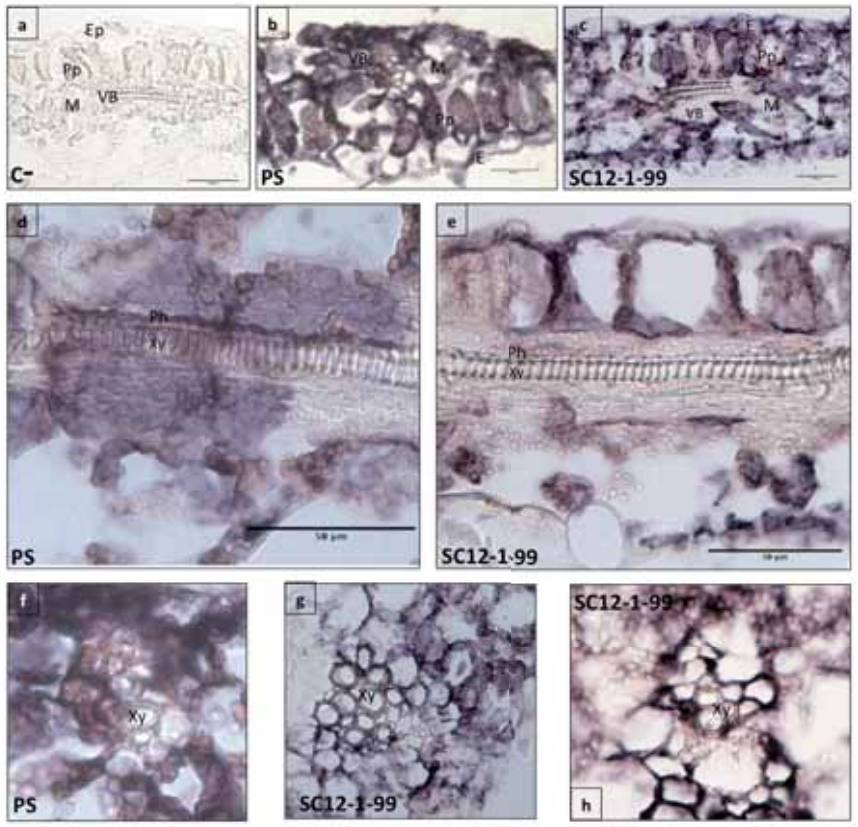


Figure CII.5. Microscopic imaging of the cellular localization of CMV-LS by *in situ* hybridization in inoculated leaves of melon plants. The images show longitudinal (a, c, d, e) and transversal (b, f, g, h) sections of symptomatic areas in the leaves, whereby the blue colour indicates the presence of viral RNA as detected with an RNA probe specific for CMV. Samples were taken at 6 dpi. C-: negative control, PS: susceptible line, SC12-1-99: resistant line. Ep: epidermal cells; Pp: palisade parenchyma; M: mesophyll cells; VB: vascular bundle; Ph: phloem; Xy: xylem.

Anatomy of melon minor veins

Minor veins (type V-VI, figure CII.6a) of PS and SC12-1-99 leaves were studied by transmission electron microscopy (TEM) to identify the different cell types of the vascular bundle (Figures CII.6b-i). First leaves of both cell lines were inoculated with CMV-LS. Foci present in

inoculated leaves were sampled and included as detailed in Material and Methods for ultra-structural study. The first observation was that there were no intercellular spaces inside the vein or between the cells of the vein and the bundle sheath, as there are in the mesophyll cells. Minor veins are surrounded by a single layer of bundle sheath (BS) cells that are in direct contact with the minor vein. Bundle sheath cells cannot be distinguished morphologically from the mesophyll cells; they have large vacuoles and contain a high amount of chloroplasts mostly with starch and a big nucleus (figure CII.6b).

Intermediary cells (IC) are specialized companion cells specific to cucurbits, which are always adjacent to the sieve elements (SE). The cytoplasm of the ICs contains numerous free ribosomes, showing a dense cytoplasm interrupted by many small vacuoles (Figures CII.6b-h). Mitochondria are quite abundant and distributed all over the cell. In contrast, we couldn't observe plastids in any of the studied IC (Figure CII.6). Vascular parenchyma (VP) cells are often located between BS and IC but frequently they were not visible in the sections examined. A large number of simple or complex plasmodesmata connect the IC and the BS (Figure CII.6h) as well as the IC with the VP (Figures CII.6e, f) and between ICs. These complex plasmodesmata seem to occur in clusters at points where the walls are slightly thickened. Intensive symplastic connection with the BS and VP is a characteristic of intermediary cells. PPU, the connections between the sieve elements (SE) and IC, were present, the single pore on the SE cell wall and the branching towards the adjacent IC could be visualised (Figure CII.6i). Sieve elements are enucleate and lack tonoplast and ribosomes.

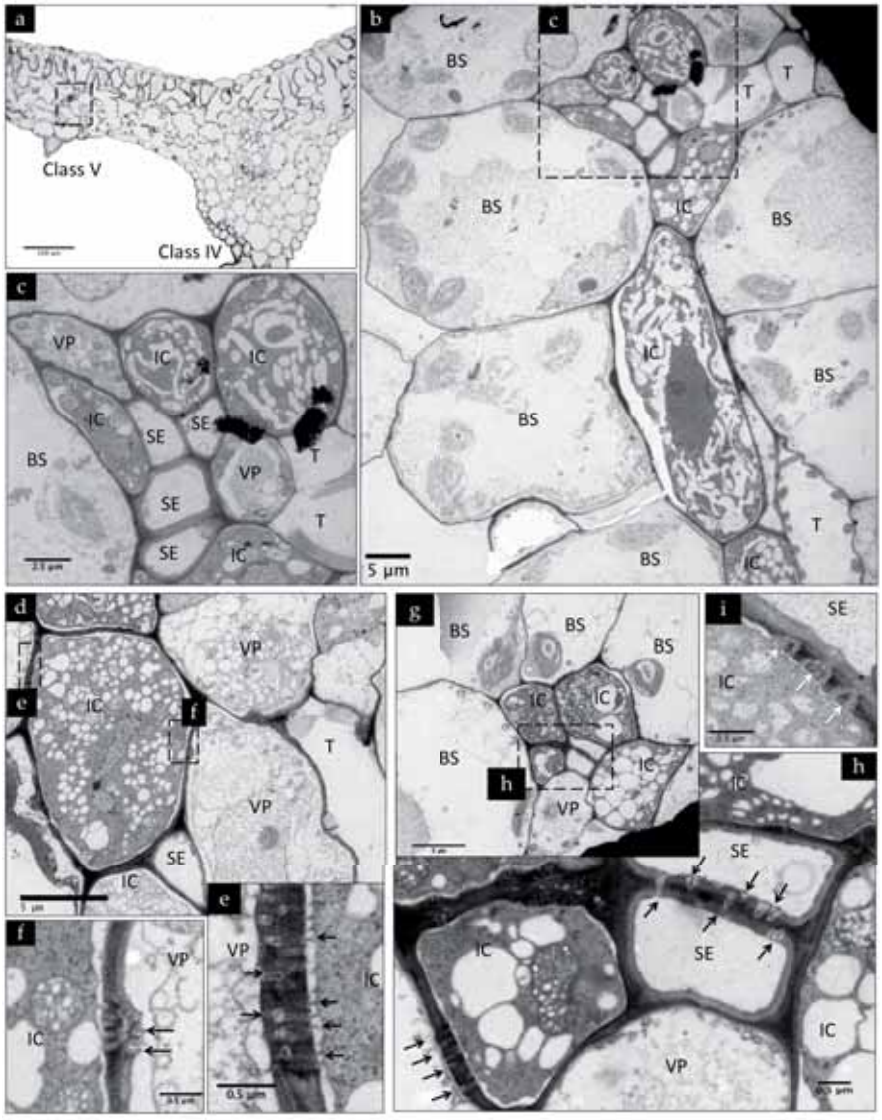


Figure CII.6. Anatomy of melon minor veins of PS and SC12-1-99 lines. Images from TEM of minor veins of SC12-1-99 (a-f, i) and PS (g,h). a) Light microscopy cross section of a melon leaf including a class IV and a class V minor vein. b) Corresponding TEM image of the class V vein in (a). Minor vein is surrounded by bundle sheath cells (BS). Tracheids (T) of the xylem and intermediary cells (IC) can be easily identified. c) Magnification of the minor vein of (b), in which IC and vascular parenchyma cells (VP) are surrounding the SE. d) TEM image of a minor

vein in which an IC is symplastically connected with VPs. e) and f) are higher magnifications of (d) to observe simple and complex PD connecting VP with IC. g) Minor vein and enlargement (h) of the PD connecting SEs and IC-BS. i) PPU connecting IC and SE. BS: bundle sheath; VP: vascular parenchyma; IC: intermediary cell; SE: sieve element; T: tracheid; black arrows point to simple or complex PD; white arrows point to PPU.

Anatomical differences between the susceptible and the resistant line, neither in the PD connecting the different cell types nor in the content of the mesophyll, BS, VP or IC, were not observed. We have determined that the TEM is a very useful tool to identify all the different vascular bundle cell types and provide us with the basic cell anatomy knowledge needed to carry out further experiments to localize the virus by immunogold labelling in both resistant and susceptible lines.

CMV-LS is restricted from entering minor veins at the bundle sheath cells.

Once we were able to distinguish all the vascular cell types by studying them with the TEM, our objective was to determine either the presence or absence of the virus in the different cell types of PS and SC12-1-99 that constitute the veins in order to identify the cell type in which virus movement is blocked.

First, leaves of both cell lines were inoculated with CMV-LS. Foci present in inoculated leaves were sampled, fixated and included, slice cut and probed with anti-CP antibody, as indicated in Material and Methods. As seen in figure CII.7, in both lines, the virus observed in the epidermal and mesophyll cells was uniformly distributed in the cytoplasm and few particles were present in the vacuole, chloroplasts, mitochondria or peroxisomes. At the minor veins, both PS and SC12-1-

99, showed a uniform distribution of the CP labelling in BS cells (Figure CII.7a), while gold particles present in VP (Figure CII.7b), and IC (Figure CII.7c), seemed to be lower and mainly distributed in their cytoplasm. Despite a background of gold particles in VP and IC of the resistant line, differences of gold particles accumulation in VP and IC between PS and SC12-1-99 lines were still evident (Figure CII.7).

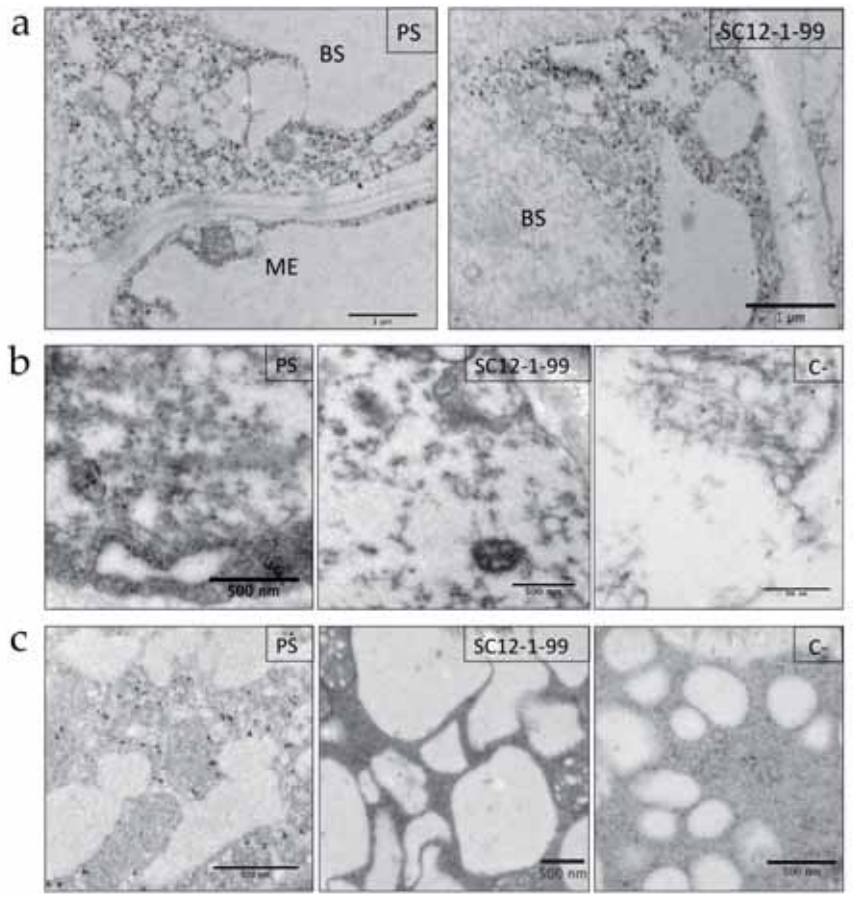


Figure CII.7. Transmission electron microscope immunocytochemistry of PS and SC12-1-99 lines inoculated with CMV-LS. Presence of gold particles in: **a)** Bundle-sheath (BS), mesophyll (ME) **b)** Vascular parenchyma cells **c)** intermediary cells are shown. C- is the negative control from a non-inoculated plant, Piel de Sapo (PS) and SC12-1-99 as susceptible and resistant lines respectively.

The BS cells of both types were heavily infected, but it was necessary to determine if the labelling differences between VP and IC of resistant and susceptible lines was significant. To do that, we counted the gold particles in 8 IC and 8 VP of PS, 8 IC and 10 VP of SC12-1-99 and 6 IC and 5 VP of healthy plants (negative control), which indicated the nonspecific gold labelling. The number of gold particles in IC and VP are represented in figure CII.8 as Au particles/μm^2. The data were analysed using T-student test with $P \leq 0.05$. Results from IC and VP are similar and indicate that in the susceptible line there is significantly higher number of gold particles than in the resistant line (pvalue of 0.0026 with IC and 0.0003 with VP) or in healthy plants (pvalue of 0.013 with IC and 0.063 with VP). The amount of gold particles in the resistant line is not significantly different from that in healthy plants (pvalue of 0.14 in IC and 0.25 in VP), indicating that these particles constitute the background of the IGL.

Therefore, our results indicate that CMV-LS is blocked in the line SC12-1-99 in the bundle sheath cells and accumulates there as much as in the susceptible line. However, in SC12-1-99 we were not able to significantly detect gold particles in the IC or VP indicating that the virus is unable to enter both cell types. Therefore the barrier to the systemic infection is located between the bundle sheath and the vascular bundle.

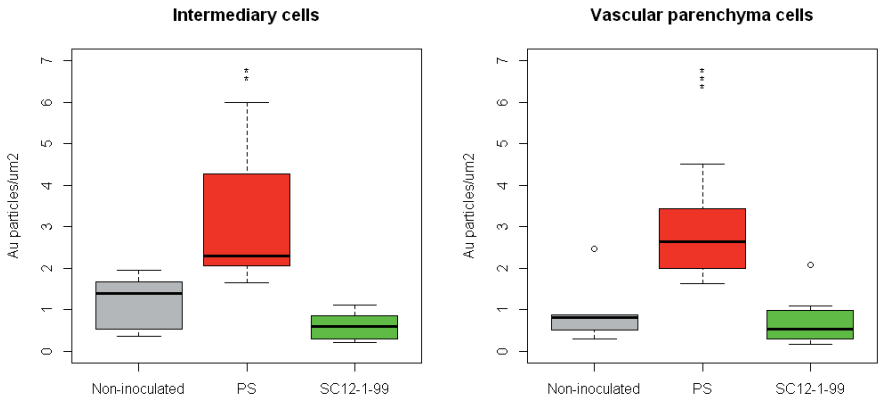


Figure CII.8. Number of gold particles per um² in intermediary cells (IC) and vascular parenchyma (VP) of PS and SC12-1-99 lines. Non-inoculated plants were used as negative control to detect non specific labelling. Results are represented in box plot. The bottom and the top of the box are the first and third quartiles. The band inside the box is the median, the second quartile. Outliers or individual points represent the variability outside upper and lower quartiles. Significant differences between PS and SC12-1-99 are represented in asterisks,** represent pvalue ≤ 0.01, *** represents pvalue ≤ 0.001.

Subcellular distribution of CMV-MP by confocal imaging

The Movement protein of CMV localizes to plasmodesmata (PD) in all the cell types (Blackman *et al.*, 1998) and increases their size exclusion limit (SEL) to allow the cell-to-cell spread of the virus (Vaquero *et al.*, 1994). Since the determinant of virulence with respect to *cmv1* is localized in the MP (Guiu-Aragonés et al., submitted), and CMV-LS is not able to enter the VP and the IC of the SC12-1-99, it may be possible that *cmv1* somehow impairs the localization of the MP of CMV-LS to the PD in the BS and, subsequently, this might affect the spread of LS to the other cells.

To study the subcellular localization of the movement protein, we fused MPs of both strains either with green fluorescent protein

(MP:GFP) or with red fluorescent protein (MP:RFP) at its C terminus. Agroinfiltrations were performed together with RFP-tagged PDLP1 (Plasmodesmata-located protein 1), a PD marker (Amari *et al.*, 2010). Confocal microscopy observations confirmed that in *N.benthamiana* leaves, MP:GFP co-localized with PDLP1, indicating that both localise to PD (Figure CII.9). These results were extrapolated for the constructions of MP:RFP since they had the same pattern of distribution (data not shown).

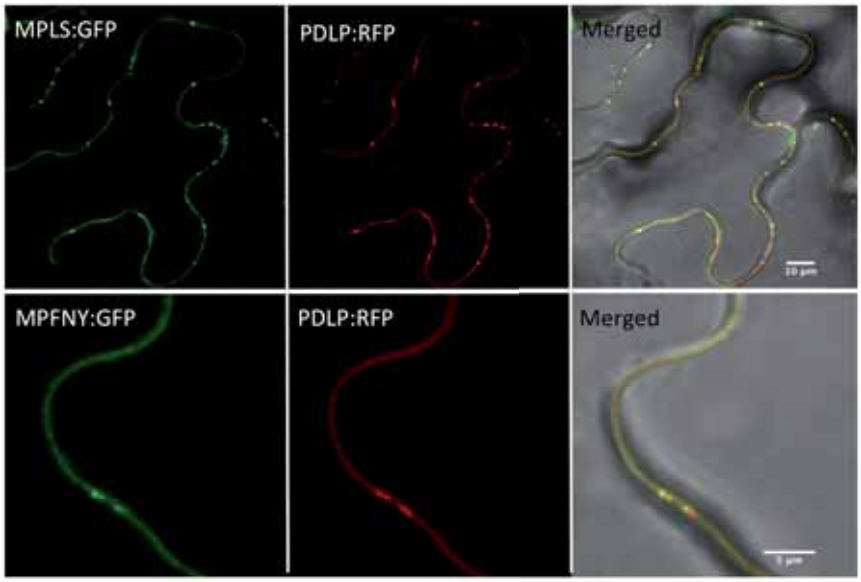


Figure CII.9. Localization of the MP in *N.benthamiana* cells. Localization of the GFP fused MP of CMV-LS and CMV-FNY (MPLS:GFP, MPFNY:GFP; green) to PD labelled with RFP-fused PDLP (PDLP:RFP; red). The "Merged" image shows the co-localization of the two proteins in yellow.

The same procedure was performed to determine the localization of CMV-MPLS in PS cells. Expression of proteins by agroinfiltration was inefficient in melon, since only a few cells appeared transformed. However, the results showed that the MP of CMV-LS indeed co-

localized with PDLP1 in PD of PS (Figure CII.10A) and therefore, it was localizing to the PDs. Using the same approach, RFP-fused MPs (MP:RFP) of CMV-LS and CMV-FNY were expressed in leaves of PS and SC12-1-99 line, to compare their capacity to localize in PDs in the susceptible and resistant lines. As shown in Figure CII.10B, both MPs localized to PD in epidermal cells irrespective of the host being resistant or susceptible. The fact that confocal microscope can not visualise cells below epidermis, like mesophyll or vascular bundle cells impede us to determine the localization of MP in BS. The observation that both MPs target PD irrespective of resistance is somehow expected, given that the CMV-LS and CMV-FNY viruses were able to move cell-to-cell in both hosts and given that this assay monitors the ability of MP to target PD in epidermal tissue rather than in BS cells in which the virus is restricted.

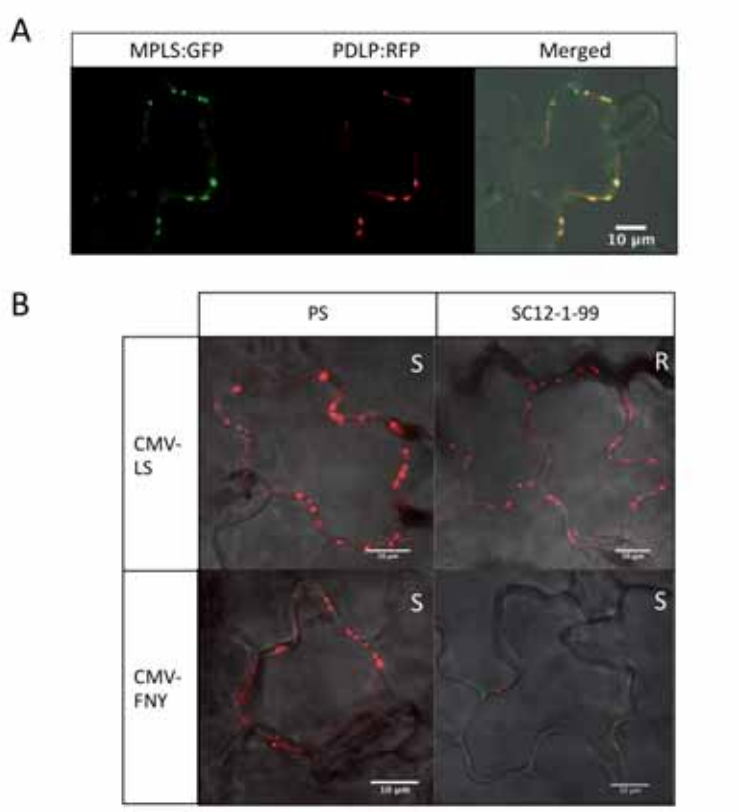


Figure CII.10. Localization of the CMV-MP in melon cells. A: Localization of the GFP fused MP of CMV-LS (MPLS:GFP; green) to PD labelled with RFP-fused PDLP (PDLP:RFP; red). The "Merged" image shows the co-localization of the two proteins in yellow. B: Localization of the MPs of CMV-LS and CMV-FNY fused to RFP in Piel de Sapo (PS) and in line SC12-1-99. Pictures were taken at 3 days post agroinfiltration. R: resistant, S: susceptible.

CMV-LS cannot infect systemically graft-inoculated SC12-1-99 plants.

The experimental evidence presented indicate extensive replication and cell-to-cell movement of CMV-LS in inoculated leaves of SC12-1-99, but no systemic infection, so *cmv1* seems to confer a blockage in vascular spread, possibly based on the absence of suitable host factors

that support entrance of the virus into the VP/IC-SE cells. To determine if CMV-LS was able to systemically infect SC12-1-99 plants once it is already in the phloem, we performed graft inoculations. This experiment would indicate us if *cmv1* was also having a role in the movement through the phloem or in the unloading of the virus from the sieve elements to the new leaves. Healthy SC12-1-99 scions were grafted onto CMV-LS-infected PS rootstocks, permitting a direct contact of CMV-LS with the phloem of SC12-1-99. In a total of 3 experiments, about 60 healthy plants of SC12-1-99 were grafted onto PS stocks that were visually infected with CMV-LS. We had a low rate of survival stocks, surviving at the end only 14 plants. This high rate of mortality can be caused because stocks were very small, due to the infection, and also in the process of acclimation some scions died. Out of 14 SC12-1-99 scions, 11 did not show any symptoms of virus even 60 days post grafting (dpg). Foliar discs of scion leaves were collected and tissue printing was performed determining that the virus was absent in the scion of those 11 symptom free plants (Figure CII.11b). The other 3 scions presented very weak viral symptoms in the leaves proximal to the graft junction, but leaves from middle and apical part of the scion were totally asymptomatic. RT-PCR from basal, middle and apical leaves indicated presence of virus in the basal samples and in one from the middle part, but the other two samples of the middle and all the apical leaves were free of CMV-LS (Figure CII.11c), indicating that SC12-1-99 is resistant to CMV-LS even when virus is already in the phloem. In some grafted plants, the stock continued growing, emerging a branch that became infected and therefore, producing virus that could be a continuous supplier of viral inoculum (branch 4 showed in figure CII.11c). However, even in this situation the scion (SC12-1-99) continued resistant. Positive control consisted on the same grafts whose scion was PS. Only one scion survived, clearly showing viral symptoms, which were confirmed by RT-PCR (Figure

CII.11a). Therefore, *cmv1* has a role in the movement through the phloem in the unloading of the virus to the new leaves.

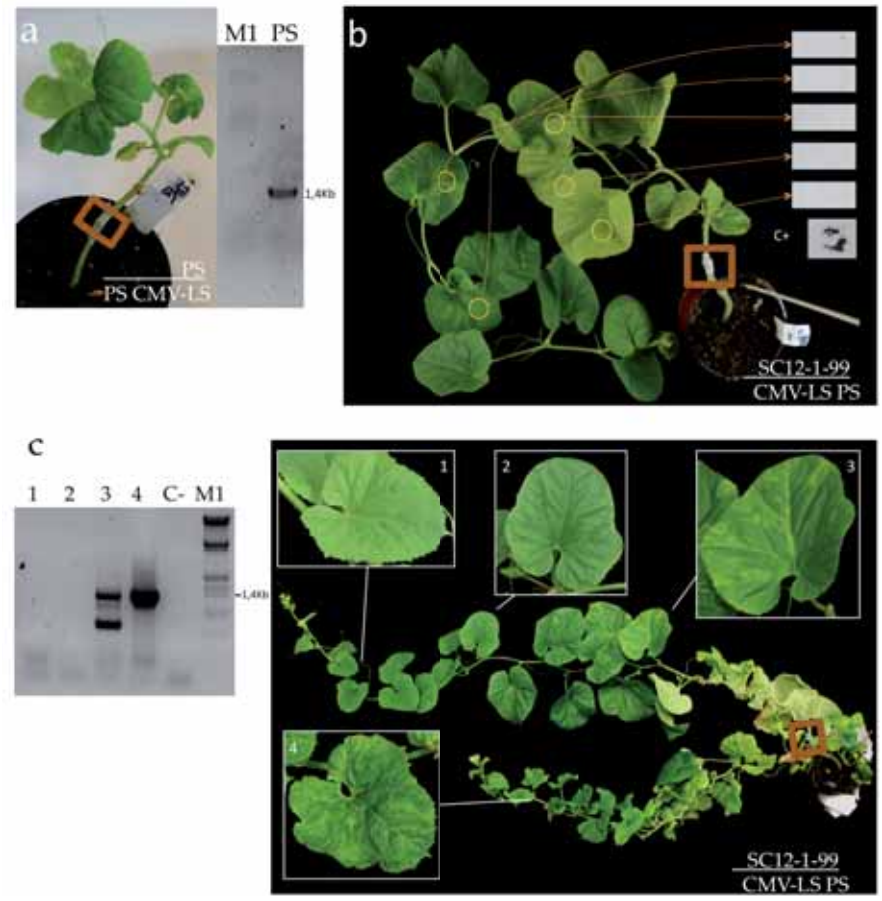


Figure CII.11. Graft inoculation of SC12-1-99 (scion) on infected CMV-LS PS (rootstock). In each picture, the grafting is represented as the rootstock under the grafted junction (red box) and the scion above the junction. **a**, Graft inoculation of PS on infected CMV-LS PS was used as positive control of graft inoculations. CMV-LS was detected by RT-PCR. **b,** Representative healthy, asymptomatic graft of SC12-1-99 on infected CMV-LS PS. Virus was detected by tissue printing. **c**, Representative symptomatic graft of SC12-1-99 on CMV-LS PS. Apical leaves (1), medium (2) and basal leaves (3), were sampled for virus detection by RT-PCR with specific primers of LS. Infected leaves of the emerged branched from the rootstock (4) and a leaf from a non-infected (C-) plant were used as positive and negative control, respectively for the RT-PCR.

CMV-MP is not a suppressor of gene silencing

Even though we have demonstrated that *cmv1*-mediated resistance mechanism consists of a block of phloem entry, we couldn't discard the possibility that the CMV-MP, the determinant of virulence for *cmv1*-mediated resistance (Guiu-Aragonés, submitted and Chapter I), could have silencing suppressor activity, making possible that the lack of phloem entry could be related to a suppressor activity of FNY MP but not of LS MP.

To determine if the MP of CMV has gene silencing suppressor activity we performed an agroinfiltration patch assay in GFP-transgenic *Nicotiana benthamiana* 16C plants (Brigneti *et al.*, 1998). We agroinfiltrated fully expanded leaves of the plants for transient over expression of GFP, either alone or together with the MP of either CMV-LS or CMV-FNY. As positive control, we agroinfiltrated the tissue for expression of GFP together with a very effective silencing suppressor, P19, a protein encoded by *Tomato bushy stunt virus* (TBSV) (Voinnet *et al.*, 1999). As negative control, the GFP was co-expressed with RFP. Results showed that at 3 days post agroinfiltration (dpa), all the agroinfiltrated areas were fluorescent, whereas at 6 dpa all agroinfiltrated patches had lost GFP fluorescence except the patch expressing GFP together with P19 (positive control). This indicated that, neither the MP of CMV-LS nor the MP of CMV-FNY act as a suppressor of RNA silencing (Figure CII.12). The possibility of the CMV-MP acting as a virulence determinant involved in phloem entry by suppressing or enhancing silencing was, therefore, rejected.

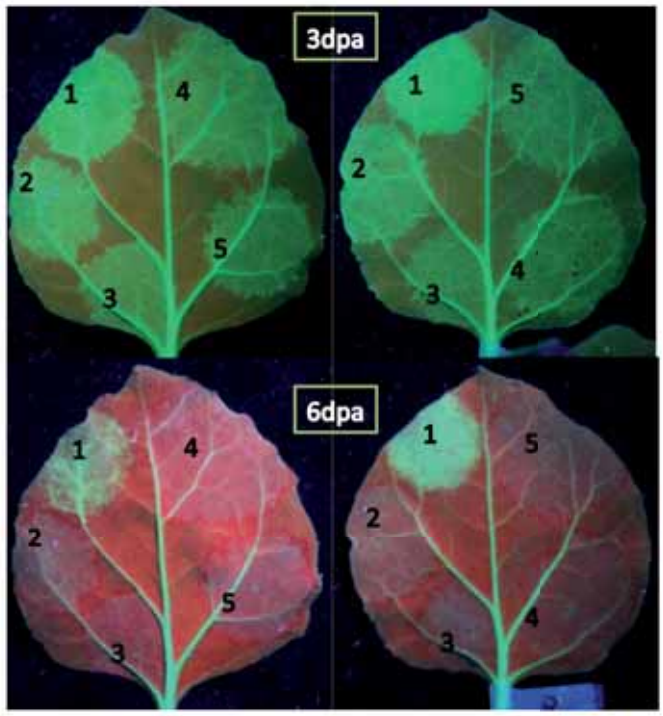


Figure CII.12. Agroinfiltrated *N. benthamiana* 16C leaves expressing: 1. GFP+P19 (positive control), 2. GFP+RFP, 3. GFP, 4. GFP+MPLS, 5. GFP+MPFNY. Images were taken at 3 dpa and 6 dpa.

Discussion

In this chapter, we have studied the major steps in the systemic infection of CMV-LS and FNY: replication, cell-to-cell movement and long distance movement both in SC12-1-99 and susceptible control line PS, to envisage which step is involved in the inability of CMV-LS to infect systemically the line SC12-1-99, carrying the recessive resistant gene *cmv1*. The results we report indicate that CMV-LS can replicate and move cell to cell in the resistant line showing no differences with CMV-FNY, that produce a systemic infection. The restriction to the systemic infection is produced at the level of entrance to the vascular bundle and results from immunocytochemistry at the TEM indicate that the virus is restricted in the bundle sheath cells of minor veins, being unable to enter the vascular parenchyma (VP) or intermediary cells (IC). To our knowledge, almost all the described recessive resistance genes, are eukaryotic translation initiation factors (eIFs), and are involved in the inhibition of the replication or the short-distance movement (Robaglia and Caranta, 2006; Yoshii *et al.*, 2004). There are also other two recessive resistance genes reported that are not eIFs, but still their mechanism of action involve impairing of translation. In Arabidopsis, a helicase-like protein, AtRH8, interacts with the VPg of TEV and co-localizes with the virus in membrane-associated vesicles where the virus is translated/replicated (Huang *et al.*, 2010). Also in Arabidopsis, a TOR factor, affects the internal re-initiation of translation in long Open Reading Frames via binding a viral re-initiation factor, TAV, from Cauliflower mosaic virus. This interaction is critical for the ability of *Tobacco etch virus* to survive. Consequently, plants deficient in TOR are resistant to TEV infection (Schepetilnikov *et al.*, 2011).

The ability of CMV-LS and CMV-FNY to replicate in both melon lines was demonstrated comparing the viral accumulation in the melon

cotyledons. Levels of accumulation seem to be higher in PS than in SC12-1-99 independently of the strain (Figure CII.1). This observation suggest the possibility that *cmv1*-mediated resistance would partially impede replication or cell-to-cell movement but not inhibit them. Alternatively, among the other gene(s) from SC carried by SC12-1-99 in the introgression, some of them could slightly impair these processes of CMV independently of the resistance provided by *cmv1*, since it affects equally to both strains. However, in this experiment we are not able to differentiate if the variation on the accumulation is due to a lower efficiency in cell-to-cell movement or a decrease in the level of replication.

Cell-to-cell movement distribution was studied localizing the virus in the inoculated leaf by tissue printing. We had a modified CMV encoding a reporter protein, GFP, (kindly given by T. Canto, from CIB, Madrid) but it is able to move only few cells and is unable to move long distance (Canto *et al.*, 1997). Therefore, it could not be used to follow the virus in our system. Results from tissue printing showed similar distributions, independently if the virus was able to produce a systemic infection or not. Both CMV-LS and FNY were detected in the entire foliar disc accumulating mainly in the veins. Interestingly, the resistant parental SC also permits cell-to-cell movement of both strains. Therefore, none of the described resistant genes (QTLs) also present in SC (Guiu-Aragonés *et al.*, 2014) are able to inhibit either the replication or the cell-to-cell movement. These QTLs should also be involved in restricting long distance movement of the virus. Other reports that studied the distribution of CMV in the inoculated leaf using the same technique, observed that when the virus was unable to produce a systemic infection, it was distributed only in few spots in the leaf and the accumulation in the veins only occurs when systemic infection is

established (Ohnishi *et al.*, 2011; Choi *et al.*, 2005; Kobori *et al.*, 2000; Hong *et al.*, 2007).

Tissue print hybridizations of petioles or stem sections and *in situ* hybridization experiments determined that CMV-LS was absent in the phloem of the resistant line. This indicated that the resistance conferred by *cmv1* operates at the level of phloem entry. In situ hybridization has been used for detection of CMV movement in several studies (Takeshita *et al.*, 2009; Xu and Roossinck, 2000; Havelda and Maule, 2000; Ziebell *et al.*, 2007; Takeshita *et al.*, 2004). This technique allows visualizing the virus and identifying the cell types that form the vascular bundle, in cucurbits (Moreno *et al.*, 2004; Gosalvez-Bernal *et al.*, 2008; Kobori *et al.*, 2000) and in other species (Kobori *et al.*, 2003; Goodrick *et al.*, 1991). In our ISH sections we were not able to identify all the cell types that constitute the vascular bundle, probably because our sections were too thick and different cell types could be mixed. Additionally, we were analysing vein type IV, in which there are many cells compared with the type V that are minor veins and have fewer cells, easier to identify. Observation of the samples with the transmission electron microscope (TEM) facilitates the identification of the cell types of the vascular bundle, since it permits to visualise all the intracellular structures. Intermediary cells, very vacuolated, with a dense cytoplasm and highly connected by PDs with BS and VP confirm that there is a symplastic connection between these cells.

Immunocytochemistry at the TEM allows determining that in both, susceptible and resistant lines, there is a high accumulation of gold particles in the epidermal, mesophyll cells and BS. However, there was a reduction of gold particles in IC and VP of both lines, This decrease had also been previously described in cucumber infected

with *Cucumber green mottle mosaic virus* (CGMMV), a symplastic loader plant like melon (Moreno *et al.*, 2004). Therefore, it seems that there could be an impairment of virus entry at the boundary BS-vascular bundle even in the susceptible plant. Despite that, the accumulation of gold particles was clearly lower in the VP and in the IC of the resistant line than in PS. To ensure that this was true, gold particles were counted in these cells and a statistical analysis confirmed that the abundance of gold particles in the susceptible line was significantly higher than in the resistant line. The number of gold particles in the VP and IC of the resistant line was not significantly different from that of healthy plants, taken as background. This presence of gold particles in the negative control can be explained because we, erroneously, had fixed the samples with osmium tetroxide, which is reported to increase non specific gold labelling (Roberts, 1994). Even with this background, the number of gold particles is significantly higher in the ICs or VPs of the susceptible than in the resistant line and both of them show similar accumulation of gold particles in the BS, indicating that *cmv1* is restricting the entrance of the virus in the VP or IC in the line SC12-1-99. With one possible exception, in which virus was detected in companion cells (Schaad and Carrington, 1996), the BS-phloem interface was also found to be a barrier for systemic virus movement in soybean lines resistant to systemic infection with *Cowpea chlorotic mottle bromovirus* (Goodrick *et al.*, 1991) and in transgenic tobacco plants resistant to CMV (Wintermantel *et al.* (1997). Since Cowpea and tobacco are apoplastic phloem loaders, it was speculated that the resistance to systemic infection was a consequence of having fewer PDs between BS-VP and BS-CC (van Bel *et al.*, 1992). Later studies with cucurbits, which are symplastic loaders with abundant PD between all the cell types, showed that this restriction at the BS-phloem interface was maintained. This is the case of the systemic infection in studies with TAV in cucumber (Thompson and García-

Arenal, 1998) and also in *Cucumis figarei,* that presents systemic resistance to CMV at the boundary of BS at 24ºC but when the temperature is 36ºC, the virus spreads systemically (Kobori *et al*., 2000). Considering that bundle sheath cells don't differ from mesophyll cells, the accumulation of CMV in BS would suggest a different viral cell-to-cell movement mechanism between epidermis/ mesophyll cells, than the mechanism controlling the entrance into the cells that constitute the veins: VP and IC. Ding *et al.* (1992) observed that TMV movement protein in transgenic tobacco plants increased SEL for PD between mesophyll and BS cells of tobacco, but not between BS and VP or CC cells. These results would indicate that other mechanisms would be involved for the increase of the SEL between these cell types. In our study, one of these other mechanisms can be related with *cmv1*. Results from our laboratory indicate that *cmv1* might be related to vacuole trafficking, since one of the candidate genes is a Vacuolar Sorting protein. It could be then involved either in the intracellular transport of the movement protein to the PD (but only in the bundle sheath cells) or be important for the ability of MP to interact with plasmodesmata in a functional manner to increase the SEL in the boundary BS/VP-IC. When *cmv1* is mutated (*cmv1*) these processes can be directly or indirectly affected impeding the entrance of the virus to VP and IC. To a deeper characterization of the mechanism in which *cmv1* can be involved, localization of the MP fused to a gene reporter (GFP or RFP) was studied by confocal imaging. The results showed that MP locates in PD in both lines, susceptible and resistant. Unfortunately, this technique has the limitation that it can visualise epidermal cells but not the vascular bundle cells, in which we are interested. Future experiments with TEM using the recently obtained MP antibody will provide us the information of the subcellular localization of the MP in all the cell types and will provide more information about the mechanism of *cmv1*-mediated resistance.

Viruses encode suppressors of silencing to support their propagation. In CMV, the 2b is the only protein reported as suppressor of posttranscriptional gene silencing (Brigneti *et al.*, 1998) but since the silencing signal spreads between cells through plasmodesmata like the viruses themselves, movement proteins (MP) may have a central role in compatible virus-host interactions by suppressing or enhancing the spread of the signal. There are other viruses in which movement proteins act as a silencing suppressor, like the P25 of *Potato Virus X* (Voinnet *et al.*, 2000) or HcPro that is encoded by Potyviruses and has several functions including movement and PTGS (Plisson *et al.*, 2003). Furthermore, the MP of *Tobacco mosaic virus,* does not enhance the silencing pathway but enhances the transport of the signal through plasmodesmata (Vogler *et al.*, 2008). Our results indicate that neither FNY MP nor LS MP of CMV have silencing suppressor activity, excluding a relation between this mechanism and *cmv1*- mediated resistance.

Grafting experiments with resistant plants as scions grafted onto CMV-LS-infected PS rootstocks showed that even if CMV-LS is in the phloem, the SC12-1-99 line is resistant, growing free of virus in most cases. As shown in Marco *et al.* (2003) even though some viral RNA of CYSDV is detected in the resistant scion of graft inoculated melon plants, systemic colonization was impeded. In our grafted plants, we detected presence of CMV-LS in the first leaves of 3 out of 14 scions. This presence might be due to cell-to-cell movement through the stem or because the virus could be unloaded from the phloem to the first leaves but then, after multiplication in the leaf, it couldn't be loaded to the phloem again because this step would be restricted in the bundle sheath cells as described before. This way, there would not be additional supply of virus to the phloem to infect the new growth.

Chapter III

Sequencing of the CMV strain M6 and generation of the infectious clones

Introduction

Availability of methodologies to obtain and manipulate infectious clones of plant viruses has been a very useful tool to improve the knowledge of different aspects in viral biology during lasts decades. The largest number of plant viruses are RNA (Scholthof *et al.*, 2011), therefore, full-length cDNA infectious clones corresponding to complete genomes of RNA plant viruses have an important impact on virology studies. Subsequently this cDNA requires *in vitro* transcription, using promoters of RNA polymerases in bacteriophages like SP6, T3 or T7, or *in vivo* transcription, using constitutive promoters like promoter 35S of *Cauliflower mosaic virus* (CaMV). The firsts infectious clones were developed in 1980s. Infectious RNAs were obtained from *in vitro* transcription of the cDNA clones of *Brome mosaic virus* clones that were able to infect barley plants (Ahlquist and Janda, 1984; Ahlquist *et al.*, 1984) and infectious clones of *Tobacco mosaic virus* infecting *N.tabacum* and tomato (Dawson *et al.*, 1986; Meshi *et al.*, 1986). Afterwards, infectivity of *in vivo* transcripts under 35S CaMV (Cauliflower mosaic virus) promoter (Kay *et al.*, 1987) was demonstrated by mechanical inoculation in *Chenopodium hybridum* of plasmids containing individual full-length cDNAs of the three RNAs of *Brome mosaic virus* (Mori *et al.*, 1991). Functionality of 35S promoter permits to use *Agrobacterium tumefaciens* as a delivery agent to transfer DNA from bacterial cells to plants. Agrobacterium permit to introduce in plants the viral cDNA cloned in binary vectors, which provided *in vivo* transcription of RNAs (Grimsley *et al.*, 1987). Since then, the manipulation of infectious clones has increased the knowledge about replication and genetic expression of viruses, since it has allowed the use of site-directed mutagenesis or complementation experiments. Moreover, it is a very useful tool to study areas such as viral

movement in plants and the interaction between plants, viruses and their vectors (Boyer and Haenni, 1994).

Cucumber mosaic virus (CMV) is an important model for research because its features facilitate the manipulation. These include strong accumulation in infected hosts, which allows easy purification, mechanical transmission and infectious cDNAs available for several different strains for a reverse genetic approach. The firsts infectious clones of CMV strains were generated in the decade of 90s (Rizzo and Palukaitis, 1990; Hayes and Buck, 1990b; Zhang *et al.*, 1994; Boccard and Baulcombe, 1992) and nowadays it is a useful tool and new infectious clones are still being generated (Ohnishi *et al.*, 2011; Kang *et al.*, 2012; Takeshita *et al.*, 2009; Phan *et al.*, 2014). The CMV clones can be modified to CMV-based expression vectors in which a foreign sequence codifying for an interested protein is introduced and it is expressed with the virus. This approach has been an important tool for production of different proteins like pharmaceutical proteins (Matsuo *et al.*, 2007). Modified CMV clones can also be a system for virus-induced gene silencing (VIGS), in which the virus vectors carry a portion derived from the host gene sequence of a target that want to be silenced (Otagaki *et al.*, 2006).

The complete nucleotide sequences of 24 isolates of CMV have been reported in the data banks and partial sequences have been deposited for several CMV isolates. CMV shows a high degree of diversity, as revealed by a large number of isolates differing in both biological and molecular properties, being classified in two main subgroups, I and II. The percentage of identity in the nucleotide sequences between these subgroups ranges from 73% to 78% (Roossinck, 2001). Subgroup I can be divided into IA and IB depending on the sequence of the open reading frame 3b and on the 5'-noncoding region of RNA3 and

sharing 92-94% sequence identity between them (Roossinck *et al.*, 1999).

In our laboratory, with the aid of a near-isogenic line (NIL) collection between Piel de Sapo (PS) and Songwhan Charmi PI 161375 (SC) as susceptible and resistant parents respectively (Eduardo *et al.*, 2005), we described a single recessive gene, *cmv1*, which confers monogenic resistance to strains of subgroup II. Other strains from subgroup I, like CMV-FNY, CMV-TL or CMV-M6, were able to overcome *cmv1*-mediated resistance (Essafi *et al.*, 2009) (Guiu-Aragonés, submitted). Screening of a DHL population between the same parents (Gonzalo *et al.*, 2011) allowed us to describe that resistance to CMV-M6 is governed by at least three QTLs, one of them, *cmvqw12.1*, co-locating with *cmv1* in linkage group (LG) XII and others mapping in LGIII and LGX. DHL lines that contain the combination of the three resistance QTLs from SC, are totally resistant to CMV-M6 (Guiu-Aragonés *et al.*, 2014). Recent experiments performed in our laboratory revealed that line 2012 from the DHL population, which is resistant to CMV-M6 (Guiu-Aragonés *et al.*, 2014), is susceptible to CMV-FNY (unpublished data). To study in more detail the resistance to CMV-M6 present in the DHL 2012 and to search for the determinant of virulence between strains M6 and FNY, the availability of infectious clones of M6 would be a very useful tool.

In this work, we obtained the complete sequence of CMV-M6 and the infectious clones of cDNA of the three RNAs (RNA1, RNA2 and RNA3) of M6 strain. The cloned strain is completely functional producing a systemic infection in *N.benthamiana* and *C.melo*.

Material and methods

Virus, plants and inoculations

Strains of CMV used in this study were two strains from subgroup I, CMV-M6 (Diaz *et al.*, 2003) and CMV-FNY (Rizzo and Palukaitis, 1990). Agroinfectious clones of CMV-FNY were provided by Prof. Kook-Hyung Kim (Seo *et al.*, 2009).

Melon line used was the Spanish Piel de Sapo type line T111 (PS). Seeds were pre-germinated and plants were grown as described in previous chapters.

CMV-M6 from Diaz *et al.* (2003) was rub-inoculated as in previous chapters into zucchini squash Chapin F1 (Semillas Fito SA, Barcelona, Spain) to produce fresh viral inoculum. Infectious clones of CMV-M6 (M6.1 M6.2 M6.3) generated in this chapter and pseudo recombinants between FNY (pCR1 pCR2 pCR3) and M6 were inoculated to *N. benthamiana*. Availability of agroinfectious clones of CMV-FNY and CMV-M6.3 permitted agroinoculation of these ones. M6.1 and M6.2 were transcribed with T7 and SP6 RNA polymerase respectively, as previously described (Chapter I- Materials and methods p.73). Mixed inoculations, using RNA and *Agrobacterium*, were optimised determining two steps: first, we performed a common agroinfiltration in the abaxial side of the leaf and 45 minutes after, we rub-inoculated the RNAs obtained from the in vitro transcription in the adaxial side. When *N.benthamiana* plants showed systemic infection (2-3 weeks post inoculation), systemic leaves were used to produce sap to rub-inoculated melon cotyledons.

Amplification of M6 RNAs

Viral M6 RNA was isolated from infected zucchini squash using TriReagent (SIGMA-ALDRICH, St Louis, MO, USA) following manufacturer's protocol. Three independent RT reactions from the same RNA were made using primers F109-3'R, F209-3'R and F309-3'R primers and Prime Script Reverse Transcriptase (Takara Biotechnology, Dalian, China). Pfu Taq polymerase (Promega Corporation, Madison, WI) was used for PCR reactions according to manufacturer's instructions. Primers of CMV-FNY strain were used for the RT-PCR since both strains belong to subgroup I. M6 RNA1 was amplified with FNY1-2 1F and F109-3'R, M6 RNA2 with FNY1-2 1F and F209-3'R and finally, M6 RNA3 was amplified with F309-1F and F309-3'R (Table CIII.1).

Table CIII.1 Primers used to synthesize the cDNA and amplify full-length cDNA from CMV-M6

FNY1-2 1F: (5' GTTTATTTACAAGAGCGTACG)
F109-3'R: (5' TGGTCTCCTTTTAGAGACCC)
F209-3'R: (5' TGGTCTCCTTTTGGAGGC)
F309-1F: (5' GTAATCTTACCACTGTGTGTG)
F309-3'R: (5' TGGTCTCCTTTTGGAGGCC)

The three full-length PCR products corresponding to M6.1 (RNA1 from M6), M6.2 (RNA2 from M6) and M6.3 (RNA3 from M6) were sequenced with FNY primers using Sanger's technology. Sequences were analysed using Sequencher TM version 4.8 (Gene Codes Corporation; Ann Arbor, MI).

5’RACE and 3’RACE sequencing

To obtain the real sequence of the lasts 30 nucleotides of each M6 RNA end, 5’RACE and 3’RACE were performed using 5'/3' RACE Kit 2n generation (Roche Applied Science, Barcelona, Spain) following manufacturer’s instructions. Briefly, for 5’ RACE, cDNAs were synthesized from total RNA using specific primers (SP) for each M6 RNA: M6.1-SP1R, M6.2-SP1R and M6.3-SP1R (Table CIII.2). After that, purified cDNAs were tailed with dATP and PCR-amplified using Oligo (dT)-Anchor primer (provided by the supplier) and specific primer 2 (SP2R) for each cDNA. Nested PCRs were performed using PCR Anchor primer (provided by the supplier) and specific primer 3 (SP3R). 5’RACE products were then sequenced using PCR-Anchor primer and SP3R. For 3’RACE, since CMV RNA doesn’t have poly(A)+ tail in the 3’ end, we polyadenilated the RNA using *E.coli* Poly (A) Polymerase (Ambion, Life Technologies, Carlsbad, CA, USA) following manufacturer’s protocol. Then, cDNAs were synthesized using oligo d(T) anchor primer, and purified cDNAs were amplified using specific primer 4 (SP4F) and PCR anchor primer. PCRs were sequenced with SP4F. All the sequences were performed using Sanger’s technology and were analysed using Sequencher TM version 4.8 (Gene Codes Corporation; Ann Arbor, MI).

Table CIII.2 Primers used for 5’RACE and 3’RACE of CMV-M6

M6.1-SP1R: (5’GGCGCATCTTATCGCGGAT)
M6.1-SP2R: (5’TTCACACAAGCGGAGGGCA)
M6.1-SP3R: (5’GATTCCGGATGACCTCGGA)
M6.1-SP4F: (5’GATGTTGTACCGCTTGTGCGTT)
M6.2-SP1R: (5’ATAGCCGCGACCAGGTCTT)
M6.2-SP2R: (5’GGCACCATAGTTGTCCGATA)
M6.2-SP3R: (5’ACCGCTTCAGCGGGAGCT)
M6.2-SP4F: (5’ATTCAGATCGTCGTCAGTGCG)

M6.3-SP1R: (5'GTTGGAAAGACACCAAAGCG)
M6.3-SP2R: (5'AACTGTTCGAGTAACAGCACA)
M6.3-SP3R: (5'TATCAGCGCGCATCCAATGA)
M6.3-SP4F: (5'CGGTACTGGTTTATCAGTATGC)

Cloning of CMV-M6

For cloning CMV-M6 into a molecular vector, a long PCR was performed for each RNA from the previously synthetized cDNA using Expand High Fidelity PCR System (Roche Applied Science, Barcelona, Spain), and specific primers introducing a restriction enzyme site. (Table CIII.3). We amplified M6.1 with 109BamF2 and 109Eco-R, M6.2 with 109BamF2 and 209Sac-R and M6.3 with 309Bam-F and 309Eco-R.

Table CIII.3 Primers used to amplify CMV-M6 from cDNA for cloning into pGEM. Restriction enzyme sites are in bold.

109 BamF2 (5'GGGG**GGATCC**GTTTATTTACAAGAGCGTACG)
109 Eco-R (5'CTCT**GAATTC**TGGTCTCCTTTTAGAGACCC)
209 Sac-R (5'GGGG**GAGCTC**TGGTCTCCTTTTGGAGGC)
309 Bam-F (5'GGGG**GGATCC**GTAATCTTACCACTGTGTGTG)
309 Eco-R (5'GGGG**GAATTC**TGGTCTCCTTTTGGAGGCC)

PCR products M6.1, M6.2 and M6.3 were inserted into pGEM®-T vector (Promega Corporation, Madison, WI) following manufacturer's protocol. M6.2 and M6.3 were transformed into *E.coli* DH5α, M6.1 into *E.coli* SURE (Agilent Technologies), both by electroporation. *E.coli* strain DH5α are: F-, endA1, glnV44, thi-1, recA1, relA1, gyrA96, deoR, nupG, Φ80dlacZΔM15 Δ(lacZYA-argF)U169, hsdR17(r_K^- m_K^+), λ–, while *E.coli* strain SURE are: endA1 glnV44 thi-1 gyrA96 relA1 lac recB recJ sbcC umuC::Tn5 uvrC e14- Δ(mcrCB-hsdSMR-mrr)171 F'[proAB+

lacI^{q} lacZΔM15 Tn10].

Insert orientations were checked using a specific primer for the vector and another one for each cDNA. For M6.1 we used M13F and F109-1400R. Orientation of M6.2 was determined with M13F and F209-400F, and M6.3 with M13R and F309-1400R (Table CIII.4).
Full-length clones were sequenced (as described above) to analyse if any mutation occurred during the cloning process.

Table CIII.4 Primers used to determine the insert orientation of M6 clones in pGEM ®-T vector

M13F: (5'GTAAACGACGGCCAGT)
M13R: (5'CAGGAAACAGCTATGAC)
F109-1400R: (5'TTTCCAAGTTGTTCGTACTTC)
F209-400F: (5'CGCTTTTGAGACGATGGTC)
F309-1400R (5'GTTAATAGTTGGACGACCAG)

M6.3 was liberated from pGEM®-T vector digesting with *BamHI* and *EcoRI* and cloned into the binary vector pGREENII61, which had been digested with same enzymes. This vector is a pGREEN II (Hellens *et al.*, 2000) where a 35S cassette from pJIT61 has been cloned removing the whole previous pGreenII polylinker (Martín-Hernández, personal communication). After ligation, pGreenII-M6.3 was transformed to *E.coli* DH5α, then, the purified plasmid DNA was transformed into *Agrobacterium* GV3101.

Comparison of CMV-M6 with other strains

Total RNA, nucleotide sequences of untranslated regions (UTR) and amino acid sequences of open reading frames (ORF) from CMV-M6 were compared to a strain from subgroup IA, CMV-FNY, a strain from subgroup IB, CMV-Pl1, and a strain from subgroup II, CMV-LS. Sequences were obtained from GenBank, strain FNY: NC_002034.1, D00355.1 and D10538.1; strain Pl1: AM183114.1, AM183115.1, AM183116.1; strain LS: AF416899.1, AF416900.1, AF127976.1 corresponding to RNA 1, 2 and 3 respectively.
All the alignments were performed using UniProt (http://www.uniprot.org).

Results

Complete sequence of CMV-M6

In this chapter we obtained the complete sequence of CMV-M6, a strain of CMV belonging to subgroup I (Diaz *et al.*, 2003). Taking into consideration that CMV-FNY is also a strain from subgroup I, and, in our laboratory there is a collection of primers for FNY strain, we used those primers to synthetize the full length cDNA of the three genomic RNAs of CMV-M6 and also to obtain the complete sequence. The inconvenience of using CMV-FNY primers to synthesize the cDNA and perform the full-length PCR was that changes between M6 and FNY in the regions where primers hybridise were undetectable. To solve this problem and obtain the real sequence of the 5′ and 3′ ends of the three RNAs, we used 5′ and 3′ RACE as mentioned in Materials and Methods section.

The results obtained from sequencing the complete cDNAs showed that the size of genomic RNAs of CMV-M6 is 3360, 3046 and 2202 nucleotides corresponding to RNA1, RNA2 and RNA3 respectively. According to Palukaitis and Garcia-Arenal (2003), this is the appropriate length for a subgroup I strain, which also confirmed the previously described classification by Diaz *et al.* (2003).

The complete nucleotide sequence of CMV-M6 was compared to three strains of CMV to have a general vision of identity percentages between strains and also to classify M6 into subgroup IA or IB. The representative strains used to perform the comparison analysis were CMV-FNY from subgroup IA, CMV-Pl1 from IB and CMV-LS from subgroup II. Sequences were obtained from GenBank. (http://www.ncbi.nlm.nih.gov) and were compared using UniProt (www.uniprot.org). Table 1 shows the comparisons between total

RNAs and between the 3 RNAs separately. We observed that the total genome of CMV-M6 shared a 98.5% identity with CMV-FNY, 91.2% with CMV-Pl1 and only 72.7% with CMV-LS (Table CIII.5).

Table CIII.5. Nucleotide sequence identity between CMV-M6 and CMV-FNY, CMV-Pl1 and CMV-LS

	CMV-FNY (IA)	CMV-Pl1 (IB)	CMV-LS (II)
RNA1	98.2%	91.4%	75.9%
RNA2	98.6%	90.9%	69.9%
RNA3	98.6%	91.3%	72.2%
Total RNA	98.5%	91.2%	72.7%

Comparing separately the amino acid sequences of the five ORFs of CMV-M6 with those of each strain, we obtained similar percentages of identity to those showed in Table CIII.5 for the comparison of total RNA genomes (Table CIII.6). Identity of all M6 ORFs with CMV-FNY is about 99.5%, the highest identity, whereas CMV-Pl1 also had a high percentage of identity with M6 compared to CMV-LS. ORF 2b is the most variant ORF region, sharing only 45.5% with CMV-LS and 80.2% with Pl1, whereas for FNY the identity is 99.1%. ORF 3b encodes for the coat protein (CP) and its amino acid sequence is used for the distribution of CMV subgroups (Verma *et al.*, 2006). Comparison of the CP of M6 with the other strains, represented in Figure CIII.1A, showed that CMV-M6 belongs to subgroup IA since it is 100% identical to CMV-FNY.

Table CIII.6. Comparison between CMV-M6 and CMV-FNY, CMV-Pl1 and CMV-LS. Open reading frames (ORFs) and untranslated regions (UTRs) were compared using the amino acid sequence and the nucleotide sequence respectively.

ORFs	CMV-FNY (IA)	CMV-PL1 (IB)	CMV-LS (II)
1a	99.5%	97.8%	86%
2a	98.7%	93.2%	74.6%
2b	99.1%	80.2%	45.5%
3a	100%	94.7%	83.3%
3b	100%	98.2%	81.7%

UTRs	CMV-FNY (IA)	CMV-PL1 (IB)	CMV-LS (II)
RNA1-5'UTR	98.9%	96.8%	77.8%
RNA1-3'UTR	99.3%	89.0%	60.1%
RNA2-5'UTR	98.8%	83.7%	73.1%
RNA2-3'UTR	97.3%	88.1%	61.5%
RNA3-5'UTR	85.2%	82.0%	42.5%
RNA3-mUTR	98.6%	82.4%	64.5%
RNA3-3'UTR	99.0%	90.5%	64.0%

Comparisons of nucleotide sequences of the UTRs are represented in Table CIII.6. CMV-FNY shows an identity of more than 98.5% with CMV-M6 in all the UTRs with the exception of 5'UTR from RNA3, which has 85.2% identity. This region is also the less conserved UTR with Pl1 and LS sharing 82% and 42.5% respectively. 5'UTR sequence from RNA3 is used, as CP, to divide subgroup I into two groups: IA and IB (Roossinck *et al.*, 1999). Its alignment is shown in figure 1B and, according to the length of the GT repetition starting at the nucleotide 14, CMV-M6 belongs to subgroup IA, confirming the result previously obtained with the alignment of CPs. Additionally, M6 has an interesting deletion of 11 nucleotides at position 82, which was not present in the other IA strains (Roossinck *et al.*, 1999). Strains from subgroup IB and II presented a deletion in this region but not in the same positions (Figure CIII.1B), even though some of the deleted nucleotides of M6 were overlapping with the deletions present also in Pl1 and LS.

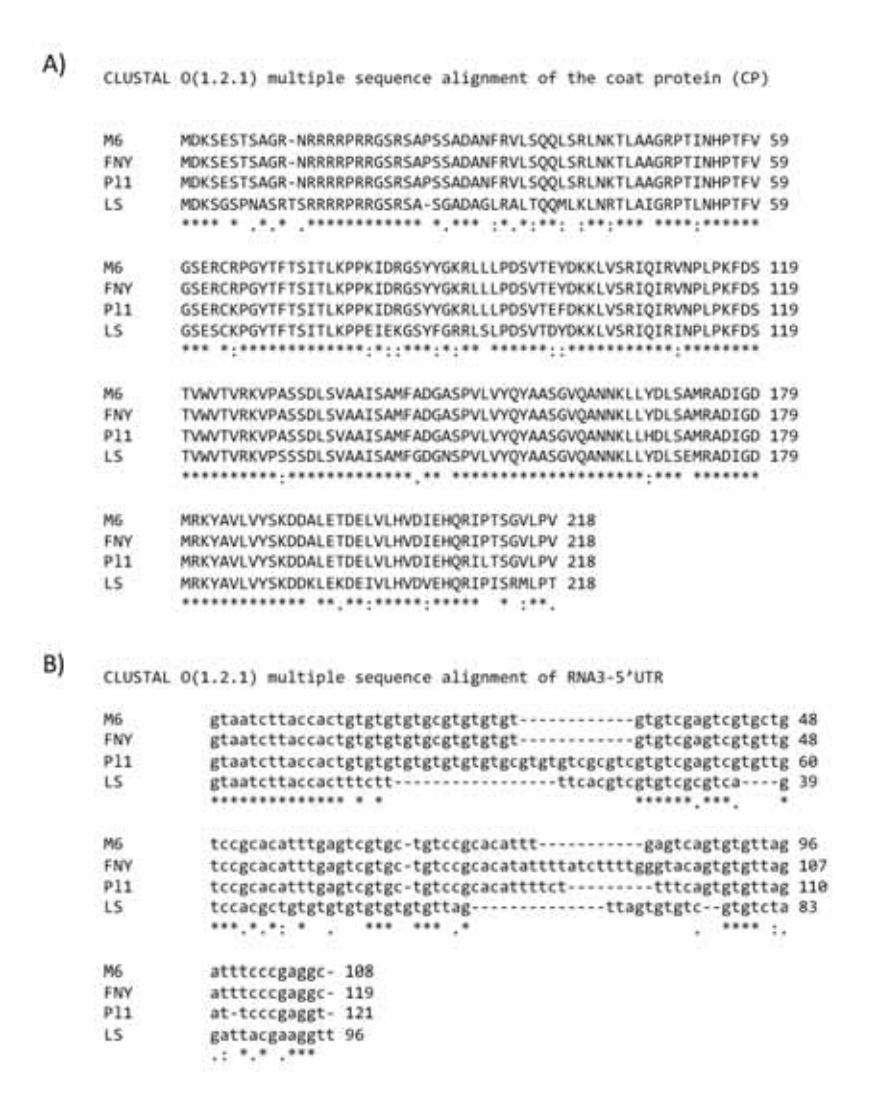
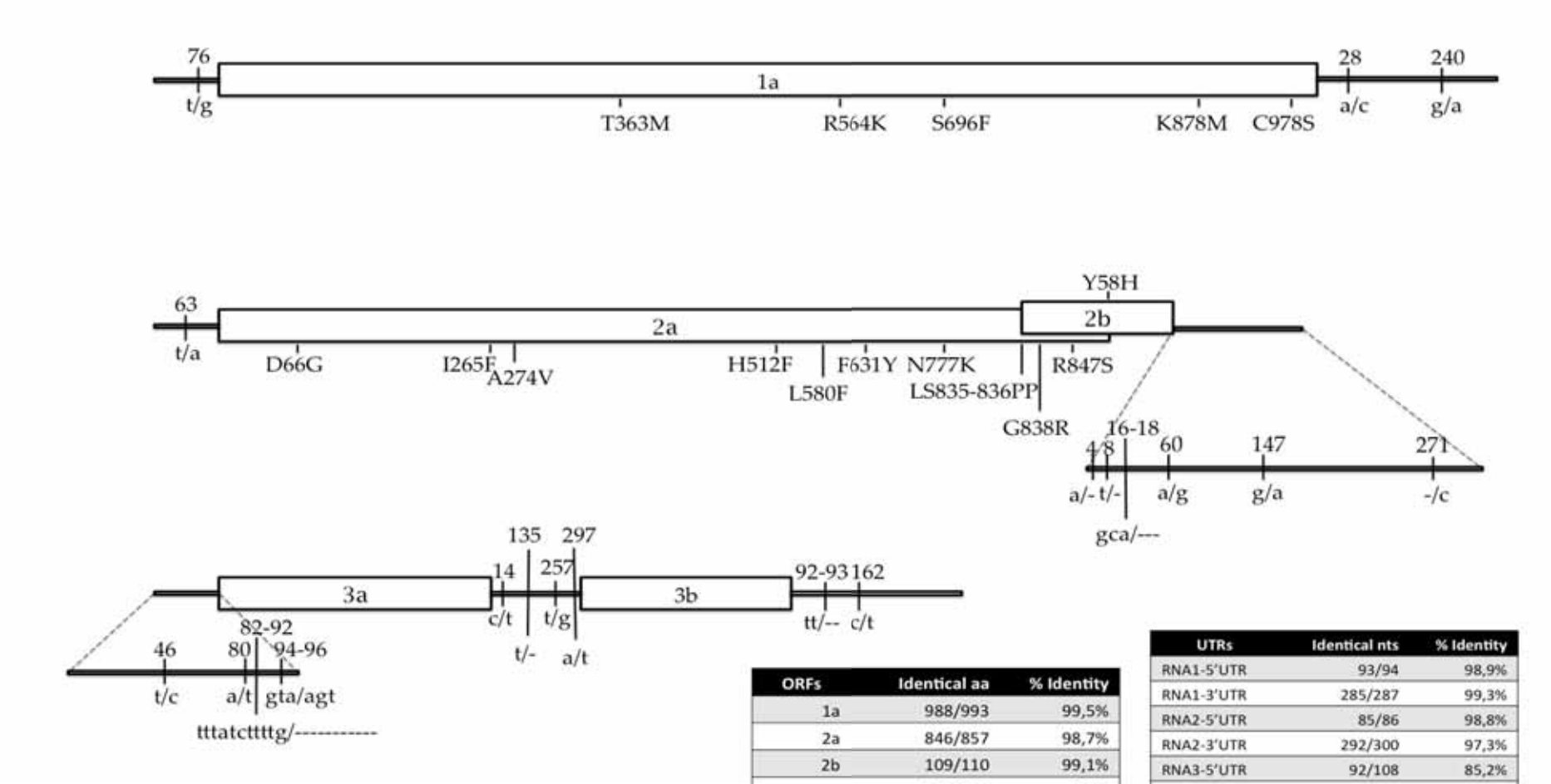


Figure CIII.1. Alignment of CPs (A) and RNA3-5'UTRs (B) from CMV-M6, CMV-FNY representing subgroup IA, CMV-Pl1 (IB) and CMV-LS (II)

Our results are similar to those previously described by Roossinck (2001), who determined that between subgroups IA and IB there was a

close sequence relationship (92–95% identity), while subgroup-II were quite distantly related to subgroup I with only 75% sequence identity.

As we have determined, CMV-M6 and CMV-FNY are members of subgroup IA. We have compared in more detail both strains and show schematically all the nucleotides and amino acids changes in figure CIII.2. Briefly, RNA1 had the lowest nucleotide identity rate (98.2%) having 3333 identical nucleotides out of 3360. Complete RNA2 had 98.6% identity with CMV-FNY and it was 4 nucleotides shorter than CMV-FNY because in the 3'UTR region there was a five-nucleotide deletion and one nucleotide insertion. Regarding RNA3 that share 98.6% of nucleotides with FNY has all the mismatches in UTR regions, and 14 of them were deletions. The ORFs of both strains are 100% identical.



ORFs	Identical aa	% Identity
1a	988/993	99,5%
2a	846/857	98,7%
2b	109/110	99,1%
3a	279/279	100%
3b	218/218	100%

UTRs	Identical nts	% Identity
RNA1-5'UTR	93/94	98,9%
RNA1-3'UTR	285/287	99,3%
RNA2-5'UTR	85/86	98,8%
RNA2-3'UTR	292/300	97,3%
RNA3-5'UTR	92/108	85,2%
RNA3-mUTR	296/300	98,6%
RNA3-3'UTR	303/306	99,0%

Figure CIII.2. Representation of CMV-M6 genome considering CMV-FNY as a reference. Nucleotide and amino acid changes are represented in minor and capital letters respectively. Changes between CMV-FNY and M6 in all the UTRs and ORFs are summarized in both tables.

Infectious clones of M6

Once known the complete sequence of M6, we developed a genetic system of infectious clones of CMV-M6. We followed the strategy based on obtaining full-length cDNAs of the three viral RNAs, cloning them into a plasmid under a promoter for *in vitro* transcription and inoculating the viral RNAs onto plants to test the infection.

cDNAs of CMV-M6 RNAs 1, 2 and 3 were synthesized and PCRs were amplified and cloned into pGEM®-T vector (Promega) as described in Materials and Methods. Ligations containing the constructs with full-length cDNA clones of M6.1, M6.2 and M6.3 were transformed into *E.coli* DH5α cells. M6.2 and M6.3 produced correct full-length clones, but attempts to get full-length clones from M6.1 in *E.coli* DH5α were always unsuccessful. M6.1 (*E.coli* DH5α) colonies from M6.1 were screened by PCR with specific primers of M6.1 and even though some were positive, after the DNA isolation, none of them had the expected length (6.4 kb, including vector and insert), indicating some reorganizations and deletions (Figure 4). We also attempted to get stable clones by incubating the cells at 30ºC instead of 37ºC obtaining the same results. Then, the ligation was transformed into *E.coli* strain SURE (Agilent Technologies), which are endonuclease and recombination deficient, obtaining a colony with full-length size. Figure CIII.3 shows the DNA of expected size extracted from SURE cells.

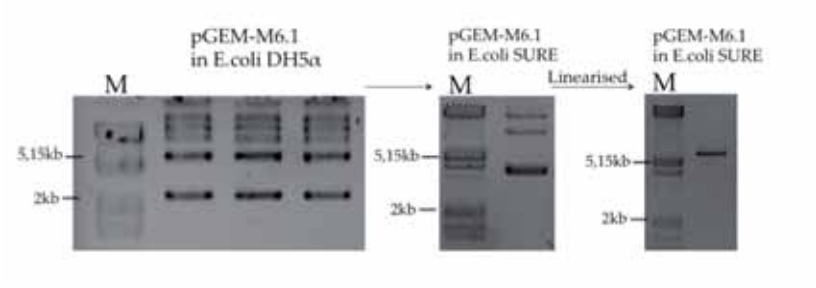


Figure CIII.3. DNA preparation miniprep from pGEM-M6.1 transformed in *E.coli* DH5α and *E.coli* SURE. Expected size of plasmid was 6.4kb (3kb from pGEM and 3.4kb from M6.1). pGEM-M6.1 in SURE was linearized with *PstI.*

Orientation of the inserts was analysed by PCR with specifics primers, determining that M6.1 is cloned to be transcribed from the T7 promoter and M6.2 and M6.3, from SP6 promoter as shown in figure CIII.4. Infective clones were sequenced to analyse if there were mutations with respect to the original sequences obtained from original sequencing of PCR products (pg.142). Sequences were completely identical, indicating that no mutations were introduced during the cloning process.

Figure CIII.4. Representation of the infective clones of M6. Orientations are represented with the direction of the green arrow. Arrows outside the plasmids represent the open reading frames (ORF). Introduced restriction sites are also represented.

To facilitate the inoculation and perform agroinfiltration instead of rub-inoculation of the viral RNAs from in vitro transcription, we tried to clone the three viral RNAs in a binary vector pGREENII61 (Hellens *et al.*, 2000)(M. Martín-Hernández, unpublished). In this thesis, we only could obtain the binary construct containing M6.3. Cloning of the cDNA of M6.2 was unsuccessful after three attempts and was temporarily abandoned. Cloning of cDNA of M6.1 in the binary vector was never attempted. To determine the infectivity of the clones, we used *N.benthamiana* and melon as experimental hosts. M6.1 and M6.2 were linearized with *PstI* and *SphI* respectively to perform the transcription. M6.3 was agroinfiltrated into *N.benthamiana* and, after 45 minutes, the RNA of M6.1 and M6.2 resulting from the in vitro transcription was rub-inoculated into the same leaves. *N.benthamiana* showed systemic symptoms of infection between 10 and 21 dpi. Taking the infected symptomatic leaf as source of inoculum we rub-inoculated melon cotyledons of Piel de Sapo (PS) plants. At 14 dpi, 6 out of 6 melons were showing clear and severe symptoms of CMV (Figure CIII.5). This result confirmed that the developed clones of CMV-M6 were infectious both in *N. benthamiana* and in melon.

Figure CIII.5. Symptoms of CMV-M6 in plants inoculated with infective clones. Systemic infection in Piel de Sapo (PS) infected with CMV-M6. Mock-inoculated plant (PS mock) was used as negative control.

Pseudo-recombinants of CMV-FNY and CMV-M6

Pseudo-recombinants combining CMV-FNY and CMV-M6 RNAs were generated for further experiments on CMV resistance. Infectious clones of CMV-M6 (M6.1, M6.2, M6.3) and binary clones of CMV-FNY (pCR1, pCR2, pCR3) were used to produce the pseudo-recombinants. In all experiments, two RNAs from CMV-FNY and one from CMV-M6 were combined to generate M6.1/pCR2/ pCR3, pCR1/M6.2/pCR3 and pCR1/pCR2/M6.3. Combinations were inoculated into *N.benthamiana* plants causing systemic symptoms at 21 dpi (Figure CIII.6). Systemically infected leaves were used as origin of inoculum to rub-inoculate melon cotyledons of Piel de Sapo (PS) which 2 weeks after inoculation were systemically infected with all the pseudo-recombinants. Results indicated that all the performed combinations of the pseudo-recombinants were viable and able to produce a systemic infection both in *N. benthamiana* and melon.

Figure CIII.6. *N.benthamiana* and Piel de Sapo inoculated with CMV-M6 and CMV-FNY pseudo-recombinants. Symptomatic infection in *N.benthamiana* (N.b) and Piel de Sapo (PS) melon of pseudo-recombinants between CMV-FNY (pCR1, pCR2, pCR3) and CMV-M6 (M6.1, M6.2, M6.3). Mock: mock-inoculated plant.

Discussion

In this study, we obtained the sequence of M6, a strain of CMV from subgroup IA. In order to acquire the wildtype M6 sequence, we have to take in consideration that during the process of cDNA synthesis and PCR amplification unintended mutations can be generated affecting the final sequence. To minimize these mutations, three independent reactions for synthesis of the cDNA were performed, then, the obtained cDNAs were mixed to perform the PCR. To avoid errors during the PCR process, a thermostable polymerase enzyme, *Pfu* DNA polymerase, was used since it exhibits 3' →5' exonuclease (proofreading) activity that enables the polymerase to correct errors introduced. Moreover, the sequence was obtained from the population represented in the PCR product, and therefore, represents the whole viral population, not single sequences. Only if a mutation had been introduced in the first cycle, it could have been detected since 50% of the sample would carry it. If it was introduced after that, it would have been very low represented and therefore, not detected in the final sequence. Accordingly, the infectious clones induce systemic symptoms that are similar to that of wildtype CMV-M6. Sequence analysis comparing with CMV-FNY genomic RNAs showed that coding open reading frames (ORFs) of CMV-M6 are very conserved and start and stop codons of the five proteins are conserved.

Focusing on the sequence, in the *Cucumovirus* genus there is a highly conserved 40 nucleotides domain that is present in the 3'UTR of all cucumovirus RNA sequences examined. This region is: 5'GAACGGGUUGUCCAUCCAGCU(N)ACGGCUAAAAUGGUCAG U 3'. The sequence is invariant with the exception of (N) that is a uracil (U) in most of cases. In CMV, this uracil (U) is replaced by an adenine (A) only in RNA1 of two strains: CMV-FNY and CMV-Y (McGarvey *et*

al., 1995). Analysing the 3'UTR of CMV-M6, this domain is conserved, having an adenine (A) in the (N) position of RNA1 3'UTR, as CMV-FNY and unlike CMV-PL1 or CMV-LS. Amino acid sequences of proteins 1a and 2a, which are mainly involved in replication, have a high identity with CMV-FNY, 99.5% and 98.7% respectively and are exchangeable between M6 and FNY, as we have seen by generating pseudo recombinants between both strains. The amino acid sequence of protein 2b is the most highly divergent among the viral proteins sharing 99.1% amino acid identity with CMV-FNY, 80.2% with CMV-Pl1 and only 45.5% with CMV-LS. It has been reported that the high heterogeneity of the 2b gene, in the *Cucumovirus* genus, may give adaptation to various host plants and it was thought that it was required for systemic virus spread and disease induction in its hosts (Ding *et al.*, 1995; Ding *et al.*, 1996a), then, Brigneti *et al.* (1998) discovered that the 2b protein of CMV is a suppressor of post-transcriptional gene silencing, and this gene encodes an arginine-rich amino acid sequence ((22)KRRRRR(27)) that is involved in targeting the protein into the nucleus of the host plant (Lucy *et al.*, 2000). The obtained sequence of CMV-M6 conserves the nuclear localization signal without modifications, as we expected since CMV-FNY and CMV-LS maintain the domain. Interestingly, the amino acid sequence of Pl1 is not exactly conserved ((22)KRQRRR(27)). RNA3 encode for the movement protein and for the coat protein, both proteins have the same amino acid sequence than CMV-FNY and also share a high similarity with CMV-Pl1 and CMV-LS. However, there are some mutations in the three UTR regions in RNA3, although all the domains are conserved. The 5'UTR contains the conserved UG tract, known to be required for the accumulation of CMV RNA3 (Boccard and Baulcombe, 1993). Moreover if we compare RNA3 5'UTR region with CMV-FNY, M6 isolate has a deletion of 11 nucleotides at position 82 (Figure CIII.1). Those 11 nucleotides are present in all the strains of

subgroup IA (Roossinck *et al.*, 1999), however, subgroup IB and II have a deletion from 9 to 14 nucleotides at the same positions. The deletions are neither at the same positions nor the same nucleotides, therefore, a possible recombination of M6 with other strains of other subgroups could not have happenned. Rather, a deletion of 11 nucleotides with respect to strains of subgroup IA is more likely. RNA3 middle UTR (mUTR) region has a conserved motif in CMV and BMV that is necessary for synthesis of subgenomic RNA4 (Boccard and Baulcombe, 1993; Smirnyagina *et al.*, 1994) and this region is conserved in CMV-M6 indicating a normal synthesis of the RNA encoding the coat protein. To sum up, CMV-M6 conserve all the motifs present in other cucumoviruses.

The three infectious clones of CMV-M6 were developed under a promoter suitable for "in vitro" transcription. M6.2 and M6.3 were cloned and transformed into *E.coli* without difficulties, but in the case of M6.1, this clone was repeatedly unstable in *E.coli*. Some clones containing complex viral genomes are unstable when they are propagated in *E.coli*, being the sequence modified with point mutations or deletions produced during the growth of the culture (Boyer and Haenni, 1994). However, these problems in plasmid instability can be potentially solved using simple strategies like transforming the constructs in different strains of *E.coli*, using low copy number plasmid vectors (Stoker *et al.*, 1982), modifying culture growth temperature (Hanahan *et al.*, 1991) or introducing an intron in the viral sequence to block putative cryptic promoters that can express some products potentially harmful for the bacteria. Once in the plant, the intron is processed and the virus, normally expressed (Johansen, 1996). In this assay, incubating the culture at 30ºC instead at 37ºC did not allow us to obtain a full length clone of CMV-M6.1 in *E.coli* DH5α. The use of SURE strain, specifically designed for unstable plasmids

allowed us to get the full length clone. Some studies have also showed that transformation with this strain reduce the probability of deletions in unstable sequences (Singh and Singh, 1995).

Infectious clones of CMV-M6 and CMV-FNY provide us a tool to study CMV resistance. DHL line 2012, from a population of Piel de Sapo (PS) and Songwhan Charmi PI 161375 (SC) as susceptible and resistant parental lines, respectively (Gonzalo *et al.*, 2011), carries at least three QTLs that confer resistance to CMV-M6 (Guiu-Aragonés *et al.*, 2014). Even though, CMV-M6 and CMV-FNY are 98.5% identical in nucleotide sequence, CMV-FNY is able to infect systemically DHL 2012, meaning that CMV-FNY has one or more determinant of virulence that overcome the resistance conferred by the three QTLs. Pseudo recombinants between CMV-FNY and CMV-M6 were performed in this study and we confirmed that the combinations are viable. Cillo *et al.* (2009) described that exchange of the 3' sequence of CMV RNA2 between strains affect the accumulation of specific viral RNAs and the disease phenotype. In these pseudo recombinants we combined two RNAs from CMV-FNY and one from CMV-M6. The combinations between FNY and M6 will be tested in the future in the DHL 2012 as a starting point to identify the determinant of virulence that is different between these two strains. Since both strains have a very high percentage of identity, once found the determinant of virulence between FNY and M6, it should be easy to determine the responsible mutation(s).

Previous results from the tissue printing experiments on inoculated leaf showed that both CMV-FNY and CMV-LS from subgroup IA and II respectively are able to move cell-to-cell in both the *cmv1*-carrying line SC12-1-99 and the resistant parent SC (Guiu-Aragonés, Chapter II). Since CMV-M6 is a member from subgroup IA and DHL2012 has the

SC QTLs that confer resistance to M6, we believe that the virus would be able to replicate and move cell-to-cell in this line. Therefore the mechanism of resistance would be involved in long distance movement: entrance into the phloem, movement though the phloem or unloading the virus from the phloem. All the CMV proteins have been described as determinants of virulence (Mochizuki and Ohki, 2012). If we try to hypothesize for important changes between CMV-FNY and CMV-M6 which can be important for virulence, we can discard MP and CP because there are no differences in amino acid sequence between both strains. Protein 2b, the suppressor of RNA silencing that suppress the silencing signal (Brigneti *et al.*, 1998) has only one amino acid change, from a tyrosine (Y) to a histidine (H), an important change that might modify the protein structure allowing the interaction with PS host factors but impeding the interaction with SC ones. In 2a protein, although it is the viral replicase, Kim and Palukaitis (1997) described that an amino acid substitution in 631 F→T results from a local, hypersensitive response to a systemic mosaic phenotype in cowpea. In CMV-M6 the residue in this position is a tyrosine, a conserved change, which, nevertheless could also a role in interaction with host factors. Regarding to protein 1a, which is the other protein reported to be involved in viral replication, none of the previous reports of 1a as a determinant of virulence (Diveki *et al.*, 2004; Kang *et al.*, 2012) had described the same amino acid changes present between CMV-FNY and CMV-M6, but it can be interesting to study the helicase domain of 1a because it has been reported to affect the systemic infection in pepper (Kang *et al.*, 2012). Also, sometimes host factors are able to modify viral proteins and these modifications allow a different behaviour of the virus. For example, a methyltransferase present in tobacco plants is able to methylate CMV 1a protein and promote the systemic spread of the virus (Kim *et al.*, 2008).

Even though MP amino acid sequences are identical between both strains, changes in RNA3 5′ UTR may be able to affect the level of the MP protein in infected cells; Yoshii *et al.* (2004) previously reported that *Arabidopsis* mutations in eIF4E and 4G actually affected the 3a translation efficiency in a 5′ UTR-sequence dependent manner. CMV-M6 present a deletion of 11 nucleotides in that region, which doesn't impede the systemic infection in Piel de Sapo, but it could be affected in the DHL2012 carrying QTLs from SC that confer resistance to CMV-M6.

Future experiments, with the aid of the obtained CMV-M6 infectious clones, will increase the knowledge of the mechanisms of resistance to CMV.

General discussion

cmv1 was described as a gene conferring recessive resistance to some strains of CMV in melon (Essafi *et al.*, 2009). In this thesis we have carried out a further study with CMV strains and we have described that *cmv1* confers resistance to strains of CMV from subgroup II and not from subgroup I.

The determinant of virulence against *cmv1* is the MP. We have also identified a combination of four positions in the MP, one of them involving a group of five amino acids, which permit the complete gain-of-function of the LS MP in the resistant melon. These residues are maintained between subgroups, indicating that we can associate them with cmv1 resistance. Association of subgroup strains with a trait is scarce. It has only been described for two traits. One of them is the temperature of infection, since strains belonging to subgroup I are called heat-resistant because virus can complete their cycle at higher temperatures, while strains belonging to subgroup II are heat-sensitive, since they can only propagate at lower temperatures. The second trait is the capacity to produce different symptoms in tobacco plants. While subgroup II cause typical etch symptoms, subgroup I strains cause severe systemic mosaic symptoms (Zhang *et al.*, 1994). To our knowledge, in this thesis we have described the first report of a phenotype of resistance associated with subgroups of CMV. The introduction of *cmv1* gene to breeding programs would let to obtain plants resistant to strains of subgroup II but not to subgroup I. Distribution of both subgroups is worldwide and they both infect a high amount of plant hosts. As subgroup II strains are heat-sensitive, they have a higher fitness when temperatures are below 24ºC. The majority of melon varieties need more than 24ºC to grow and ripe. Nevertheless, *cmv1*-mediated resistance can be important for early melon varieties or short-season melons like cantaloupe or charentais. Melon fruits from SC12-1, a NIL that contains *cmv1* in an introgression

larger than the line SC12-1-99 in linkage group XII, are significantly similar in terms of weight and sugar contents to those of Piel de Sapo cultivar (J. Argyris, personal communication) indicating that the *cmv1* gene is not altering these traits.

All CMV proteins have been found in different systems to act as determinant of virulence (Mochizuki and Ohki, 2012), but a role of RNA itself as determinant of virulence in CMV has not been reported. In this study, among the gain of function mutants generated, those with a combination of four residue changes resulted infectious in SC12-1-99, but mutants with fewer changes did not. A comparison of the RNA structures of the whole RNA3 of all the constructs generated using the program RNAalifold (http://rna.tbi.univie.ac.at/cgi-bin/RNAalifold.cgi) showed that the structure of LS RNA 3 presented many more loops than that of FNY and the mutants varied between the LS-like structure, and a more FNY-like. However, the virulent mutant L3-MP3/4/6/7 with 22 nucleotides exchanged (see figure CI.4, in chapter I) acquired the same RNA conformation than the mutant L3-MP3/4 with 16 nts exchanged, which is non virulent in the resistant plant (figure D.1.). Therefore, there was no correlation between a given conformation of the RNA3 and capacity to overcome the resistance, suggesting that the RNA does not have a role as determinant of virulence to overcome *cmv1*-mediated resistance.

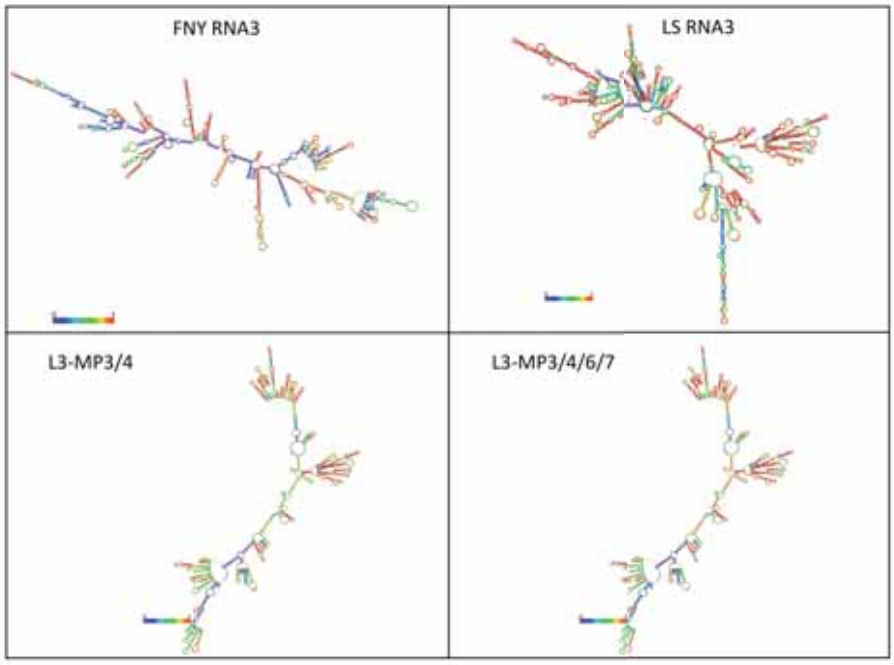


Figure D.1. RNA secondary structure prediction of RNA3 of CMV-LS, CMV-FNY, and the mutants L3-MP3/4 and L3-MP3/4/6/7.

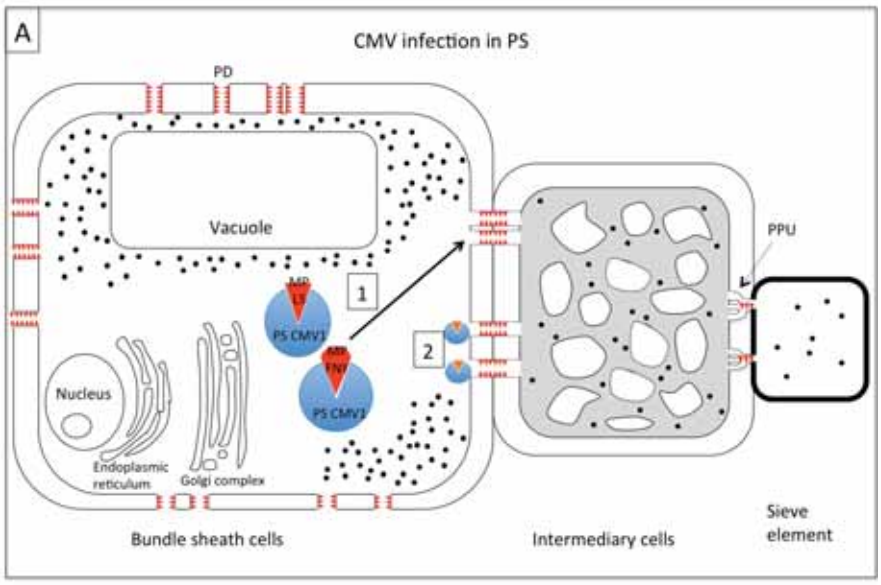
The absence of a published tertiary structure for the MP of CMV makes the search of the important amino acids affecting the structure more difficult. However, the program SCRATCH protein predictor permit to differentiate two domains in the MP. The amino acid residues determinants for virulence against the *cmv1* gene are located in the domain 1. The domain 2 includes from residue 226 to 279. The last 33 C-terminal amino acids of the MP interact with CP for movement (Kim *et al.*, 2004; Nagano *et al.*, 2001), which would let the first domain of the MP free for possible interactions with host factors that can be directly or indirectly related to the *cmv1* gene.

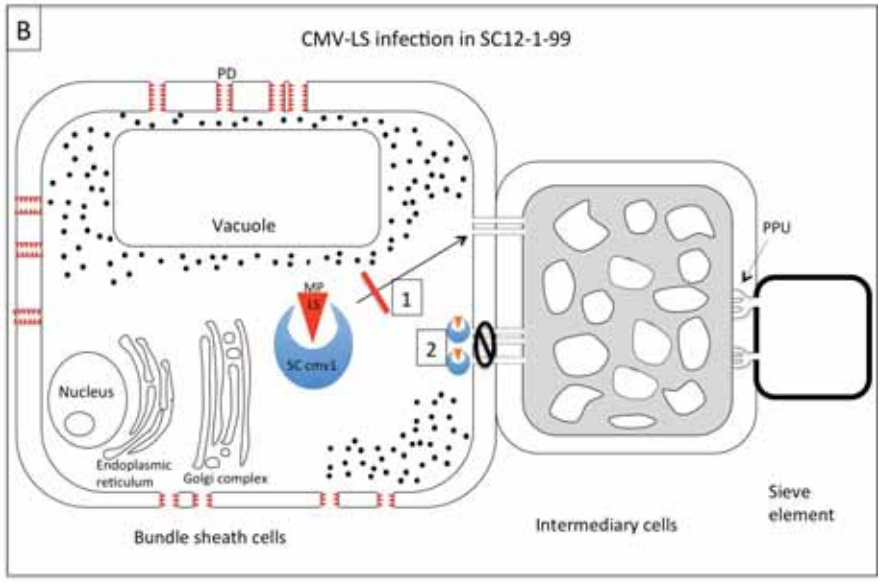
Results from the characterization of line SC12-1-99 (*cmv1*), allow us to locate the barrier for the systemic infection in the bundle sheath cells, indicating that CMV-LS is not present neither in the vascular

parenchyma cells nor intermediary cells. Considering that the most likely candidate gene for *cmv1* is a vacuolar sorting protein (VPS) (A. Giner, personal communication) and that the determinant of the virulence for the *cmv1*-mediated resistance is the movement protein, we should locate the role of *cmv1* in the bundle sheath cells and involved in the transport of CMV to VP or CC. Results from studies of the expression of *CMV1* in PS and *cmv1* in SC indicate that both alleles are equally expressed in the leaf tissue (A. Giner, personal communication). Therefore, the phenotype of resistance to CMV in SC is not due a lack of expression of *cmv1* in the leaf. Still, there could be a differential expression of both alleles only in BS cells that could be masked when a bigger area of the leaf is examined. However, the fact that introducing four mutations into MP LS can mimic MP FNY, suggests that MP LS structure impedes the accommodation of SC alleles of cmv1, whereas it can accommodate CMV1. This might be the mechanism involved in *cmv1*-mediated resistance, rather than a specific lack of expression of *cmv1* in BS cells. Alternatively, if *cmv1* was not being expressed in BS cells, MPFNY still could interact with other gene products (*cmvqw3.1* or *cmvqw10.1*), for instance (Guiu-Aragonés *et al.*, 2014) instead of with *cmv1*, to invade the phloem. Consequently, the gain of function MPLS might also acquire the ability to interact with those other gene products (*cmvqw3.1* or *cmvqw10.1*).

Focusing in the BS cells, PS CMV1 protein could be directly or indirectly interacting with the MP of CMV-LS permitting the virus to infect the minor veins cells (VP and IC). Movement from ME to ME, ME to BS, and BS to BS requires only viral MP to increase the SEL of PD. Movement from BS to VP, VP to CC, or BS to CC requires additional viral and host factors apart from the MP to increase the SEL because it occurs by a different pathway that is still unclear

(Waigmann *et al.*, 2004; Ding *et al.*, 1992). In the case that CMV-LS or CMV-FNY infects PS, CMV1 protein could be needed in the PD of the BS cells, to increase the SEL of PD (figure D.2A-2), or it might be involved in the intracellular transport of the MP to PD (figure D.2A-1). This interaction would permit the virus to infect VP and IC entering the SE to finally cause a systemic infection (figure D.2A). In the case of SC12-1-99 infected by CMV-LS, SC cmv1 protein would not interact with the MP LS in the BS cells, and the virus would be blocked there (figure D.2B). The limitation in this cell type can be because SC cmv1 is not functional as a protein that contributes to increase the SEL of the PD (figure D.2B-2), or that cmv1 protein can be impeding the intracellular transport of the MP LS to the PD (figure D.2B-1). SC12-1-99 can only be systemically infected by CMV-FNY or by CMV-LS when the MP is mutated in the four identified sequence positions from CMV-FNY (L3-MP3/4/6/7 from chapter 1). The introduced mutations from FNY in the MP LS would permit its interaction with SC cmv1 protein establishing a systemic infection in the SC12-1-99 line (figure D.2C).


A
CMV infection in PS
PD
Vacuole
PPU
MP
LS
PS CMV1
1
2
MP
PNY
PS CMV1
Nucleus
Endoplasmic reticulum
Golgi complex
Bundle sheath cells
Intermediary cells
Sieve element


B
CMV-LS infection in SC12-1-99
PD
Vacuole
PPU
MP
LS
SC cmv1
1
2
Nucleus
Endoplasmic reticulum
Golgi complex
Bundle sheath cells
Intermediary cells
Sieve element

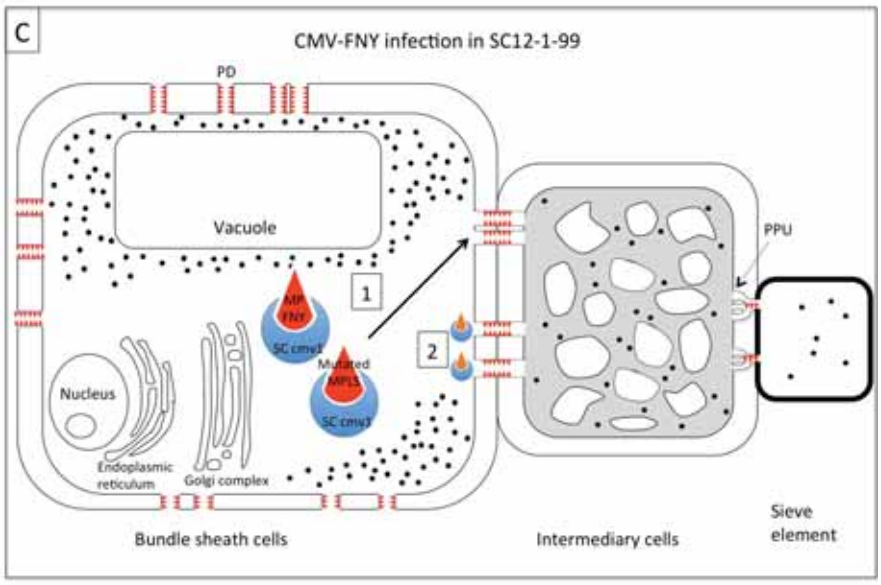


Figure D.2. Hypothetical model of CMV-LS and CMV-FNY infecting PS and SC12-1-99. In the PD surrounding the BS, MP (red triangles) is opening the SEL. In all the cases, virus (black dots) is accumulated in the bundle sheath cells (BS). For simplicity, only traffic between BS and ICs is represented. The model would be the same for traffic between BS and VP. A) Situation in which CMV infect PS. In the BS, the protein PS CMV1 can interact directly or indirectly with movement proteins of LS and FNY either to be transported to PDs (1) or to open PDs (2). PD between BS-IC can be opened permitting the systemic spread of the virus. B) SC12-1-99 infected with CMV-LS. MP LS is not able to interact with SC cmv1 protein impeding the intracellular traffic of the virus to the PD (1) or through the PD (2) between BS-IC. C) CMV-FNY infection of SC12-1-99. MP FNY or mutated MP LS with the relevant FNY residues (L3-MP3/4/6/7), are able to interact with the SC protein of cmv1 and permit the intracellular trafficking to PDs (1) or the aperture of the PD (2) and the trafficking of CMV to the IC and SE.

The resistant parental line SC, which carries at least three QTLs involved in the resistance to CMV (Guiu-Aragonés *et al.*, 2014), permits replication and cell-to-cell movement of the virus as well as SC12-1-99 (figure D2). This line is, therefore, an interesting model to

study. The resistance conferred by these new QTLs, presumably will also involve restriction of the viral movement, as *cmv1* does. The characterization of this resistance will involve the use of the strain M6, together with the strains LS and FNY, used in this thesis. The use of infectious clones of CMV-M6 developed, in this thesis, will be of most importance both for a deeper study of the mechanisms involved in CMV resistance present in the accession SC and for the analysis and identification of additional determinants of virulence in CMV.

Conclusions

- The analysis of 11 strains of CMV subgroups I and II in the melon line SC12-1-99 has permitted to establish that *cmv1* confers resistance to strains of CMV subgroup II but not to strains of subgroup I.

- The generation of pseudo recombinants and chimeric viruses between CMV-FNY (subgroup I) and CMV-LS (subgroup II) has allowed us to identify the MP as determinant of virulence.

- A combination of four residue positions in the MP (the group 64-68 (SNNLL) to (HGRIA), and the point mutations R81C, G171T and A195I) are necessary for a complete gain of function of the LS MP in the line SC12-1-99.

- Both the susceptible PS line and the resistant SC12-1-99 allow to the CMV-LS strain the replication and the cell-to-cell movement in the inoculated leaf. Therefore, the gene *cmv1* does not interfere with these two processes.

- CMV-LS has not been detected in the phloem of the resistant line SC12-1-99.

- CMV-LS cannot infect SC12-1-99 even when it is provided from the phloem in grafting experiments.

- The restriction to enter the phloem of the SC12-1-99 line is not related to RNA silencing, since the MP does not have a suppressor of silencing activity.

- The movement of CMV-LS in the resistant line SC12-1-99 (*cmv1*) is blocked in the BS cells, being unable to enter the VP cells and IC. Therefore, this boundary between BS and VP or IC impedes the systemic infection in the line carrying *cmv1*.

- The sequence of CMV-M6 reveals that it is a strain of subgroup IA with high similarity with CMV-FNY strain.

- We have generated molecular clones able to infect systemically *N.benthamiana* and melon.

Bibliography

Adams, I. P., Glover, R. H., Monger, W. A., Mumford, R., Jackeviciene, E., Navalinskiene, M., Samuitiene, M. & Boonham, N. (2009). Next-generation sequencing and metagenomic analysis: a universal diagnostic tool in plant virology. *Mol Plant Pathol* 10(4): 537-545.

Agrios, G. (2005). *Plant Pathology.* London, Elsiver Academic Press, Inc.

Ahlquist, P., Dasgupta, R. & Kaesberg, P. (1981). Near identity of 3- RNA secondary structure in bromoviruses and cucumber mosaic virus. *Cell* 23(1): 183-189.

Ahlquist, P., French, R., Janda, M. & Loesch-Fries, L. S. (1984). Multicomponent RNA plant virus infection derived from cloned viral cDNA. *Proceedings of the National Academy of Sciences of the United States of America* 81(22 I): 7066-7070.

Ahlquist, P. & Janda, M. (1984). cDNA cloning and in vitro transcription of the complete brome mosaic virus genome. *Molecular and Cellular Biology* 4(12): 2876-2882.

Albar, L., Bangratz-Reyser, M., Hebrard, E., Ndjiondjop, M. N., Jones, M. & Ghesquiere, A. (2006). Mutations in the eIF(iso)4G translation initiation factor confer high resistance of rice to Rice yellow mottle virus. *Plant J* 47(3): 417-426.

Amano, M., Mochizuki, A., Kawagoe, Y., Iwahori, K., Niwa, K., Svoboda, J., Maeda, T. & Imura, Y. (2013). High-resolution mapping of zym, a recessive gene for Zucchini yellow mosaic virus resistance in cucumber. *Theor Appl Genet* 126(12): 2983-2993.

Amari, K., Boutant, E., Hofmann, C., Schmitt-Keichinger, C., Fernandez-Calvino, L., Didier, P., Lerich, A., Mutterer, J., Thomas, C. L., Heinlein, M., Mély, Y., Maule, A. J. & Ritzenthaler, C. (2010). A family of plasmodesmal proteins with receptor-like properties for plant viral movement proteins. *PLoS Pathogens* 6(9).

Andreev, I. A., Hyon Kim, S., Kalinina, N. O., Rakitina, D. V., Fitzgerald, A. G., Palukaitis, P. & Taliansky, M. E. (2004). Molecular interactions between a plant virus movement protein and RNA: force spectroscopy investigation. *J Mol Biol* 339(5): 1041-1047.

Baulcombe, D. C. (2000). Molecular biology. Unwinding RNA silencing. *Science* 290(5494): 1108-1109.

Ben-Chaim, A., Grube, R., Lapidot, M., Jahn, M. & Paran, I. (2001). Identification of quantitative trait loci associated with tolerance to Cucumber mosaic virus in Capsicum annuum. *Theor. Appl. Genet* 102: 1213.

Blackman, L. M., Boevink, P., Santa Cruz, S., Palukaitis, P. & Oparka, K. J. (1998). The movement protein of *Cucumber mosaic virus* traffics into sieve elements in minor veins of *Nicotiana clevelandii*. *Plant Cell* 10(4): 525-537.

Boccard, F. & Baulcombe, D. (1993). Mutational analysis of cis-acting sequences and gene function in RNA3 of cucumber mosaic virus. *Virology* 193(2): 563-578.

Boccard, F. & Baulcombe, D. C. (1992). Infectious in vitro transcripts from amplified cDNAs of the Y and Kin strains of cucumber mosaic virus. *Gene* 114(2): 223-227.

Bonnet, J., Fraile, A., Sacristan, S., Malpica, J. M. & Garcia-Arenal, F. (2005). Role of recombination in the evolution of natural populations of Cucumber mosaic virus, a tripartite RNA plant virus. *Virology* 332(1): 359-368.

Boulton, M. I., Maule, A. J. & Wood, K. R. (1985). Effect of actinomycin D and UV irradiation on the replication of cucumber mosaic virus in protoplasts isolated from resistant and susceptible cucumber cultivars. *Physiological Plant Pathology* 26(3): 279-287.

Boyer, J. C. & Haenni, A. L. (1994). Infectious transcripts and cDNA clones of RNA viruses. *Virology* 198(2): 415-426.

Brigneti, G., Voinnet, O., Li, W. X., Ji, L. H., Ding, S. W. & Baulcombe, D. C. (1998). Viral pathogenicity determinants are suppressors of transgene silencing in *Nicotiana benthamiana*. *EMBO Journal* 17(22): 6739-6746.

Brodersen, P. & Voinnet, O. (2006). The diversity of RNA silencing pathways in plants. *Trends Genet* 22(5): 268-280.

Brotman, Y., Normantovich, M., Goldenberg, Z., Zvirin, Z., Kovalski, I., Stovbun, N., Doniger, T., Bolger, A. M., Troadec, C., Bendahmane, A., Cohen, R., Katzir, N., Pitrat, M., Dogimont, C. & Perl-Treves, R. (2012). Dual Resistance of Melon to Fusarium oxysporum Races 0 and 2 and to Papaya ring-spot virus is Controlled by a Pair of Head-to-Head-Oriented NB-LRR Genes of Unusual Architecture. *Molecular Plant*.

Brown, D. J. & MacFarlane, S. A. (2001). 'Worms' that transmit viruses. *Biologist (London)* 48(1): 35-40.

Bruun-Rasmussen, M., Moller, I. S., Tulinius, G., Hansen, J. K., Lund, O. S. & Johansen, I. E. (2007). The same allele of translation initiation factor 4E mediates resistance against two Potyvirus spp. in Pisum sativum. *Mol Plant Microbe Interact* 20(9): 1075-1082.

Buschges, R., Hollricher, K., Panstruga, R., Simons, G. & Wolter, M. (1997). The barley Mlo gene: a novel control element of plant pathogen resistance. *Cell* 88: 695.

Campbell, R. N. (1996). Fungal transmission of plant viruses. *Annu Rev Phytopathol* 34: 87-108.

Canto, T., Choi, S. K. & Palukaitis, P. (2001). A subpopulation of RNA 1 of Cucumber mosaic virus contains 3′ termini originating from RNAs 2 or 3. *Journal of General Virology* 82(4): 941-945.

Canto, T. & Palukaitis, P. (1999). Are Tubules Generated by the 3a Protein Necessary for Cucumber Mosaic Virus Movement? *Molecular Plant-Microbe Interactions* 12(11): 985-993.

Canto, T., Prior, D. A., Hellwald, K. H., Oparka, K. J. & Palukaitis, P. (1997). Characterization of cucumber mosaic virus. IV. Movement protein and coat protein are both essential for cell-to-cell movement of cucumber mosaic virus. *Virology* 237(2): 237-248.

Caranta, C., Pflieger, S., Lefebvre, V., Daubeze, A. M., Thabuis, A. & Palloix, A. (2002). QTLs involved in the restriction of cucumber mosaic virus (CMV) long-distance movement in pepper. *Theor Appl Genet* 104(4): 586-591.

Carmo-Sousa, M., Moreno, A., Garzo, E. & Fereres, A. (2013). A non-persistently transmitted-virus induces a pull-push strategy in its aphid vector to optimize transmission and spread. *Virus Res*.

Carrington, J., Kasschau, K., Mahajan, S. & Schaad, M. (1996). Cell-to-cell and long-distance transport of viruses in plants. *Plant Cell* 8: 1669.

Carrington, J. C. (2000). RNA silencing. Moving targets. *Nature* 408(6809): 150-151.

Celebi-Toprak, F., Slack, S. A. & Russo, P. (2003). Potato resistance to Cucumber Mosaic Virus is temperature sensitive and virus-strain specific. *Breed Sci* 53(1): 69-75.

Chen, Y., Chen, J., Zhang, H., Tang, X. & Du, Z. (2007). Molecular evidence and sequence analysis of a natural reassortant between Cucumber mosaic virus subgroup IA and II strains. *Virus Genes*.

Cheng, N. H., Su, C. L., Carter, S. A. & Nelson, R. S. (2000). Vascular invasion routes and systemic accumulation patterns of tobacco mosaic virus in Nicotiana benthamiana. *Plant J* 23(3): 349-362.

Choi, S. K., Palukaitis, P., Min, B. E., Lee, M. Y., Choi, J. K. & Ryu, K. H. (2005). Cucumber mosaic virus 2a polymerase and 3a movement proteins independently affect both virus movement and the timing of symptom development in zucchini squash. *J Gen Virol* 86(Pt 4): 1213-1222.

Cillo, F., Mascia, T., Pasciuto, M. M. & Gallitelli, D. (2009). Differential effects of mild and severe Cucumber mosaic virus strains in the perturbation of MicroRNA-regulated gene expression in tomato map to the 3' sequence of RNA 2. *Mol Plant Microbe Interact* 22(10): 1239-1249.

Cillo, F., Roberts, I. M. & Palukaitis, P. (2002). In situ localization and tissue distribution of the replication-associated proteins of Cucumber mosaic virus in tobacco and cucumber. *J Virol* 76(21): 10654-10664.

Citovsky, V., Knorr, D., Schuster, G. & Zambryski, P. (1990). The P30 movement protein of *tobacco mosaic virus* is a single-strand nucleic acid binding protein. *Cell* 60(4): 637-647.

Codoner, F. M. & Elena, S. F. (2008). The promiscuous evolutionary history of the family Bromoviridae. *J Gen Virol* 89(Pt 7): 1739-1747.

Coudriet, D. L., Kishaba, A. N. & Bohn, G. W. (1981). Inheritance of resistance to *Muskmelon necrotic spot virus* in a melon aphidresistant breeding line of muskmelon. *Euphytica* 93: 331-337.

Crafts, A. S. (1932). Phloem anatomy, exudation and transport of organic nutrients in cucurbits. *Plant Physiol* 7(2): i4-225.

Dahmani-Mardas, F., Troadec, C., Boualem, A., Leveque, S., Alsadon, A. A., Aldoss, A. A., Dogimont, C. & Bendahmane, A. (2010). Engineering melon plants with improved fruit shelf life using the TILLING approach. *PLoS ONE* 5(12): e15776.

Dangl, J. L. & Jones, J. D. (2001). Plant pathogens and integrated defence responses to infection. *Nature* 411(6839): 826-833.

Daryono, B. S., Wakui, K. & Natsuaki, K. T. (2010). Linkage analysis and mapping of scar markers linked to CMV-B2 resistance gene in melon. *Sabrao Journal of Breeding and Genetics* 42(1): 35-45.

Dawson, W. O., Beck, D. L., Knorr, D. A. & Grantham, G. L. (1986). cDNA cloning of the complete genome of tobacco mosaic virus and production of

infectious transcripts. *Proceedings of the National Academy of Sciences of the United States of America* 83(6): 1832-1836.

De Ronde, D., Butterbach, P. & Kormelink, R. (2014). Dominant resistance against plant viruses. *Frontiers in Plant Science* 5.

De Storme, N. & Geelen, D. (2014). Callose homeostasis at plasmodesmata: Molecular regulators and developmental relevance. *Frontiers in Plant Science* 5(APR).

de Vos, M. & Jander, G. (2010). Volatile communication in plant-aphid interactions. *Curr Opin Plant Biol* 13(4): 366-371.

Deom, C., Murphy, J. & Paguio, O. (1997). Resistance to Tobacco etch virus in Capsicum annuum: inhibition of virus RNA accumulation. Mol. *Plant Microbe Interact* 10: 917.

Dhillon, N. P. S., Ranjana, R., Singh, K., Eduardo, I., Monforte, A. J., Pitrat, M., Dhillon, N. K. & Singh, P. P. (2007). Diversity among landraces of Indian snapmelon (Cucumis melo var. momordica). *Genetic Resources and Crop Evolution* 54(6): 1267-1283.

Diaz, A., Fergany, M., Formisano, G., Ziarsolo, P., Blanca, J., Fei, Z., Staub, J., Zalapa, J., Cuevas, H., Dace, G., Oliver, M., Boissot, N., Dogimont, C., Pitrat, M., Hofstede, R., van Koert, P., Harel-Beja, R., Tzuri, G., Portnoy, V., Cohen, S., Schaffer, A., Katzir, N., Xu, Y., Zhang, H., Fukino, N., Matsumoto, S., Garcia-Mas, J. & Monforte, A. (2011). A consensus linkage map for molecular markers and Quantitative Trait Loci associated with economically important traits in melon (Cucumis melo L.). *BMC Plant Biology* 11(1): 111.

Diaz, J. A., Mallor, C., Soria, C., Camero, R., Garzo, E., Fereres, A., Alvarez, J. M., Gómez-Guillamón, M. L., Luis-Arteaga, M. & Moriones, E. (2003). Potential sources of resistance for melon to nonpersistently aphid-borne viruses. *Plant Disease* 87(8): 960-964.

Diaz-Pendon, J., Truniger, V., Nieto, C., Garcia-Mas, J., Bendahmane, A. & Aranda, M. (2004). Advances in understanding recessive resistance to plant viruses. *Mol. Plant Pathol* 5: 223.

Ding, B., Haudenshield, J. S., Hull, R. J., Wolf, S., Beachy, R. N. & Lucas, W. J. (1992). Secondary plasmodesmata are specific sites of localization of the tobacco mosaic virus movement protein in transgenic tobacco plants. *The Plant Cell Online* 4(8): 915-928.

Ding, S. W., Anderson, B. J., Haase, H. R. & Symons, R. H. (1994). New overlapping gene encoded by the cucumber mosaic virus genome. *Virology* 198(2): 593-601.

Ding, S. W., Li, W. X. & Symons, R. H. (1995). A novel naturally occurring hybrid gene encoded by a plant RNA virus facilitates long distance virus movement. *EMBO J* 14(23): 5762-5772.

Ding, S. W., Shi, B. J., Li, W. X. & Symons, R. H. (1996a). An interspecies hybrid RNA virus is significantly more virulent than either parental virus. *Proc Natl Acad Sci U S A* 93(15): 7470-7474.

Ding, X., Shintaku, M. H., Carter, S. A. & Nelson, R. S. (1996b). Invasion of minor veins of tobacco leaves inoculated with tobacco mosaic virus mutants defective in phloem-dependent movement. *Proc Natl Acad Sci U S A* 93(20): 11155-11160.

Diveki, Z., Salanki, K. & Balazs, E. (2004). The necrotic pathotype of the cucumber mosaic virus (CMV) ns strain is solely determined by amino acid 461 of the 1a protein. *Mol Plant Microbe Interact* 17(8): 837-845.

Dogimont, C., Leconte, L., Perin, C., Thabuis, A., Lecoq, H. & Pitrat, M. (2000). Identification of QTLs contributing to resistance to different strains of cucumber mosaic cucumovirus in melon. *Acta Hortic.* 510: 391-398.

Doolittle, S. P. (1916). A new infectious mosaic disease of cucumber. *Phytopathology* 6: 145-147.

Eduardo, I., Arus, P. & Monforte, A. J. (2005). Development of a genomic library of near isogenic lines (NILs) in melon (Cucumis melo L.) from the exotic accession PI161375. *Theor Appl Genet* 112(1): 139-148.

Edwards, M. C., Gonsalves, D. & Provvidenti, R. (1983). Genetic analysis of cucumber mosaic virus in relation to host resistence: location of determinants for pathogenicity to certain legumes and Lactuca saligna. *Phytopathology* 73: 269-273.

Edwardson, J. R. & Christie, R. G. (1991).Cucumoviruses. . In *CRC Handbook of viruses infecting legumes*, 293-319: Boca Raton, FL, CRC Press.

Ehlers, K. & Kollmann, R. (2001). Primary and secondary plasmodesmata: Structure, origin, and functioning. *Protoplasma* 216(1-2): 1-30.

Esau, K., Cronshaw, J. & Hoefert, L. L. (1967). Relation of beet yellows virus to the phloem and to movement in the sieve tube. *J Cell Biol* 32(1): 71-87.

Escriu, F., Perry, K. L. & Garcia-Arenal, F. (2000). Transmissibility of Cucumber mosaic virus by Aphis gossypii correlates with viral accumulation and is affected by the presence of its satellite RNA. *Phytopathology* 90(10): 1068-1072.

Eshed, Y. & Zamir, D. (1995). An introgression line population of Lycopersicon pennellii in the cultivated tomato enables the identification and fine mapping of yield-associated QTL. *Genetics* 141(3): 1147-1162.

Essafi, A., Diaz-Pendon, J. A., Moriones, E., Monforte, A. J., Garcia-Mas, J. & Martin-Hernandez, A. M. (2009). Dissection of the oligogenic resistance to *Cucumber mosaic virus* in the melon accession PI 161375. *Theor Appl Genet* 118(2): 275-284.

Fergany, M., Kaur, B., Monforte, A. J., Pitrat, M., Rys, C., Lecoq, H., Dhillon, N. P. S. & Dhaliwal, S. S. (2011). Variation in melon (Cucumis melo) landraces adapted to the humid tropics of southern India. *Genetic Resources and Crop Evolution* 58(2): 225-243.

Finch, J. T., Klug, A. & van Regenmortel, M. H. V. (1967). The structure of cucumber mosaic virus. *Journal of Molecular Biology* 24(2): 303-"304,IN315-IN316,305".

Flor, H. (1971). Current status of gene-for-gene concept. *Annu. Rev. Phytopathol* 9: 275.

Foucart, C., Boualem, A., Lasseur, B., Eleblu, J., Fahraj, I. & Bendahmane, A. (2012). Sex determination in cucurbits. *Biol Aujourdhui* 206(1): 57-62.

Fraser, R. (1990). The genetics of resistance to plant viruses. *Annu Rev Phytopathol* 28: 179-200

Gamalei, Y. (1989). Structure and function of leaf minor veins in trees and herbs A taxonomic review. *Trees-Structure and Function* 3(2): 96-110.

Gao, Z., Johansen, E., Eyers, S., Thomas, C. L., Noel Ellis, T. H. & Maule, A. J. (2004). The potyvirus recessive resistance gene, sbm1, identifies a novel role for translation initiation factor eIF4E in cell-to-cell trafficking. *Plant J* 40(3): 376-385.

Garcia-Arenal, F. & Palukaitis, P. (1999). Structure and functional relationships of satellite RNAs of cucumber mosaic virus. *Curr Top Microbiol Immunol* 239: 37-63.

García-Cano, E., Resende, R. O., Fernández-Muñoz, R. & Moriones, E. (2006). Synergistic Interaction Between Tomato chlorosis virus and Tomato

spotted wilt virus Results in Breakdown of Resistance in Tomato. *Phytopathology* 96(11): 1263-1269.

Garcia-Mas, J., Benjak, A., Sanseverino, W., Bourgeois, M., Mir, G., Gonzalez, V. M., Henaff, E., Camara, F., Cozzuto, L., Lowy, E., Alioto, T., Capella-Gutierrez, S., Blanca, J., Canizares, J., Ziarsolo, P., Gonzalez-Ibeas, D., Rodriguez-Moreno, L., Droege, M., Du, L., Alvarez-Tejado, M., Lorente-Galdos, B., Mele, M., Yang, L., Weng, Y., Navarro, A., Marques-Bonet, T., Aranda, M. A., Nuez, F., Pico, B., Gabaldon, T., Roma, G., Guigo, R., Casacuberta, J. M., Arus, P. & Puigdomenech, P. (2012). The genome of melon (Cucumis melo L.). *Proc Natl Acad Sci U S A* 109(29): 11872-11877.

Garcia-Mas, J., Oliver, M., Gómez-Paniagua, H. & de Vicente, M. C. (2000). Comparing AFLP, RAPD and RFLP markers for measuring genetic diversity in melon. . *Theor Appl Genet* 101: 860-864.

Golecki, B., Schulz, A. & Thompson, G. A. (1999). Translocation of structural P proteins in the phloem. *Plant Cell* 11(1): 127-140.

Gonzalez, M., Xu, M., Esteras, C., Roig, C., Monforte, A. J., Troadec, C., Pujol, M., Nuez, F., Bendahmane, A., Garcia-Mas, J. & Pico, B. (2011). Towards a TILLING platform for functional genomics in Piel de Sapo melons. *BMC Res Notes* 4: 289.

Gonzalez, V. M., Garcia-Mas, J., Arus, P. & Puigdomenech, P. (2010). Generation of a BAC-based physical map of the melon genome. *BMC Genomics* 11: 339.

Gonzalo, M. J., Claveria, E., Monforte, A. J. & Dolcet-Sanjuan, R. (2011). Parthenogenic Haploids in Melon: Generation and Molecular Characterization of a Doubled Haploid Line Population. *Journal of the American Society for Horticultural Science* 136(2): 145-154.

Goodrick, B., Kuhn, C. & Hussey, R. (1991). Restricted systemic movement of Cowpea chlorotic mottle virus in soybean with nonnecrotic resistance. *Phytopathology* 81: 1426.

Gosalvez-Bernal, B., Genoves, A., Navarro, J. A., Pallas, V. & Sanchez-Pina, M. A. (2008). Distribution and pathway for phloem-dependent movement of *Melon necrotic spot virus* in melon plants. *Mol Plant Pathol* 9(4): 447-461.

Grimsley, N., Hohn, T., Davies, J. W. & Hohn, B. (1987). Agrobacterium-mediated delivery of infectious maize streak virus into maize plants. *Nature* 325(6100): 177-179.

Guiu-Aragonés, C., Monforte, A. J., Saladié, M., Corrêa, R. X., Garcia-Mas, J. & Martín-Hernández, A. M. (2014). The complex resistance to cucumber mosaic cucumovirus (CMV) in the melon accession PI161375 is governed by one gene and at least two quantitative trait loci. *Molecular Breeding*.

Habili, N. & Francki, R. I. (1974). Comparative studies on tomato aspermy and cucumber mosaic viruses. II. Virus stability. *Virology* 60(1): 29-36.

Hammond-Kosack, K. & Jones, J. (1997). Plant disease resistance genes. *Annu. Rev. Plant Physiol. Plant Mol. Biol* 48: 575.

Hammond-Kosack, K. & Parke, r. J. (2003). Deciphering plant-pathogen communication: fresh perspectives for molecular resistance breeding. *Curr. Opin. Biotechnol* 14: 177.

Hampton, R. O. & Francki, R. I. (1992). RNA 1 dependent seed transmissibility of Cucumber mosaic virus in Phaseolus vulgaris. *Phytopathology* 82: 127-130.

Hanahan, D., Jessee, J. & Bloom, F. R. (1991).Plasmid transformation of Escherichia coli and other bacteria. In *Methods in Enzymology*, Vol. Volume 204, 63-113 (Ed H. M. Jeffrey). Academic Press.

Haritatos, E., Keller, F. & Turgeon, R. (1996). Raffinose oligosaccharide concentrations measured in individual cell and tissue types in Cucumis melo L. leaves: Implications for phloem loading. *Planta* 198(4): 614-622.

Harries, P. & Ding, B. (2011). Cellular factors in plant virus movement: At the leading edge of macromolecular trafficking in plants. *Virology* 411(2): 237-243.

Harries, P. A., Schoelz, J. E. & Nelson, R. S. (2010). Intracellular transport of viruses and their components: Utilizing the cytoskeleton and membrane highways. *Molecular Plant-Microbe Interactions* 23(11): 1381-1393.

Havelda, Z. & Maule, A. J. (2000). Complex spatial responses to cucumber mosaic virus infection in susceptible Cucurbita pepo cotyledons. *Plant Cell* 12(10): 1975-1985.

Hayes, R. J. & Buck, K. W. (1990a). Complete replication of a eukaryotic virus RNA in vitro by a purified RNA-dependent RNA polymerase. *Cell* 63(2): 363-368.

Hayes, R. J. & Buck, K. W. (1990b). Infectious cucumber mosaic virus RNA transcribed in vitro from clones obtained from cDNA amplified using the polymerase chain reaction. *J Gen Virol* 71 (Pt 11): 2503-2508.

Hellens, R. P., Edwards, E. A., Leyland, N. R., Bean, S. & Mullineaux, P. M. (2000). pGreen: a versatile and flexible binary Ti vector for Agrobacterium-mediated plant transformation. *Plant Mol Biol* 42(6): 819-832.

Hipper, C., Brault, V., Ziegler-Graff, V. & Revers, F. (2013). Viral and cellular factors involved in Phloem transport of plant viruses. *Front Plant Sci* 4: 154.

Hong, J. S., Ohnishi, S., Masuta, C., Choi, J. K. & Ryu, K. H. (2007). Infection of soybean by cucumber mosaic virus as determined by viral movement protein. *Arch Virol* 152(2): 321-328.

Huang, T. S., Wei, T., Laliberte, J. F. & Wang, A. (2010). A host RNA helicase-like protein, AtRH8, interacts with the potyviral genome-linked protein, VPg, associates with the virus accumulation complex, and is essential for infection. *Plant Physiol* 152(1): 255-266.

Huh, S. U., Kim, M. J., Ham, B. K. & Paek, K. H. (2011). A zinc finger protein Tsip1 controls Cucumber mosaic virus infection by interacting with the replication complex on vacuolar membranes of the tobacco plant. *New Phytol* 191(3): 746-762.

Hull, R. (2002). *Matthews' Plant Virology.* London, Academic Press.

Hwang, M. S., Kim, S. H., Lee, J. H., Bae, J. M., Paek, K. H. & Park, Y. I. (2005). Evidence for interaction between the 2a polymerase protein and the 3a movement protein of Cucumber mosaic virus. *J Gen Virol* 86(Pt 11): 3171-3177.

Ishihama, A. & Barbier, P. (1994). Molecular anatomy of viral RNA-directed RNA polymerases. *Arch Virol* 134(3-4): 235-258.

Jacquemond, M. (2012).Cucumber Mosaic Virus. In *Adv Virus Res*, Vol. 84, 439-504.

Jagger, I. C. (1916). Experiments with the cucumber mosaic disease. *Phytopathology* 6: 148-151.

Javelle, M., Marco, C. F. & Timmermans, M. (2011). In situ hybridization for the precise localization of transcripts in plants. *J Vis Exp* (57).

Jeffrey, C. (1990). *Systematics of the Cucurbitaceae: An overview.* Cornell University Press, Ithaca: Cornell University Press, Ithaca.

Jeuken, M. J. & Lindhout, P. (2004). The development of lettuce backcross inbred lines (BILs) for exploitation of the Lactuca saligna (wild lettuce) germplasm. *Theor Appl Genet* 109(2): 394-401.

Ji, L. H. & Ding, S. W. (2001). The suppressor of transgene RNA silencing encoded by Cucumber mosaic virus interferes with salicylic acid-mediated virus resistance. *Mol Plant Microbe Interact* 14(6): 715-724.

Johansen, I. E. (1996). Intron insertion facilitates amplification of cloned virus cDNA in Escherichia coli while biological activity is reestablished after transcription in vivo. *Proc Natl Acad Sci U S A* 93(22): 12400-12405.

Kang, B.-C., Yeam, I., Frantz, J., Murphy, J. & Jahn, M. (2005a). The pvr1 locus in pepper encodes a translation initiation factor eIF4E that interacts with Tobacco etch virus VPg. *Plant J* 42: 392.

Kang, B.-C., Yeam, I. & Jahn, M. M. (2005b). Genetics of Plant Virus Resistance. *Annu Rev Phytopathol* 43(1): 581-621.

Kang, W. H., Hoang, N. H., Yang, H. B., Kwon, J. K., Jo, S. H., Seo, J. K., Kim, K. H., Choi, D. & Kang, B. C. (2010). Molecular mapping and characterization of a single dominant gene controlling CMV resistance in peppers (Capsicum annuum L.). *Theoretical and Applied Genetics* 120(8): 1587-1596.

Kang, W. H., Seo, J. K., Chung, B. N., Kim, K. H. & Kang, B. C. (2012). Helicase domain encoded by cucumber mosaic virus RNA1 determines systemic infection of Cmr1 in pepper. *PLoS ONE* 7(8).

Kaper, J. M. & Geelen, J. L. M. C. (1971). Studies on the stabilizing forces of simple RNA viruses. II. Stability, dissociation and reassembly of cucumber mosaic virus. *Journal of Molecular Biology* 56(2): 277-"284,IN229,285-294".

Kaplan, I. B., Shintaku, M. H., Li, Q., Zhang, L., Marsh, L. E. & Palukaitis, P. (1995). Complementation of virus movement in transgenic tobacco expressing the cucumber mosaic virus 3a gene. *Virology* 209(1): 188-199.

Karchi, Z., Cohen, S. & Govers, A. (1975). Inheritance of resistance to *Cucumber Mosaic virus* in melons. *Phytopathology* 65: 479-481.

Karimi, M., Inzé, D. & Depicker, A. (2002). GATEWAY™ vectors for Agrobacterium-mediated plant transformation. *Trends in Plant Science* 7(5): 193-195.

Kay, R., Chan, A., Daly, M. & McPherson, J. (1987). Duplication of CaMV 35S Promoter Sequences Creates a Strong Enhancer for Plant Genes. *Science* 236(4806): 1299-1302.

Keller, K., Johansen, I., Martin, R. & Hampton, R. (1998). Potyvirus genome-linked protein (VPg) determines Pea seed-borne mosaic virus pathotype-specific virulence in Pisum sativum. *Mol. Plant Microbe Interact* 11: 124.

Kempers, R., Ammerlaan, A. & Van Bel, A. J. E. (1998). Symplasmic Constriction and Ultrastructural Features of the Sieve Element/Companion Cell Complex in the Transport Phloem of Apoplasmically and Symplasmically Phloem-Loading Species. *Plant Physiology* 116(1): 271-278.

Kempers, R., Prior, D. A. M., Van Bel, A. J. E. & Oparka, K. J. (1993). Plasmodesmata between sieve element and companion cell of extrafascicular stem phloem of Cucurbita maxima permit passage of 3 kDa fluorescent probes. *The Plant Journal* 4(3): 567-575.

Kempers, R. & van Bel, A. E. (1997). Symplasmic connections between sieve element and companion cell in the stem phloem ofVicia faba L. have a molecular exclusion limit of at least 10 kDa. *Planta* 201(2): 195-201.

Kerje, T. & Grum, M. (2000). The origin of melon, Cucumis melo: a review of the literature. *Acta Horticulturae* 510: 37-44.

Kim, C. H. & Palukaitis, P. (1997). The plant defense response to cucumber mosaic virus in cowpea is elicited by the viral polymerase gene and affects virus accumulation in single cells. *EMBO J* 16(13): 4060-4068.

Kim, M. J., Huh, S. U., Ham, B. K. & Paek, K. H. (2008). A novel methyltransferase methylates Cucumber mosaic virus 1a protein and promotes systemic spread. *J Virol* 82(10): 4823-4833.

Kim, M. J., Kim, H. R. & Paek, K. H. (2006). Arabidopsis tonoplast proteins TIP1 and TIP2 interact with the cucumber mosaic virus 1a replication protein. *J Gen Virol* 87(Pt 11): 3425-3431.

Kim, S. H., Kalinina, N. O., Andreev, I., Ryabov, E. V., Fitzgerald, A. G., Taliansky, M. E. & Palukaitis, P. (2004). The C-terminal 33 amino acids of the cucumber mosaic virus 3a protein affect virus movement, RNA binding and inhibition of infection and translation. *J Gen Virol* 85(Pt 1): 221-230.

Kim, S. H., Palukaitis, P. & Park, Y. I. (2002). Phosphorylation of cucumber mosaic virus RNA polymerase 2a protein inhibits formation of replicase complex. *EMBO J* 21(9): 2292-2300.

King, A. M. Q., Adams, M. J., Carstens, E. B. & Lefkowitz, E. J. (2012). *Virus taxonomy: classification and nomenclature of viruses: Ninth Report of the International Committee on Taxonomy of Viruses.* Elsevier Academic Press.

Kobayashi, I. & Kobayashi, Y. (2008).Microtubules and pathogen defence. In *Plant Cell Monographs*, Vol. 11, 121-140.

Kobori, T., Miyagawa, M., Nishioka, K., Ohki, S. T. & Osaki, T. (2002). Amino acid 129 of Cucumber mosaic virus coat protein determines local symptom expression and systemic movement in Tetragonia expansa, Momordica charantia and Physalis floridana. *Journal of General Plant Pathology* 68(1): 81-88.

Kobori, T., Ohki, S. T. & Osaki, T. (2000). Movement of Cucumber Mosaic Virus Is Restricted at the Interface between Mesophyll and Phloem Pathway in Cucumis figarei. *Journal of General Plant Pathology* 66(2): 159-166.

Kobori, T., Osaki, T. & Ohki, S. T. (2003). Cucumber mosaic virus Establishes Systemic Infection at Increased Temperature Following Viral Entrance Into the Phloem Pathway of Tetragonia expansa. *Phytopathology* 93(11): 1445-1451.

Koumproglou, R., Wilkes, T. M., Townson, P., Wang, X. Y., Beynon, J., Pooni, H. S., Newbury, H. J. & Kearsey, M. J. (2002). STAIRS: a new genetic resource for functional genomic studies of Arabidopsis. *Plant J* 31(3): 355-364.

Kurata, T., Okada, K. & Wada, T. (2005). Intercellular movement of transcription factors. *Current Opinion in Plant Biology* 8(6): 600-605.

Kyle, M. & Palloix, A. (1997). Proposed revision of nomenclature for potyvirus resistance genes in Capsicum. *Euphytica* 97: 183.

Lalonde, S., Franceschi, V. R. & Frommer, W. B. (2001).Companion Cells. In *eLS*: John Wiley & Sons, Ltd.

Lazarowitz, S. G. & Beachy, R. N. (1999). Viral movement proteins as probes for intracellular and intercellular trafficking in plants. *Plant Cell* 11(4): 535-548.

Lecoq, H., Wisler, G. & Pitrat, M. (1998). Cucurbit viruses: the classics and the emerging. In Cucurbitaceae 98. Evaluation and enhancement of Cucurbit Germplasm. e.d J.D. McCreight, Alezandria, VA:ASHS Press.: 126-142.

Lee, D. K. & Sieburth, L. E. (2010). Plasmodesmata formation: Poking holes in walls with ise. *Current Biology* 20(11): R488-R490.

Lee, J. H., Muhsin, M., Atienza, G. A., Kwak, D. Y., Kim, S. M., De Leon, T. B., Angeles, E. R., Coloquio, E., Kondoh, H., Satoh, K., Cabunagan, R. C., Cabauatan, P. Q., Kikuchi, S., Leung, H. & Choi, I. R. (2010). Single nucleotide polymorphisms in a gene for translation initiation factor (eIF4G) of rice (Oryza sativa) associated with resistance to Rice tungro spherical virus. *Mol Plant Microbe Interact* 23(1): 29-38.

Leisner, S. M. & Turgeon, R. (1993). Movement of virus and photoassimilate in the phloem: A comparative analysis. *BioEssays* 15(11): 741-748.

Levy, A., Guenoune-Gelbart, D. & Epel, B. L. (2007). β-1,3-Glucanases: Plasmodesmal gate keepers for intercellular communication. *Plant Signaling and Behavior* 2(5): 404-407.

Lewsey, M., Surette, M., Robertson, F. C., Ziebell, H., Choi, S. H., Ryu, K. H., Canto, T., Palukaitis, P., Payne, T., Walsh, J. A. & Carr, J. P. (2009). The role of the Cucumber mosaic virus 2b protein in viral movement and symptom induction. *Molecular plant-microbe interactions : MPMI* 22(6): 642-654.

Li, Q. & Palukaitis, P. (1996). Comparison of the nucleic acid- and NTP-binding properties of the movement protein of cucumber mosaic cucumovirus and tobacco mosaic tobamovirus. *Virology* 216(1): 71-79.

Li, Q., Ryu, K. H. & Palukaitis, P. (2001). Cucumber mosaic virus-plant interactions: identification of 3a protein sequences affecting infectivity, cell-to-cell movement, and long-distance movement. *Mol Plant Microbe Interact* 14(3): 378-385.

Liu, S., He, X., Park, G., Josefsson, C. & Perry, K. L. (2002). A conserved capsid protein surface domain of Cucumber mosaic virus is essential for efficient aphid vector transmission. *J Virol* 76(19): 9756-9762.

Lot, H. & Kaper, J. M. (1976). Physical and chemical differentiation of three strains of cucumber mosaic virus and peanut stunt virus. *Virology* 74(1): 209-222.

Lough, T. J. & Lucas, W. J. (2006). Integrative plant biology: role of phloem long-distance macromolecular trafficking. *Annu Rev Plant Biol* 57: 203-232.

Lucas, J. (1998). *Plant Pathology and Plant Pathogens.* Oxford, Blackwell Science.

Lucas, W. J. & Franceschi, V. R. (1982). Organization of the sieve-element walls of leaf minor veins. *J Ultrastruct Res* 81(2): 209-221.

Lucy, A. P., Guo, H. S., Li, W. X. & Ding, S. W. (2000). Suppression of post-transcriptional gene silencing by a plant viral protein localized in the nucleus. *EMBO J* 19(7): 1672-1680.

Maia, I. G. & Haenni, A. L. (1994). Early events in virus-plant interactions. *Braz J Med Biol Res* 27(11): 2521-2532.

Malik, A., Vashisht, V. K., Singh, K., Sharma, A., Singh, D. K., Singh, H., Monforte, A. J., McCreight, J. & Dhillon, N. S. (2014). Diversity among melon

(Cucumis melo L.) landraces from the Indo-Gangetic plains of India and their genetic relationship with USA melon cultivars. *Genetic Resources and Crop Evolution* 61(6): 1189-1208.

Marco, C. F., Aguilar, J. M., Abad, J., Gomez-Guillamon, M. L. & Aranda, M. A. (2003). Melon Resistance to Cucurbit yellow stunting disorder virus Is Characterized by Reduced Virus Accumulation. *Phytopathology* 93(7): 844-852.

Martin, A., Troadec, C., Boualem, A., Rajab, M., Fernandez, R., Morin, H., Pitrat, M., Dogimont, C. & Bendahmane, A. (2009). A transposon-induced epigenetic change leads to sex determination in melon. *Nature* 461(7267): 1135-1138.

Más, P. & Pallás, V. (1995). Non-isotopic tissue-printing hybridization: a new technique to study long-distance plant virus movement. *Journal of Virological Methods* 52(3): 317-326.

Mascarell-Creus, A., Canizares, J., Vilarrasa-Blasi, J., Mora-Garcia, S., Blanca, J., Gonzalez-Ibeas, D., Saladie, M., Roig, C., Deleu, W., Pico-Silvent, B., Lopez-Bigas, N., Aranda, M., Garcia-Mas, J., Nuez, F., Puigdomenech, P. & Cano-Delgado, A. (2009). An oligo-based microarray offers novel transcriptomic approaches for the analysis of pathogen resistance and fruit quality traits in melon (Cucumis melo L.). *BMC Genomics* 10(1): 467.

Masuta, C., Ueda, S., Suzuki, M. & Uyeda, I. (1998). Evolution of a quadripartite hybrid virus by interspecific exchange and recombination between replicase components of two related tripartite RNA viruses. *Proc Natl Acad Sci U S A* 95(18): 10487-10492.

Mathews, R. E. F. (1991). *Plant Virology* San Diego, CA.

Matsuo, K., Hong, J. S., Tabayashi, N., Ito, A., Masuta, C. & Matsumura, T. (2007). Development of Cucumber mosaic virus as a vector modifiable for different host species to produce therapeutic proteins. *Planta* 225(2): 277-286.

Maule, A. J. (2008). Plasmodesmata: structure, function and biogenesis. *Current Opinion in Plant Biology* 11(6): 680-686.

McGarvey, P., Tousignant, M., Geletka, L., Cellini, F. & Kaper, J. M. (1995). The complete sequence of a cucumber mosaic virus from Ixora that is deficient in the replication of satellite RNAs. *J Gen Virol* 76 (Pt 9): 2257-2270.

Meshi, T., Ishikawa, M., Motoyoshi, F., Semba, K. & Okada, Y. (1986). In vitro transcription of infectious RNAs from full-length cDNAs of tobacco mosaic virus. *Proceedings of the National Academy of Sciences* 83(14): 5043-5047.

Mochizuki, T. & Ohki, S. T. (2005). Amino acid 129 in the coat protein of Cucumber mosaic virus primarily determines invasion of the shoot apical meristem of tobacco plants. *Journal of General Plant Pathology* 71(4): 326-332.

Mochizuki, T. & Ohki, S. T. (2012). Cucumber mosaic virus: viral genes as virulence determinants. *Mol Plant Pathol* 13(3): 217-225.

Monforte, A. J., Garcia-Mas, J. & Arus, P. (2003). Genetic variability in melon based on microsatellite variation. *Plant Breeding* (122): 153-157.

Monforte, A. J., Oliver, M., Gonzalo, M. J., Alvarez, J. M., Dolcet-Sanjuan, R. & Arus, P. (2004). Identification of quantitative trait loci involved in fruit quality traits in melon (Cucumis melo L.). *Theor Appl Genet* 108(4): 750-758.

Monforte, A. J. & Tanksley, S. D. (2000). Development of a set of near isogenic and backcross recombinant inbred lines containing most of the Lycopersicon hirsutum genome in a L. esculentum genetic background: a tool for gene mapping and gene discovery. *Genome* 43(5): 803-813.

Moreno, I., Thompson, J. & Garcia-Arenal, F. (2004). Analysis of the systemic colonization of cucumber plants by Cucumber green mottle mosaic virus. *J. Gen. Virol* 85: 749.

Mori, M., Mise, K., Kobayashi, K., Okuno, T. & Furusawa, I. (1991). Infectivity of plasmids containing brome mosaic virus cDNA linked to the cauliflower mosaic virus 35S RNA promoter. *J Gen Virol* 72 (Pt 2): 243-246.

Murphy, J., Blauth, J., Livingstone, K., Lackney, V. & Jahn, M. (1998). Genetic mapping of the pvr1 locus in Capsicum spp. and evidence that distinct potyvirus resistance loci control responses that differ at the whole plant and cellular levels. *Mol. Plant Microbe Interact* 11: 943.

Murphy, J. & Kyle, M. (1995). Alleviation of restricted systemic spread of pepper mottle potyvirus in Capsicum annuum vs. Avelar by coinfection with a Cucumovirus. *Phytopathology* 85: 561.

Nagano, H., Mise, K., Furusawa, I. & Okuno, T. (2001). Conversion in the requirement of coat protein in cell-to-cell movement mediated by the cucumber mosaic virus movement protein. *J Virol* 75(17): 8045-8053.

Nasu, Y., Karasawa, A., Hase, S. & Ehara, Y. (1996). Cry, the resistance locus of cowpea to cucumber mosaic virus strain Y. *Phytopathology* 86(9): 946-951.

Nelson, R. & van Bel, A. E. (1998).The mystery of virus trafficking into, through and out of vascular tissue. In *Progress in Botany*, Vol. 59, 476-533 (Eds H. D. Behnke, K. Esser, J. W. Kadereit, U. Lüttge and M. Runge). Springer Berlin Heidelberg.

Ng, J. C., Josefsson, C., Clark, A. J., Franz, A. W. & Perry, K. L. (2005). Virion stability and aphid vector transmissibility of Cucumber mosaic virus mutants. *Virology* 332(1): 397-405.

Ng, J. C., Liu, S. & Perry, K. L. (2000). Cucumber mosaic virus mutants with altered physical properties and defective in aphid vector transmission. *Virology* 276(2): 395-403.

Nicaise, V., German-Retana, S., Sanjuan, R., Dubrana, M. P., Mazier, M., Maisonneuve, B., Candresse, T., Caranta, C. & LeGall, O. (2003). The eukaryotic translation initiation factor 4E controls lettuce susceptibility to the Potyvirus Lettuce mosaic virus. *Plant Physiol* 132(3): 1272-1282.

Nieto, C., Morales, M., Orjeda, G., Clepet, C., Monfort, A., Sturbois, B., Puigdomenech, P., Pitrat, M., Caboche, M., Dogimont, C., Garcia-Mas, J., Aranda, M. A. & Bendahmane, A. (2006). An eIF4E allele confers resistance to an uncapped and non-polyadenylated RNA virus in melon. *Plant J* 48(3): 452-462.

Nieto, C., Piron, F., Dalmais, M., Marco, C. F., Moriones, E., Gomez-Guillamon, M. L., Truniger, V., Gomez, P., Garcia-Mas, J., Aranda, M. A. & Bendahmane, A. (2007). EcoTILLING for the identification of allelic variants of melon eIF4E, a factor that controls virus susceptibility. *BMC Plant Biol* 7: 34.

Nunez-Palenius, H. G., Gomez-Lim, M., Ochoa-Alejo, N., Grumet, R., Lester, G. & Cantliffe, D. J. (2008). Melon fruits: genetic diversity, physiology, and biotechnology features. *Crit Rev Biotechnol* 28(1): 13-55.

O'Reilly, E. K., Wang, Z., French, R. & Kao, C. C. (1998). Interactions between the structural domains of the RNA replication proteins of plant-infecting RNA viruses. *J Virol* 72(9): 7160-7169.

Ohnishi, S., Echizenya, I., Yoshimoto, E., Boumin, K., Inukai, T. & Masuta, C. (2011). Multigenic system controlling viral systemic infection determined by the interactions between Cucumber mosaic virus genes and quantitative trait loci of soybean cultivars. *Phytopathology* 101(5): 575-582.

Olczak-Woltman, H., Marcinkowska, J. & Niemirowicz-Szczytt, K. (2011). The genetic basis of resistance to downy mildew in Cucumis spp.--latest developments and prospects. *J Appl Genet* 52(3): 249-255.

Omid, A., Malter, D., Peleg, G. & Wolf, S. (2008). Long-distance trafficking of macromolecules in the phloem. *Plant Signaling and Behavior* 3(4): 260-262.

Orzaez, D., Monforte, A. J. & Granell, A. (2010). Using genetic variability available in the breeder's pool to engineer fruit quality. *GM Crops* 1(3): 120-127.

Otagaki, S., Arai, M., Takahashi, A., Goto, K., Hong, J.-S., Masuta, C. & Kanazawa, A. (2006). Rapid induction of transcriptional and post-transcriptional gene silencing using a novel Cucumber mosaic virus vector. *Plant Biotechnology* 23(3): 259-265.

Owen, J. & Palukaitis, P. (1988). Characterization of cucumber mosaic virus. I. Molecular heterogeneity mapping of RNA 3 in eight CMV strains. *Virology* 166(2): 495-502.

Palukaitis, P. & Garcia-Arenal, F. (2003). Cucumoviruses. *Adv Virus Res* 62: 241-323.

Palukaitis, P., Roossinck, M. J., Dietzgen, R. G. & Francki, R. I. B. (1992). Cucumber mosaic virus. *Adv. Virus Res.* 41: 281-348.

Parella, G., Ruffel, S., Moretti, A., Morel, C., Palloix, A. & Caranta, C. (2002). Recessive resistance genes against potyviruses are localized in colinear genomic regions of the tomato (Lysopersicon spp.) and pepper (Capsicum spp.) genomes. Theor. *Appl. Genet* 105: 855.

Pech, J. C., Bouzayen, M. & Latché, A. (2008). Climacteric fruit ripening: Ethylene-dependent and independent regulation of ripening pathways in melon fruit. *Plant Science* 175(1–2): 114-120.

Peleman, J. D. & Van der Voort, J. R. (2003). Breeding by Design. *Trendos in Genetics* 19(6): 303-306.

Phan, M. S. V., Seo, J.-K., Choi, H.-S., Lee, S.-H. & Kim, K.-H. (2014). Molecular and Biological Characterization of an Isolate of Cucumber mosaic virus from Glycine soja by Generating its Infectious Full-genome cDNA Clones. *Plant Pathol. J.* 30(2): 159-167.

Pierrugues, O., Guilbaud, L., Fernandez-Delmond, I., Fabre, F., Tepfer, M. & Jacquemond, M. (2007). Biological properties and relative fitness of inter-subgroup cucumber mosaic virus RNA 3 recombinants produced in vitro. *J Gen Virol* 88(Pt 10): 2852-2861.

Pink, D. A. C. (1987). Genetic control of resistance to cucumber mosaic virus in Cucurbita pepo. *Annals of Applied Biology* 111(2): 425-432.

Pitrat, M. (2002). Gene list for melon. *Cucurbit Genet. Coop. Rep.* 25: 76-93.

Pitrat, M. (2008). *Melon (Cucumis melo L.).* Handbook of plant breeding: Springer.

Pitrat, M. & Lecoq, H. (1980). Inheritance of resistance to cucumber mosaic virus transmission by Aphis gossypii in Cucumis melo. *Phytopathology* 70: 958-961.

Plisson, C., Drucker, M., Blanc, S., German-Retana, S., Le Gall, O., Thomas, D. & Bron, P. (2003). Structural characterization of HC-Pro, a plant virus multifunctional protein. *J Biol Chem* 278(26): 23753-23761.

Porta, C., Devergne, J. C., Cardin, L., Briand, J. P. & Van Regenmortel, M. H. (1989). Serotype specificity of monoclonal antibodies to cucumber mosaic virus. *Arch Virol* 104(3-4): 271-285.

Pruss, G., Ge, X., Shi, X. M., Carrington, J. C. & Bowman Vance, V. (1997). Plant viral synergism: the potyviral genome encodes a broad-range pathogenicity enhancer that transactivates replication of heterologous viruses. *Plant Cell* 9(6): 859-868.

Qu, F. & Morris, T. J. (2005). Suppressors of RNA silencing encoded by plant viruses and their role in viral infections. *FEBS Lett* 579(26): 5958-5964.

Ramsay, L. D., Jennings, D. E., Kearsey, M. J., Marshall, D. F., Bohuon, E. J., Arthur, A. E. & Lydiate, D. J. (1996). The construction of a substitution library of recombinant backcross lines in Brassica oleracea for the precision mapping of quantitative trait loci. *Genome* 39(3): 558-567.

Rao, A. L. & Francki, R. I. (1981). Comparative studies on tomato aspermy and cucumber mosaic viruses. VI. Partial compatibility of genome segments from the two viruses. *Virology* 114(2): 573-575.

Reidel, E. J., Rennie, E. A., Amiard, V., Cheng, L. & Turgeon, R. (2009). Phloem loading strategies in three plant species that transport sugar alcohols. *Plant Physiol* 149(3): 1601-1608.

Rennie, E. A. & Turgeon, R. (2009). A comprehensive picture of phloem loading strategies. *Proceedings of the National Academy of Sciences of the United States of America* 106(33): 14162-14167.

Requena, A., Simon-Buela, L., Salcedo, G. & Garcia-Arenal, F. (2006). Potential involvement of a cucumber homolog of phloem protein 1 in the long-

distance movement of Cucumber mosaic virus particles. *Mol Plant Microbe Interact* 19(7): 734-746.

Rhee, Y., Tzfira, T., Chen, M. H., Waigmann, E. & Citovsky, V. (2000). Cell-to-cell movement of tobacco mosaic virus: enigmas and explanations. *Mol Plant Pathol* 1(1): 33-39.

Rietveld, K., Pleij, C. W. & Bosch, L. (1983). Three-dimensional models of the tRNA-like 3' termini of some plant viral RNAs. *EMBO J* 2(7): 1079-1085.

Risser, G., Pitrat, M. & Rode, J. C. (1977). Etude de la résistance du melon (Cucumis melo L.) au virus de la mosaïque du concombre. . *Ann Amél Plant* 27: 509-522.

Rizzo, T. M. & Palukaitis, P. (1990). Construction of full-length cDNA clones of cucumber mosaic virus RNAs 1, 2 and 3: generation of infectious RNA transcripts. *Mol Gen Genet* 222(2-3): 249-256.

Robaglia, C. & Caranta, C. (2006). Translation initiation factors: a weak link in plant RNA virus infection. *Trends Plant Sci* 11(1): 40-45.

Roberts, A. G. & Oparka, K. J. (2003). Plasmodesmata and the control of symplastic transport. *Plant Cell Environ* 26(1): 103-124.

Roberts, I. M. (1994). Factors affecting the efficiency of immunogold labelling of plant virus antigens in thin sections. *J Virol Methods* 50(1-3): 155-166.

Roossinck, M. J. (2001). Cucumber mosaic virus, a model for RNA virus evolution. *Mol Plant Pathol* 2(2): 59-63.

Roossinck, M. J. (2002). Evolutionary history of Cucumber mosaic virus deduced by phylogenetic analyses. *Journal of Virology* 76(7): 3382-3387.

Roossinck, M. J., Zhang, L. & Hellwald, K. H. (1999). Rearrangements in the 5' nontranslated region and phylogenetic analyses of cucumber mosaic virus RNA 3 indicate radial evolution of three subgroups. *J Virol* 73(8): 6752-6758.

Roth, B. M., Pruss, G. J. & Vance, V. B. (2004). Plant viral suppressors of RNA silencing. *Virus Res* 102(1): 97-108.

Ruffel, S., Dussault, M., Palloix, A., Moury, B. & Bendahmane, A. (2002). A natural recessive resistance gene against potato virus Y in pepper corresponds to the eukaryotic initiation factor 4E (eIF4E). *Plant J* 32: 1067.

Ruffel, S., Gallois, J. L., Moury, B., Robaglia, C., Palloix, A. & Caranta, C. (2006). Simultaneous mutations in translation initiation factors eIF4E and eIF(iso)4E are required to prevent pepper veinal mottle virus infection of pepper. *J Gen Virol* 87(Pt 7): 2089-2098.

Ryu, K. H., Kim, C. H. & Palukaitis, P. (1998). The coat protein of cucumber mosaic virus is a host range determinant for infection of maize. *Molecular Plant-Microbe Interactions* 11(5): 351-357.

Saitoh, H., Fujiwara, M., Ohki, S. T. & Osaki, T. (1999). The coat protein gene is essential for the systemic infection of cucumber mosaic virus in Cucumis figarei at a high temperature. *Annual Phytopathology Society of Japan* 65: 248-253.

Salanki, K., Gellert, A., Huppert, E., Naray-Szabo, G. & Balazs, E. (2004). Compatibility of the movement protein and the coat protein of cucumoviruses is required for cell-to-cell movement. *J Gen Virol* 85(Pt 4): 1039-1048.

Salanki, K., Gellert, A., Naray-Szabo, G. & Balazs, E. (2007). Modeling-based characterization of the elicitor function of amino acid 461 of Cucumber mosaic virus 1a protein in the hypersensitive response. *Virology* 358(1): 109-118.

Salanki, K., Kiss, L., Gellert, A. & Balazs, E. (2011). Identification a coat protein region of cucumber mosaic virus (CMV) essential for long-distance movement in cucumber. *Arch Virol* 156(12): 2279-2283.

Santa Cruz, S. (1999). Perspective: phloem transport of viruses and macromolecules—What goes in must come out. *Trends Microbiol* 7: 237.

Sanz, A. I., Serra, M. T. & Garcia-Luque, I. (2000). Altered local and systemic spread of movement deficient virus in transgenic tobacco plants expressing the cucumber mosaic virus 3a protein. *Arch Virol* 145(11): 2387-2401.

Sasaki, N., Park, J. W., Maule, A. J. & Nelson, R. S. (2006). The cysteine-histidine-rich region of the movement protein of Cucumber mosaic virus contributes to plasmodesmal targeting, zinc binding and pathogenesis. *Virology* 349(2): 396-408.

Sauer, N. (1997). Sieve elements and companion cells — extreme division of labour. *Trends in Plant Science* 2(8): 285-286.

Schaad, M. C. & Carrington, J. C. (1996). Suppression of long-distance movement of tobacco etch virus in a nonsusceptible host. *J Virol* 70(4): 2556-2561.

Schepetilnikov, M., Kobayashi, K., Geldreich, A., Caranta, C., Robaglia, C., Keller, M. & Ryabova, L. A. (2011). Viral factor TAV recruits TOR/S6K1 signalling to activate reinitiation after long ORF translation. *EMBO J* 30(7): 1343-1356.

Scholthof, K. B., Adkins, S., Czosnek, H., Palukaitis, P., Jacquot, E., Hohn, T., Hohn, B., Saunders, K., Candresse, T., Ahlquist, P., Hemenway, C. & Foster, G. D. (2011). Top 10 plant viruses in molecular plant pathology. *Mol Plant Pathol* 12(9): 938-954.

Sebastian, P., Schaefer, H., Telford, I. R. & Renner, S. S. (2010). Cucumber (Cucumis sativus) and melon (C. melo) have numerous wild relatives in Asia and Australia, and the sister species of melon is from Australia. *Proc Natl Acad Sci U S A* 107(32): 14269-14273.

Sekine, K. T., Ishihara, T., Hase, S., Kusano, T., Shah, J. & Takahashi, H. (2006). Single amino acid alterations in Arabidopsis thaliana RCY1 compromise resistance to Cucumber mosaic virus, but differentially suppress hypersensitive response-like cell death. *Plant Mol Biol* 62(4-5): 669-682.

Sekine, K. T., Nandi, A., Ishihara, T., Hase, S., Ikegami, M., Shah, J. & Takahashi, H. (2004). Enhanced resistance to Cucumber mosaic virus in the Arabidopsis thaliana ssi2 mutant is mediated via an SA-independent mechanism. *Mol Plant Microbe Interact* 17(6): 623-632.

Seo, J.-K., Kwon, S.-J., Choi, H.-S. & Kim, K.-H. (2009). Evidence for alternate states of Cucumber mosaic virus replicase assembly in positive- and negative-strand RNA synthesis. *Virology* 383(2): 248-260.

Seo, Y. S., Rojas, M. R., Lee, J. Y., Lee, S. W., Jeon, J. S., Ronald, P., Lucas, W. J. & Gilbertson, R. L. (2006). A viral resistance gene from common bean functions across plant families and is up-regulated in a non-virus-specific manner. *Proc Natl Acad Sci U S A* 103(32): 11856-11861.

Silva, M. S., Wellink, J., Goldbach, R. W. & van Lent, J. W. (2002). Phloem loading and unloading of Cowpea mosaic virus in Vigna unguiculata. *J Gen Virol* 83(Pt 6): 1493-1504.

Singh, M. & Singh, R. P. (1995). Propagation of plasmid containing an unstable insert of potato virus Yo using STBL2- competent cells. *Focus* 17: 72-73.

Slewinski, T. L., Zhang, C. & Turgeon, R. (2013). Structural and functional heterogeneity in phloem loading and transport. *Frontiers in Plant Science* 4.

Smirnyagina, E., Hsu, Y. H., Chua, N. & Ahlquist, P. (1994). Second-site mutations in the brome mosaic virus RNA3 intercistronic region partially suppress a defect in coat protein mRNA transcription. *Virology* 198(2): 427-436.

Soards, A. J., Murphy, A. M., Palukaitis, P. & Carr, J. P. (2002). Virulence and differential local and systemic spread of cucumber mosaic virus in tobacco are affected by the CMV 2b protein. *Mol Plant Microbe Interact* 15(7): 647-653.

Stoker, N. G., Fairweather, N. F. & Spratt, B. G. (1982). Versatile low-copy-number plasmid vectors for cloning in Escherichia coli. *Gene* 18(3): 335-341.

Su, S., Liu, Z., Chen, C., Zhang, Y., Wang, X., Zhu, L., Miao, L., Wang, X. C. & Yuan, M. (2010). Cucumber Mosaic Virus movement protein severs actin filaments to increase the plasmodesmal size exclusion limit in tobacco. *Plant Cell* 22(4): 1373-1387.

Sunpapao, A., Nakai, T., Dong, F., Mochizuki, T. & Ohki, S. T. (2009). The 2b protein of cucumber mosaic virus is essential for viral infection of the shoot apical meristem and for efficient invasion of leaf primordia in infected tobacco plants. *J Gen Virol* 90(Pt 12): 3015-3021.

Symons, R. H. (1975). Cucumber mosaic virus RNA contains 7-methyl guanosine at the 5'-terminus of all four RNA species. *Mol Biol Rep* 2(4): 277-285.

Takahashi, H., Suzuki, M., Natsuaki, K., Shigyo, T., Hino, K., Teraoka, T., Hosokawa, D. & Ehara, Y. (2001). Mapping the virus and host genes involved in the resistance response in cucumber mosaic virus-Infected Arabidopsis thaliana. *Plant Cell Physiol* 42(3): 340-347.

Takeshita, M., Matsuo, Y., Suzuki, M., Furuya, N., Tsuchiya, K. & Takanami, Y. (2009). Impact of a defective RNA 3 from cucumber mosaic virus on helper virus infection dynamics. *Virology* 389(1–2): 59-65.

Takeshita, M., Shigemune, N., Kikuhara, K., Furuya, N. & Takanami, Y. (2004). Spatial analysis for exclusive interactions between subgroups I and II of Cucumber mosaic virus in cowpea. *Virology* 328(1): 45-51.

Takeshita, M., Suzuki, M. & Takanami, Y. (2001). Combination of amino acids in the 3a protein and the coat protein of cucumber mosaic virus determines symptom expression and viral spread in bottle gourd. *Arch Virol* 146(4): 697-711.

Taliansky, M. E. & Garcia-Arenal, F. (1995). Role of cucumovirus capsid protein in long-distance movement within the infected plant. *J Virol* 69(2): 916-922.

Tanaka, K., Akashi, Y., Fukunaga, K., Yamamoto, T., Aierken, Y., Nishida, H., Long, C. L., Yoshino, H., Sato, Y. & Kato, K. (2013). Diversification and genetic differentiation of cultivated melon inferred from sequence polymorphism in the chloroplast genome. *Breed Sci* 63(2): 183-196.

Thompson, J. R. & García-Arenal, F. (1998). The Bundle Sheath-Phloem Interface of Cucumis sativus Is a Boundary to Systemic Infection by Tomato Aspermy Virus. *Molecular Plant-Microbe Interactions* 11(2): 109-114.

Truniger, V. & Aranda, M. A. (2009). Recessive resistance to plant viruses. *Adv Virus Res* 75: 119-159.

Truniger, V., Nieto, C., Gonzalez-Ibeas, D. & Aranda, M. (2008). Mechanism of plant eIF4E-mediated resistance against a Carmovirus (Tombusviridae): cap-independent translation of a viral RNA controlled in cis by an (a)virulence determinant. *Plant J* 56(5): 716-727.

Turgeon, R., Beebe, D. U. & Gowan, E. (1993). The intermediary cell: Minor-vein anatomy and raffinose oligosaccharide synthesis in the Scrophulariaceae. *Planta* 191(4): 446-456.

Turgeon, R. & Medville, R. (2004). Phloem loading. A reevaluation of the relationship between plasmodesmatal frequencies and loading strategies. *Plant Physiology* 136(3): 3795-3803.

Turgeon, R., Webb, J. A. & Evert, R. F. (1975). Ultrastructure of minor veins in Cucurbita pepo leaves. *Protoplasma* 83(3): 217-232.

van Bel, A. E. & Kempers, R. (1997).The Pore/Plasmodesm Unit; Key Element in the Interplay Between Sieve Element and Companion Cell. In *Progress in Botany*, Vol. 58, 278-291 (Eds H. D. Behnke, U. Lüttge, K. Esser, J. Kadereit and M. Runge). Springer Berlin Heidelberg.

van Bel, A. J., Gamalei, Y. V., Ammerlaan, A. & Bik, L. P. (1992). Dissimilar phloem loading in leaves with symplasmic or apoplasmic minor-vein configurations. *Planta* 186(4): 518-525.

Vaquero, C., Turner, A. P., Demangeat, G., Sanz, A., Serra, M. T., Roberts, K. & Garcia-Luque, I. (1994). The 3a protein from *Cucumber mosaic virus* increases the gating capacity of plasmodesmata in transgenic tobacco plants. *J Gen Virol* 75(11): 3193-3197.

Verchot, J., Driskel, B. A., Zhu, Y., Hunger, R. M. & Littlefield, L. J. (2001). Evidence that soilborne wheat mosaic virus moves long distance through the xylem in wheat. *Protoplasma* 218(1-2): 57-66.

Verma, N., Mahalingam, B. K., Ram, R. & Zaidi, A. A. (2006). Coat protein sequence shows that Cucumber mosaic virus isolate from geraniums (Pelargonium spp.) belongs to subgroup II. *J Biosci* 31(1): 47-54.

Vogler, H., Kwon, M. O., Dang, V., Sambade, A., Fasler, M., Ashby, J. & Heinlein, M. (2008). *Tobacco mosaic virus* movement protein enhances the spread of RNA silencing. *PLoS Pathogens* 4(4).

Voinnet, O., Lederer, C. & Baulcombe, D. C. (2000). A viral movement protein prevents spread of the gene silencing signal in Nicotiana benthamiana. *Cell* 103(1): 157-167.

Voinnet, O., Pinto, Y. M. & Baulcombe, D. C. (1999). Suppression of gene silencing: a general strategy used by diverse DNA and RNA viruses of plants. *Proc Natl Acad Sci U S A* 96(24): 14147-14152.

von Korff, M., Wang, H., Leon, J. & Pillen, K. (2004). Development of candidate introgression lines using an exotic barley accession (Hordeum vulgare ssp. spontaneum) as donor. *Theor Appl Genet* 109(8): 1736-1745.

Waigmann, E., Ueki, S., Trutnyeva, K. & Citovsky, V. (2004). The Ins and Outs of Nondestructive Cell-to-Cell and Systemic Movement of Plant Viruses. *Critical Reviews in Plant Sciences* 23(3): 195-250.

Wang, Y., Tzfira, T., Gaba, V., Citovsky, V., Palukaitis, P. & Gal-On, A. (2004). Functional analysis of the Cucumber mosaic virus 2b protein: pathogenicity and nuclear localization. *J Gen Virol* 85(Pt 10): 3135-3147.

Wintermantel, W., Banerjee, N., Oliver, J., Paolillo, D. & Zaitlin, M. (1997). Cucumber mosaic virus is restricted from entering minor veins in transgenic tobacco exhibiting replicase-mediated resistance. *Virology* 231: 248.

Wolf, S., Deom, C. M., Beachy, R. N. & Lucas, W. J. (1989). Movement protein of tobacco mosaic virus modifies plasmodesmatal size exclusion limit. *Science* 246(4928): 377-379.

Wong, S. M., Thio, S. S. C., Shintaku, M. H. & Palukaitis, P. (1999). The rate of cell-to-cell movement in squash of cucumber mosaic virus is affected by sequences of the capsid protein. *Molecular Plant-Microbe Interactions* 12(7): 628-632.

Xu, P. & Roossinck, M. J. (2000). Cucumber mosaic virus D satellite RNA-induced programmed cell death in tomato. *Plant Cell* 12(7): 1079-1092.

Yi, S., Akashi, Y., Tanaka, K., Cho, T., Khaing, M., Yoshino, H., Nishida, H., Yamamoto, T., Win, K. & Kato, K. (2009). Molecular analysis of genetic diversity in melon landraces (Cucumis melo L.) from Myanmar and their relationship with melon germplasm from East and South Asia. *Genetic Resources and Crop Evolution* 56(8): 1149-1161.

Yoshii, M., Nishikiori, M., Tomita, K., Yoshioka, N., Kozuka, R., Naito, S. & Ishikawa, M. (2004). The Arabidopsis cucumovirus multiplication 1 and 2 loci encode translation initiation factors 4E and 4G. *J Virol* 78(12): 6102-6111.

Zamir, D. (2001). Improving plant breeding withexotic genetic libraries. *Nat Rev Genet* 2: 983-989.

Zhang, B., Tolstikov, V., Turnbull, C., Hicks, L. M. & Fiehn, O. (2010). Divergent metabolome and proteome suggest functional independence of dual phloem transport systems in cucurbits. *Proceedings of the National Academy of Sciences*.

Zhang, C. & Turgeon, R. (2009). Downregulating the sucrose transporter VpSUT1 in Verbascum phoeniceum does not inhibit phloem loading. *Proceedings of the National Academy of Sciences of the United States of America* 106(44): 18849-18854.

Zhang, C., Yu, X., Ayre, B. G. & Turgeon, R. (2012). The origin and composition of cucurbit "Phloem" exudate. *Plant Physiology* 158(4): 1873-1882.

Zhang, L., Handa, K. & Palukaitis, P. (1994). Mapping local and systemic symptom determinants of cucumber mosaic cucumovirus in tobacco. *J Gen Virol* 75 (Pt 11): 3185-3191.

Ziebell, H., Murphy, A. M., Groen, S. C., Tungadi, T., Westwood, J. H., Lewsey, M. G., Moulin, M., Kleczkowski, A., Smith, A. G., Stevens, M., Powell, G. & Carr, J. P. (2011). Cucumber mosaic virus and its 2b RNA silencing suppressor modify plant-aphid interactions in tobacco. *Sci Rep* 1: 187.

Ziebell, H., Payne, T., Berry, J. O., Walsh, J. A. & Carr, J. P. (2007). A cucumber mosaic virus mutant lacking the 2b counter-defence protein gene provides protection against wild-type strains. *Journal of General Virology* 88(10): 2862-2871.