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► To cite this version:

Mattia Dall'Ara. RNA/RNA interactions involved in the regulation of Benyviridae viral cycle. Vegetal Biology. Université de Strasbourg; Università degli studi (Bologne, Italie), 2018. English. NNT: 2018STRAJ019 . tel-02003448

HAL Id: tel-02003448

<https://theses.hal.science/tel-02003448>

Submitted on 1 Feb 2019

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Université de Strasbourg et Università di Bologna

Thèse en co-tutelle

Présentée à la

FACULTÉ DES SCIENCES DE LA VIE

En vue de l'obtention du titre de

DOCTEUR DE L'UNIVERSITÉ DE STRASBOURG

Discipline : Sciences de la Vie et de la Santé,

Spécialité : Aspects moléculaires et cellulaires de la biologie

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RNA/RNA interactions involved in the regulation of Benyviridae viral cycle

Soutenue publiquement le 18 mai 2018 devant la Commission d'Examen :

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Acknowledgments

First of all I want to thank my tutors David and Claudio. They believed in me and accepted me in their labs giving me the opportunity to work on a such interesting topic. Being a Ph.D. student is hard work but it would be even harder without their help, example and coaching. I would like to thanks all the people that worked with me or I have had the pleasure to meet during these four years of “co-tutelle”, but especially Chiara, Alice, Alyssa, Elodie, Marco, Marion, Matteo, Antonio, Mariateresa, Danièle, Salah, Veronique, Khalid, Imma (!), Roby kiwi and Roby grapevine and many others. I have a special thank for each of you.

I also want to thank the member of the jury, Dr Stéphane Blanc, Dr Renato Brandimarti, Dr Roland Marquet and Dr Mirco Iotti for evaluating this work.

Résumé / Riassunto

Les virus multipartites possèdent au moins deux sinon plusieurs segments du génome encapsidés dans des particules indépendantes. Ce groupe hétérogène de virus (ssADN circulaire, dsARN ou ssARN de polarité positive ou négative) est retrouvé majoritairement chez les plantes [1]. Actuellement, il est admis que pour assurer une infection productive, les virus doivent préserver leur intégrité génomique comme unité infectieuse fonctionnelle. Les virus monopartites et segmentés suivent cette règle car chaque particule virale renferme l'intégrité de leur génome. A l'inverse pour les virus multipartites, l'encapsulation en entités distinctes de chaque segment porteur d'information génomique rend la notion d'intégrité génomique énigmatique [2]. La condition pour assurer l'apport d'un set complet des segments génomiques dans chaque cellule infectée implique un niveau élevé de multiplicité d'infection. Cette forte multiplicité d'infection peut être considérée comme ayant un coût biologique insoutenable en termes de réplication virale. Durant l'infection, les virus multipartites organisent leur génome en une formule génomique définie, dans laquelle certains segments seraient moins nombreux que d'autres [3]. Dans la plante, la propagation indépendante et aléatoire des différentes parties génomiques virales ne peut être un modèle soutenable pour le mouvement à longue distance dans les tissus vasculaires. Les tubes criblés sont des éléments de la plante dans lesquels les virus ne peuvent se répliquer. Ils ne peuvent donc pas y augmenter leur titre viral et assurer dans chaque cellule compagne la présence de tous les éléments nécessaires pour initier une nouvelle infection.

Afin d'étudier les aspects de l'intégrité génomique des virus multipartites à ARN le *Beet necrotic yellow vein virus* BNYVV a été choisi comme modèle car son génome consiste en quatre ou cinq ssARN de polarité positive. De plus, comme la majorité des virus multipartites à ARN des plantes le BNYVV a une organisation génomique caractérisée par deux segments (i.e. ARN1 et ARN2) qui codent les gènes de ménage, indispensables pour l'infection au niveau de la feuille ainsi que des segments dit accessoires mais qui restent nécessaires pour l'infection naturelle de l'hôte, *Beta vulgaris*. Si l'ARN1 code pour la replicase virale, l'ARN2 dirige la synthèse des protéines majeures et mineures de capsid (CP et p75) nécessaires à la formation des particules en bâtonnets. Il permet également la production des protéines responsable du mouvement de cellule à cellules (*triple gene block*, TGB) et du suppresseur d'ARN interférence (p14) grâce à la production d'ARN subgénomiques. L'ARN3 est

impliqué dans le mouvement à longue distance et code la protéine p25, le déterminant de la prolifération racinaire (rhizomanie), l'un des symptômes qui est aggravé lorsque l'ARN5 est présent. L'ARN4 quant à lui est nécessaire à la transmission des particules virales par le vecteur *Polymyxa betae* [4].

L'infection de cellule à cellule implique des complexes ribonucléoprotéiques (RNP) qui implique les protéines TGB et le VSR mais pas la protéine de capsid. Cependant, si la protéine CP est nécessaire à l'infection virale à longue distance (infection systémique), l'encapsidation des ARN génomiques n'est pas essentielle pour le mouvement à longue distance du BNYVV. Nous avons donc émis l'hypothèse qu'il existait un réseau d'interaction entre les ARN génomiques viraux assurant la présence d'au moins une copie de chaque ARN génomique. Cette hypothèse rappelle l'interaction ARN/ARN démontrée comme nécessaire à l'empaquetage sélectif des segments du virus *Influenza A* [5].

Mon objectif est de déterminer l'existence des interactions entre les ARN génomiques et leur implication dans le cycle viral. J'ai donc vérifié si une formule génomique virale était maintenue dans chacun des hôtes et dans des tissus variés (chapitre 1); si l'ensemble des segments génomiques était effectivement retrouvé dans chaque cellule infectée (chapitre 2). *In fine*, j'ai identifié des séquences présentes sur les ARN1 et 2 validant l'existence d'interactions ARN/ARN entre les ARN génomiques (chapitre 3).

Dans le modèle proposé pour baisser au minimum le coût biologique de la préservation de l'intégrité génomique, un réseau d'interactions ARN/ARN détermine la reconnaissance et l'assemblage de chaque ARN génomique dans un complexe RNP modulaire. Ce complexe est considéré comme l'unité infectieuse mobile pour la propagation du virus multipartite dans la plante [6]. Si ce complexe RNP existe comme entité virale régulée et fonctionnelle, il est probable qu'il soit préservé parmi divers tissus et parmi des espèces de plantes différentes. Après avoir optimisé et validé le protocole *dual step digital droplets* (dd) RT-PCR, j'ai déterminé la formule génomique dans différents hôtes du BNYVV (*Chenopodium quinoa*, *Spinacea oleracea* and *Beta macrocarpa*) et tissus (feuilles et racines). J'ai comparé les ARN génomiques totaux avec ceux encapsidés qui représentent la forme transmissible du virus. Que ce soit pour les ARN génomiques totaux ou encapsidés, mes résultats indiquent une forte accumulation des ARN auxiliaires (ARN3 et ARN4) jusqu'à un ordre de grandeur par rapport aux ARN1 et ARN2, indépendamment de l'hôte et des tissus. Considérant la fréquence

relative des ARN3 et 4, le BNYVV semble fixer sa formule génomique de manière différente en fonction de l'hôte et du tissu systémique. Toutefois le ratio entre les ARN1 et 2 (qui expriment les protéines essentielles au cycle viral) semble être conservé et correspond à 2 ou 3 molécules d'ARN1 pour une molécule de ARN2.

Le mouvement du virus dans la plante est décrit comme une circulation non coordonnée de particules ou de complexes RNP. Toutefois, l'accumulation différentielle des ARN de BNYVV dans les tissus infectés rend insoutenable le coût de la préservation de l'intégrité génomique assurant la présence du set complet des segments génomiques à l'intérieur de chaque cellule infectée. Cet état de faits porte à la formuler deux hypothèses opposées:

1. les segments du BNYVV ne doivent pas forcément être présents simultanément dans chaque cellule infectée et la circulation des protéines virales assure la réplication et la stabilisation des ARN grâce à la continuité du symplasme.

2. un mécanisme spécifique garanti le mouvement coordonné des segments comme une unité infectieuse.

Pour vérifier l'une ou l'autre hypothèse, la présence simultanée des quatre ARN génomiques du BNYVV a été testée avec le protocole *Real time RT-PCR*, individuellement sur des protoplastes isolés provenant de plantes d'épinards systématiquement infectées. Les protoplastes ont été préparés et fixés, après dilution, avec du glutaraldehyde à 0.5%. Chaque protoplaste isolé ou le milieu dépourvu de protoplaste (utilisé comme contrôle négatif) a été échantillonné par pipetage sous microscope optique. Nous avons utilisé les échantillons comme matrice d'une réaction *triplex qualitative RT-PCR Real time* qui a comme cible deux ARN différents du BNYVV et la sous-unité majeur de la Rubisco (RbcL). La RT-PCR sur l'ARN du BNYVV a été suivie en temps réel utilisant des sondes TaqMan avec différents fluorophores (FAM et HEX) et la présence de contrôle endogène RbcL a été vérifiée après amplification (*end-point*) sur gel d'agarose. Quatre réactions ont été réalisées pour vérifier la présence de l'ARN1 avec l'ARN2, de l'ARN2 avec l'ARN3, de l'ARN1 avec l'ARN4 et enfin de l'ARN3 avec l'ARN4. Entre 30 et 50% des protoplastes analysés sont infectés par le virus. ~93% des protoplastes infecté a révélé la présence des ARN viraux testés. Ces résultats suggèrent donc bien l'existence d'un mécanisme qui orchestre le déplacement coordonné des segments génomiques du BNYVV. Etant donné que les particules virales ne diffèrent que par

leur longueur (qui dépend de la taille de l'ARN génomique encapsidés), nous avons proposé l'hypothèse selon laquelle la reconnaissance spécifique des diverses espèces génomiques a lieu grâce à des interactions ARN/ARN [6].

Afin de vérifier le modèle proposé impliquant l'interaction ARN/ARN pour la formation des unités RNP mobiles et infectieuses renfermant au moins une copie de chaque segment, nous avons utilisé la technique de gel-retard (*Electro Mobility Gel Shift Assays, EMSA*). Dans certains hôtes, la seule présence de l'ARN1 et de l'ARN2 suffit pour produire une infection dans la plante entière. Par conséquent, le complexe RNP minimal devrait impliquer l'interaction entre ces deux ARN génomiques. Pour cette raison nous avons cherché à évaluer l'existence d'interactions spécifiques et hétérologues entre l'ARN1 et l'ARN2.

La co-transcription *in vitro* des clones complets ou partiels des ARN1 ou ARN2 a été suivie par électrophorèse en gel natif ou en présence de chélateur de magnésium (Mg^{2+}). Nous avons observé la présence de duplexes d'ARN (homo et hétérodimères) aussi bien sur gel coloré au bromure d'éthidium que par hybridation moléculaire à l'aide de sondes spécifiques radiomarquées. Nous n'avons pas pu observer des décalages de bandes significatifs avec les ARN1 et ARN2 complets, probablement en raison de l'absence de facteurs viraux et cellulaires qui pourraient aider et réguler l'interaction ARN/ARN en contexte naturel. Toutefois, l'utilisation des clones partiels ARN1 (3655-4783) et ARN2 (1-2715) a démontré la formation d'un hétéro-duplex stable en gel partiellement dénaturant, une condition réduisant considérablement la stabilité des structures ARN normalement stabilisées par le magnésium. En réduisant les tailles des séquences utilisées, nous avons pu localiser les domaines d'interaction et nous avons affiné leur identification en utilisant des oligodésoxyribonucléotides compétiteurs (ODN). Ce domaine d'interaction consiste en deux séquences complémentaires de 17 nucléotides présentant un mésappariement de deux bases non complémentaires. Ces séquences sont situées respectivement à l'extrémité 5' UTR de l'ARN2, à partir du quatrième nucléotide, et dans la région de l'ARN1 codant la réplicase. Afin de démontrer le sens biologique de l'interaction ARN/ARN trouvée, nous avons créé des mutations équivalentes et compensatoires dans les clones ADNc infectieux de l'ARN1 et de l'ARN2 pour prévenir ou restaurer les appariements de bases. Nous avons effectué des expériences de gel-retard EMSA pour valider les effets des mutations sur les interactions, en utilisant des ODN compétiteurs portant les mutations indiquées. Ces ODN n'empêchent pas

les interactions des ARN sauvages contrairement à ceux utilisés initialement. Nous avons évalué le sens biologique des domaines d'interactions identifiés en contexte viral *in planta*. Nous avons réalisé des tests de vitalité des ARN génomiques mutants sur feuille de *C. quinoa* (hôte local) pour évaluer le maintien des fonctions essentielles de réplication, mouvement cellule-à-cellule, et suppression de l'interférence par ARN lorsque l'interaction ARN/ARN est interrompue ou restaurée par un appariement de bases alternatif. Les combinaisons ARN1 WT+ARN2 MUT et ARN1 MUT+ARN2 MUT n'ont pas conduit à la formation de sites d'infection au niveau des feuilles probablement en raison de l'altération fonctionnelle du promoteur de l'ARN2 MUT. Toutefois la combinaison ARN MUT avec ARN2 WT a permis la réplication et le mouvement cellule-cellule du virus avec une diminution drastique du nombre des lésions locales comparée avec la combinaison sauvage. Le BNYVV et le *Beet soil borne mosaic virus* (BSBMV) sont deux espèces différentes mais étroitement apparentés et appartenant au genre *Benyvirus* et à la famille *Benyviridae* [4]. Ils ont en commun le même hôte, vecteur de transmission et organisation génomique. L'échange artificiel des ARN génomiques entre les ARN1 et ARN2 du BNYVV et du BSBMV conduit à la formation de virus chimériques [7]. Après avoir effectué une comparaison bio-informatique, nous avons conclu qu'il existe un réseau alternatif d'interactions ARN1/ARN2 pour ces virus réassortants. Nous avons donc comparé le comportement des chimères formées par les ARN1 WT BNYVV + ARN2 BSBMV et ARN1 MUT BNYVV + ARN2 BSBMV. Le nombre comparable des lésions induites sur feuille de *C. quinoa*, tout comme les niveaux similaires d'ARN accumulés démontrent que les mutations introduites dans l'ARN1 du BNYVV ne sont pas impliquées dans la réplication (i.e stabilisation des ARN et mouvement cellule-cellule) mais effectivement dans l'interaction ARN1/ARN2 responsable de l'orchestration d'un réseau régulateur.

Pour renforcer ces résultats, la réplication et le mouvement des ARN1 et 2 sauvages ont été évalués en présence de replicon du BNYVV qui porte l'un des deux domaines d'interaction, muté ou non. L'accumulation de ces réplicons vise à surproduire des ARN entrant en compétition avec les segments génomiques et interférer dans la formation de l'interaction ARN1/ARN2. Quatre cent nucléotides de l'ARN2 ou de l'ARN1 ont été insérés dans le vecteur dérivant de l'ARN5 (Rep5, constitué du 5' et 3' UTR de l'ARN5). L'analyse de l'accumulation des ARN viraux en protoplastes de *C. quinoa* a permis de constater un effet défectif interférent (DI) du Rep5 porteur du domaine d'interaction de l'ARN2. Cet effet n'a

pas été observé avec l'ARN5 portant le domaine d'interaction de l'ARN1. Afin d'exclure l'effet DI des compétiteurs introduits, nous avons réalisé des expérimentations similaires utilisant un ODN complémentaire au domaine d'interaction de l'ARN1. En tenant compte d'une possible activité RNase H dans la cellule nous avons utilisé comme contrôle négatif d'autres ODN complémentaires à l'ARN1 et au brin négatif de l'ARN2. Lorsque la quantité de l'ODN est cinq fois plus élevée que celle de la cible l'ARN1, nous avons observé une réduction significative de l'accumulation de l'ARN viral. Cet effet n'est pas observé dans le contrôle négatif. Toutefois, en utilisant un ratio 50.1, l'activité RNase H semble activée puisque la quantité d'ARN génomique diminue de manière importante quel que soit l'ODN utilisé.

En conclusion, nous avons démontré que tous les segments génomiques du BNYVV sont présents à l'intérieur de chaque cellule infectée des tissus systémiques dans lesquels s'accumulent les ARN jusqu'à atteindre une formule génomique déterminée préservant un ratio ARN1 et ARN2 similaire mais présentant une variabilité de l'accumulation des ARN3 et 4 selon les types d'organes et d'hôtes. Dans un modèle dans lequel les ARN viraux interagissent entre eux pour former une unité mobile infectieuse, l'ARN1 et l'ARN2 interagissent au moins *in silico* et *in vitro*. L'existence d'une telle interaction *in vivo* est fortement renforcée grâce aux résultats obtenus à l'aide des virus chimères BNYVV et BSBMV. De telles interactions pourraient d'une part réguler l'accumulation des ARN génomiques et d'autre part orchestrer le cycle viral (encapsidation, régulation de l'expression des protéines et de la synthèse des ARN subgénomiques).

I virus multipartiti possiedono due o più segmenti genomici incapsidati in particelle indipendenti. Questo gruppo eterogeneo di virus (ssDNA circolare, dsRNA o ssRNA a polarità positiva o negativa) infetta prevalentemente le piante [1]. Ad oggi, è scientificamente accettato che, per assicurare una infezione produttiva, i virus preservano la loro integrità genomica come unità infettive funzionali. I virus monopartitici e segmentati seguono questa regola in quanto ogni particella virale contiene un set genomico completo. Diversamente, per i virus multipartiti, l'incapsidazione in entità distinte di ogni segmento genomico rende enigmatica la nozione di integrità genomica [2]. La condizione necessaria per assicurare la presenza di un set genomico completo del virus in ogni cellula infettata implica, infatti, un

alto livello di molteplicità di infezione il cui costo biologico in termini di replicazione virale può essere considerato insostenibile. Durante l'infezione, i virus multipartiti organizzano il loro genoma in formule genomiche definite, nelle quali alcuni segmenti sono più abbondanti di altri [3]. Nella pianta, la propagazione indipendente e aleatoria delle differenti componenti genomiche virali nei tessuti vascolari non può essere considerato un modello sostenibile di movimento a lunga distanza. I tubi cribrosi sono infatti elementi della pianta nei quali i virus non possono replicarsi e quindi aumentare il loro titolo virale e assicurare in ogni cellula compagna la presenza di tutti gli elementi necessari per iniziare una nuova infezione.

Al fine studiare differenti aspetti dell'integrità genomica dei virus multipartiti aventi genoma a RNA il *Beet necrotic yellow vein virus* (BNYVV) è stato scelto come modello in quanto il suo genoma consiste in quattro o cinque ssRNA a polarità positiva. Inoltre, come la maggior parte dei virus multipartiti a RNA delle piante il BNYVV presenta una organizzazione genomica caratterizzata da due segmenti (i.e. RNA1 e RNA2) indispensabili per l'infezione a livello della foglia, in quanto codificanti geni housekeeping, e segmenti detti accessori che restano comunque necessari per l'infezione naturale dell'ospite, *Beta vulgaris*. Se l'RNA1 codifica la replicasi virale, l'RNA2 dirige la sintesi dei costituenti proteici maggiore e minore del capsido (CP e p75) necessari per la formazione delle particelle bastoncelliformi. L'RNA2 permette altresì l'espressione delle proteine responsabili del movimento cellula a cellula (triple gene block, TGB) e del soppressore dell'RNA interference (p14) grazie alla produzione di RNA subgenomici. L'RNA3 è implicato nel movimento a lunga distanza e codifica la proteina p25, il determinante della iper proliferazione radicale (rizomania), sintomatologia aggravata dalla presenza dell'RNA5. L'RNA4 è infine necessario per la trasmissione delle particelle virali attraverso il vettore *Polomyxa betae* [4].

L'infezione cellula a cellula necessita la formazione di complessi ribonucleoproteici (RNP) che implicano le proteine TGB e il VSR ma non la proteina del capsido. Tuttavia se la CP è necessaria per l'infezione virale a lunga distanza (infezione sistemica) del BNYVV, l'incapsidazione degli RNA genomici non è essenziale per l'infezione sistemica. Abbiamo quindi ipotizzato l'esistenza di un network di interazioni tra gli RNA genomici che assicura la presenza di almeno una copia di ogni RNA genomico all'interno di un complesso macromolecolare rappresentante l'unità infettiva virale. Questa ipotesi richiama le interazioni

RNA/RNA dimostrate necessarie per l'impacchettamento selettivo dei segmenti del virus dell'*Influenza A* [5].

Il mio obiettivo è quello di determinare l'esistenza delle interazioni tra gli RNA genomici del BNYVV e la loro implicazione nel ciclo virale. Ho quindi verificato se una formula genomica è mantenuta in ospiti e organi differenti (capitolo 1); se il set completo di segmenti genomici si ritrova effettivamente in ogni cellula infetta (capitolo 2). In fine, ho identificato due sequenze presenti nell'RNA1 e nell'RNA2 che convalidano l'esistenza di interazione RNA/RNA tra gli RNA genomici del BNYVV (capitolo 3).

Nel modello proposto per minimizzare il costo del mantenimento dell'integrità genomica, un network di interazioni RNA/RNA determina il riconoscimento e l'assemblaggio di ogni RNA genomico in un complesso RNP modulare. Questo complesso è considerato come l'unità infettiva mobile per la propagazione del virus multipartito nella pianta [6]. Se questo complesso RNP esiste come entità virale regolata e funzionale, è probabile che sia conservato all'interno di organi differenti e di ospiti differenti. Dopo aver ottimizzato un protocollo di *dual step digital droplets* (dd) RT-PCR, ho determinato la formula genomica in differenti ospiti del BNYVV (*Chenopodium quinoa*, *Spinacea oleracea* e *Beta macrocarpa*) in foglie e radici. Ho quindi comparato la quantità di RNA genomico totale con quella dell'RNA incapsidato che rappresenta la forma trasmissibile del virus. Sia per gli RNA genomici totali sia per quelli incapsidati, i miei risultati indicano un forte accumulo, fino ad un ordine di grandezza degli RNA ausiliari (RNA3 e RNA4) rispetto agli RNA1 e RNA2, indipendentemente dall'ospite o organo analizzato. Considerando la frequenza relativa degli RNA3 e RNA4, il BNYVV sembra stabilire la sua formula genomica in maniera differente in funzione dell'ospite e organo sistemico. Tuttavia, il rapporto tra gli RNA1 e RNA2 (che esprimono le proteine essenziali al ciclo virale) sembra essere conservato e corrisponde a 2 o 3 molecole di RNA1 per una molecola di RNA2.

Il movimento del virus nella pianta è descritto come una circolazione non coordinata di particelle o complessi RNP. Tuttavia l'accumulo differenziale degli RNA del BNYVV nei tessuti infetti rende insostenibile il costo di assicurare all'interno di ogni cellula infetta il set completo di segmenti genomici. Questo stato di fatto porta alla formulazione delle seguenti due ipotesi:

1. I segmenti del BNYVV non devono necessariamente essere presenti simultaneamente in ogni cellula infetta e la continuità simplastica garantisce la circolazione delle proteine virali indispensabili per la replicazione e stabilizzazione degli RNA del virus.
2. Un meccanismo specifico garantisce il movimento coordinato dei segmenti che costituiscono una unità infettiva.

Per verificare una o l'altra ipotesi, la presenza simultanea dei quattro RNA genomici del BNYVV è stata testata grazie a un protocollo di *Real time RT-PCR* su singolo protoplasto isolato da tessuti infetti e sistemici provenienti da piante di spinacio. I protoplasti sono stati preparati e fissati, dopo diluizione, con gluteraldeide al 0,5%. Ogni protoplasto isolato o il buffer privo di protoplasti (utilizzato come controllo negativo) sono stati campionati per pipettaggio al microscopio ottico. Abbiamo quindi utilizzato i campioni come matrice per una reazione qualitativa di *triplex RT-PCR Real time* avente come target due RNA differenti del BNYVV e la sub-unità maggiore della Rubisco (RbcL). La RT-PCR sugli RNA del BNYVV è stata seguita in tempo reale utilizzando sonde TaqMan con differenti fluorofori (FAM ed HEX) e la presenza del controllo endogeno RbcL è stata verificata, dopo amplificazione (*end-point*), su gel di agarosio. Quattro reazioni sono state eseguite per verificare la presenza dell'RNA1 con RNA2, dell'RNA2 con l'RNA3, dell'RNA1 con l'RNA4 e dell'RNA3 con l'RNA4. Il ~93% dei protoplasti infetti ha rivelato la co-presenza degli RNA virali testati. Questi risultati suggeriscono quindi l'esistenza di un meccanismo che orchestra lo spostamento coordinato dei segmenti genomici del BNYVV. Tenendo conto che le particelle virali non differiscono tra loro se non per la loro lunghezza (che dipende dalla taglia dell'RNA genomico incapsidato), abbiamo proposto l'ipotesi secondo la quale il riconoscimento specifico delle diverse specie genomiche avviene grazie a delle interazione RNA/RNA [6].

Al fine di verificare il modello proposto che implica la formazione di interazioni RNA/RNA per la formazione delle unità RNP mobili e infettive che contengono almeno una copia di ogni segmento genomico, abbiamo utilizzato la tecnica del *Electro Mobility Gel Shift Assay* (EMSA). In alcuni ospiti la sola presenza degli RNA1 e RNA2 è sufficiente per produrre una infezione produttiva nell'intera pianta. Di conseguenza, il complesso RNP minimale dovrebbe implicare l'interazione tra questi due RNA genomici. Per questo motivo abbiamo valutato l'esistenza di interazione specifiche e eterologhe tra gli RNA1 e RNA2.

La co-trascrizione in vitro dei cloni completi o parziali degli RNA1 o RNA2 è stata seguita dalla elettroforesi su gel nativo o in presenza di chelante (EDTA) di ioni magnesio (Mg^{2+}). Abbiamo osservato la presenza di duplex di RNA (omo ed etero dimeri) sia su gel colorato con etidio bromuro sia dopo ibridazione molecolare con sonde radiomarcate specifiche. Non abbiamo tuttavia osservato band shift significativi con gli RNA1 e RNA2 completi, probabilmente a causa dell'assenza di fattori virali e dell'ospite che potrebbero aiutare al regolazione dell'interazione RNA/RNA nel contesto naturale. Tuttavia l'utilizzo dei cloni parziali RNA1 (3655-4783) e RNA2 (1-2715) ha dimostrato la formazione di un complesso etero-duplex stabile in gel parzialmente denaturante. Riducendo le dimensioni delle sequenze utilizzate, siamo stati in grado di localizzare i domini di interazione per poi perfezionare la loro identificazione utilizzando oligodeossiribonucleotidi (ODN) come competitori. Questo dominio di interazione consiste in due sequenze complementari di 17 nucleotidi con disappaiamento dovuto a due basi non complementari. Tali sequenze sono localizzate rispettivamente all'estremità 5' UTR dell'RNA2, a partire dal quarto nucleotide, e nella regione dell'RNA1 che codifica la Replicasi. Al fine di dimostrare il significato biologico dell'interazione RNA/RNA trovata, abbiamo creato mutazioni silenziose e compensatorie nei cloni di cDNA infettivi di RNA1 e RNA2 per prevenire e/o ripristinare l'accoppiamento di basi. Abbiamo eseguito altri esperimenti EMSA per convalidare gli effetti delle mutazioni sulla interazione, utilizzando ODN competitori portanti le mutazioni indicate. Questi ODN non hanno impedito agli RNA wild type di interagire tra loro, a differenza di quelli utilizzati inizialmente. Abbiamo valutato il senso biologico dei domini di interazione identificati nel contesto virale *in planta*. Abbiamo eseguito test di vitalità degli RNA genomici mutanti su foglia di *C. quinoa* (ospite locale) per valutare la preservazione delle funzioni essenziali di replicazione, movimento cellula a cellula e soppressione del silenziamento genico quando l'interazione RNA/RNA viene interrotta o ripristinata da un accoppiamento di basi alternativo. Le combinazioni RNA1 WT + RNA2 MUT e RNA1 MUT + RNA2 MUT non hanno portato alla formazione di loci di infezione fogliare, probabilmente a causa dell'alterazione funzionale del promotore dell'RNA2 MUT. Tuttavia la combinazione RNA1 MUT + RNA2 WT permette la replicazione e movimento cellula a cellula del virus seppure con una drastica riduzione del numero di lesioni locali rispetto alla combinazione wild type. BNYVV e il *Beet soil-borne mosaic virus* (BSBMV) sono due specie diverse ma strettamente correlate appartenenti al genere *Benyvirus* e famiglia *Benyviridae* [4]. Condividono lo stesso ospite,

vettore di trasmissione e organizzazione genomica. Il riassortimento artificiale tra RNA1 e RNA2 di BNYVV e BSBMV ha portato alla formazione di virus chimerici [7]. Dopo un confronto bioinformatico, abbiamo concluso l'esistenza di network di interazioni RNA1/RNA2 alternati in questi due virus riassortanti. Abbiamo, quindi, confrontato il comportamento delle chimere formate da RNA1 BNYVV WT + RNA2 BSBMV e RNA1 BNYVV MUT + RNA2 BSBMV. Il numero comparabile di lesioni indotte su foglie di *C. quinoa*, dimostra che le mutazioni introdotte nel RNA1 di BNYVV non sono coinvolte nella replicazione (i.e. la stabilizzazione dell'RNA e movimento cellula a cellula), ma, effettivamente, nell'interazione RNA1/RNA2 responsabile dell'orchestrazione di un network regolatore.

Per consolidare questi risultati, la replicazione e il movimento degli RNA wild-type 1 e 2 sono stati valutati in presenza del replicone BNYVV che porta uno dei due domini di interazione, mutato o meno. L'accumulo di questi repliconi ha lo scopo di sovra-produrre RNA che entra in competizione con i segmenti genomici e interferisce nella formazione dell'interazione RNA1/RNA2. Quattrocento nucleotidi di RNA2 o RNA1 sono stati inseriti nel vettore derivante dall'RNA5 (Rep5, costituito da 5' e 3' UTR dell'RNA5). L'analisi dell'accumulo di RNA virali nei protoplasti di *C. quinoa* ha rivelato un effetto di RNA defective interference (DI) del Rep5 che porta il dominio di interazione RNA2.

Questo effetto non è stato osservato con il Rep5 che porta il dominio di interazione dell'RNA1. Al fine di escludere l'effetto DI dei competitori introdotti, abbiamo eseguito esperimenti simili a quelli precedentemente descritti utilizzando un ODN complementare al dominio di interazione RNA1. Prendendo in considerazione una possibile attività RNasi H nella cellula, abbiamo usato come controllo negativo altri ODN complementari all'RNA1 e al filamento negativo dell'RNA2. Quando la quantità di ODN è cinque volte superiore a quella dell'RNA1 bersaglio, abbiamo osservato una significativa riduzione dell'accumulo di RNA virale. Questo effetto non è osservato nel controllo negativo. Tuttavia, utilizzando un rapporto 50:1, l'attività RNasi H sembra essere attivata poiché la quantità di RNA genomico diminuisce significativamente indipendentemente dall'ODN utilizzato.

In conclusione, abbiamo dimostrato che tutti i segmenti genomici di BNYVV sono presenti all'interno di ogni cellula infetta in tessuti sistemici dove l'RNA virale si accumula fino a raggiungere una formula genomica ospite specifica, che preserva un rapporto RNA1 e RNA2

simile ma presenta una variabilità dell'accumulo di RNA3 e 4 in base ai tipi di organo e ospite. In un modello secondo il quale gli RNA virali interagiscono l'uno con l'altro per formare un'unità mobile infettiva, l'RNA1 e l'RNA2 interagiscono almeno *in silico* e *in vitro*. L'esistenza di tale interazione *in vivo* è fortemente suggerita dai risultati ottenuti con i virus chimerici BNYVV e BSBMV. Tali interazioni potrebbero, da un lato, regolare l'accumulo di RNA genomici oppure orchestrare il ciclo virale (incapsidazione, regolazione dell'espressione proteica e sintesi di RNA subgenomici).

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Abstract

Multipartite viruses possess more than two genomic segments packaged into independent particles. Such groups of viruses infect mostly plants. They possess a wide range of genome types: circular ssDNA, dsRNA or ssRNA with either positive or negative polarity. The condition to provide a complete set of genomic segments in each infected cell implies a high level of multiplicity of infection that results in an unsustainable biological cost in terms of viral replication. *Beet necrotic yellow vein virus* (BNYVV) was chosen as a model to investigate the aspect of genome integrity of multipartite RNA viruses since such virus possesses the highest number of genomic segments (up to 5) within the Baltimore's class IV that concerns ssRNA(+) viruses. In the proposed model, to minimize the cost of the genome integrity preservation, a network of RNA/RNA interactions determines the recognition and the mobilization of at least one of each genomic RNAs in a modular RNP complex. Such complex must be considered as the mobile infectious unit of the segmented genome during viral spread in the plant. The Aim of this thesis was to experimentally determine the existence of RNA/RNA interactions between genomic BNYVV RNAs and their implication in the viral cycle. In parallel approaches, I demonstrated that all BNYVV genomic segments are present within the single infected cell in systemic tissues where they accumulate to reach set point genomic formulas. These formulas depend on organs and hosts (chapter I and II). In the model where vRNAs interact each other to form the minimal mobile infective unit, RNA1 and RNA2 interaction domain has been identified *in silico* and *in vitro*. The rationale of such an interaction has been provided *in vivo* using BNYVV and Beet soil-borne mosaic virus chimeras (chapter III).

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General Introduction

1. *Benyviridae* family: taxa characteristics

Benyviridae family belongs to group IV of the Baltimore classification [1], together with viruses possessing single-stranded positive-sense RNA genome (ssRNA (+)) that replicate through ssRNA(-) intermediates. The family possesses one single genus, *Benyvirus*, which includes four plant viral species: the type member *Beet necrotic yellow vein virus* (BNYVV), *beet soil-borne mosaic virus* (BSBMV), *burdock mottle virus* (BdMV) and *rice stripe necrosis virus* (RSNV) [2].

Initially assigned to *Tobamovirus* genus because of the virion morphology, BNYVV was included in the *Furovirus* genus characterized by the multipartite RNA genome species transmitted by fungal vectors [3]. *Furovirus* genus was then redefined in *Furovirus*, *Pomovirus*, *Pecluvirus*, *Hordeivirus* genera and *Benyvirus* genus [4] later added in the newborn *Benyviridae* family [2]. This new classification was based on the following taxa criteria: vector of transmission; number of open reading frame (ORF) encoding replicase subunits; presence or absence 3' end poly-A and presence or absence of triple gene block (TGB), a gene module involved in the cell-to-cell and long-distance movement in the host.

As the above mentioned genera, Benyviruses are non-enveloped rigid rod-shaped particles with helical symmetry, an axial channel and a diameter of about 20 nm (Figure 1). Depending on the genomic RNA encapsidated, virion lengths could reach 390 nm representing the longest rod-shaped particle ever observed within plant viruses [5]. Genome is split in up to five RNAs that, unlike the RNAs of other multipartite rod-shaped plant viruses, have a capped 5' end and a polyadenylated 3' end tail [2]. Longest RNA (RNA1) is monocistronic having one large ORF. This RNA encodes for a replication-associated polyprotein that undergoes an autocatalytic cleavage. This characteristic is a second criteria distinguishing *Benyvirus* genus from other genera whose species carry two ORFs encoding multiple replication-associated proteins [5]. Finally similarly to Pomoviruses and Pecluviruses, Benyviruses are characterized by their cell-to-cell movement function relying on a TGB and their host transmission mediated by protozoa vectors [4].

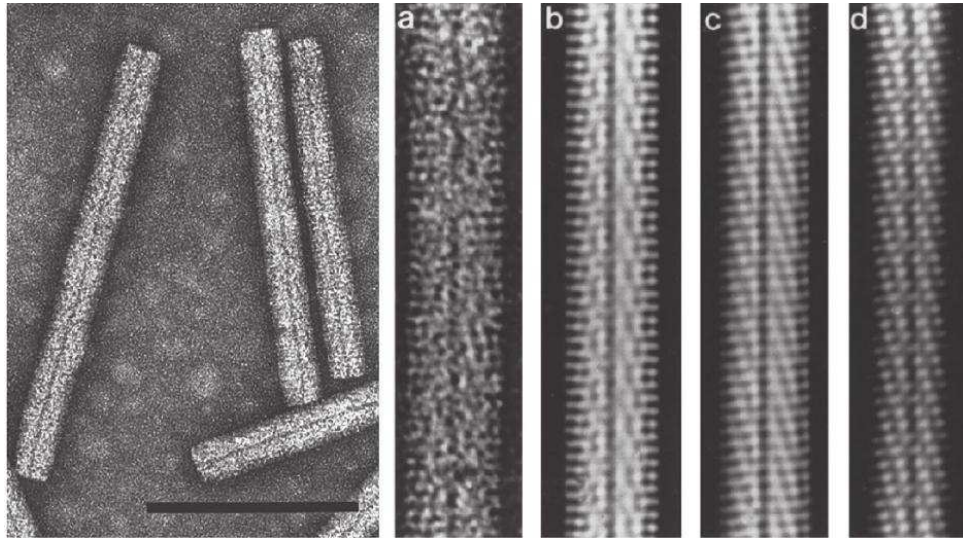


Figure 1 Negative contrast-stained BNYVV viral particles observed in transmission electron microscopy (a) and computer-filtered micrographs (b, c, d). Right-handed helix has a pitch of 2.6 nm and an axial repeat of four turns, involving 49 protein subunits [6]. One subunit covers four nucleotides (ibid). Bar represents 100 nm (modified from Gilmer and Ratti [2]).

2. Beet necrotic yellow vein virus

2.1 Types and Strains

BNYVV is a multipartite virus having up to five genomic RNAs required to complete its viral cycle in a natural context. Each RNA is separately encapsidated in independent particles. Viral isolates typically contain four particle species of 390, 265, 100 and 85 nm length. The presence of a fifth RNA species is described in European and Asian isolates giving a supplementary particle with a size ranging from 65 to 80 nm in length [7]. The five distinct encapsidated RNAs have been described and named RNA1 (6.8 kb), RNA2 (4.7 kb), RNA3 (1.8 kb), RNA4 (1.5 kb) and RNA5 (1.45 kb) [2]. Recent phylogenetic analyses based on CP, p25 and p31 nucleotide sequences [8,9] organized BNYVV isolates in A-I, A-II, A-III and B distinct types and in more than 10 strains [7]. Type and strain characteristics, such as CP, p25, and p31 clusters, presence or absence of RNA5 and geographical distribution are presented in table 1.

Type	Strain	Cluster CP	Cluster p25	Cluster p31	RNA5	Geographical distribution
A-I	China-H	A or B	I	I	+	China, Japan, UK
A-I	China-Y	A or B	I	I	+	China
A-II	P type	A	II	II	+	France, Kazakhstan, UK, Iran
A-II	Japan-D	A	II	II	+	Japan
A-II	Japan-O	A	II	I or II	+	Japan
A-II	China- B	A or B	II	II	+	China, Germany
A-II	China-L	B	II	I	+	China
A-III	Italian A type	A	I	III	-	Europe, USA, Middle East
B	China-X	A	III	I or III	+	China
B	B type	B	III	IV	-	Germany, France, Belgium, Austria, Switzerland, Czech Republic, China

Table 1 BNYVV types and derived strains. Groupings represents clusters derived from nucleotide sequence phylogenetic analyses of CP (A and B), p25 (I–III), and p31 (I–IV). + or – indicates presence of absence of RNA5 in the strain (modified from Tamada et al [7])

2.2 Vector transmission

BNYVV is persistently transmitted by the protozoan *Polymyxa betae* [10], a root obligate parasite of different plant species mainly belonging to *Chenopodiaceae* family. Four biological forms characterize the protozoan life cycle (Figure 2):

- Resting spores (sporosori) are able to remain viable and viruliferous in the soil for years. They germinate in the presence of host exudates together with suitable condition of temperature, pH and humidity [11].
- Primary biflagellate zoospores resulted from sporosori germination represent the main biological form of dissemination within *P. betae* life cycle. They reach and encyst in a rootlet cell in which their cytoplasmic content is injected through a tubular structure (Rohr) containing a dagger-like body (Stachel) [10]. BNYVV transmission in the host is thought to take place with the cytoplasmic fusion between zoospore and rootlet cell [11].
- Multinucleated plasmodium derived from the sporangial phase after several cycles of mitotic nuclear division [10].
- Zoosporangium, resulted from maturation of plasmodium, leads the production of secondary zoospores that can infect new roots once released in the rhizosphere. Multinucleate plasmodium can also mature as sporogenic stage forming clusters of sporosori then released in to the soil with the senescence of infected plant rootlets [11]. Factors determining the sporangial or sporogenic cycle are still unknown since the two

phases apparently overlap [10]. BNYVV acquisition by an aviruliferous vector seems to occur at the plasmodial level with a still unknown mechanism that determine the invagination of the plasmodial membrane around viral particles facilitated by trans-membrane motifs of minor capsid protein [12].

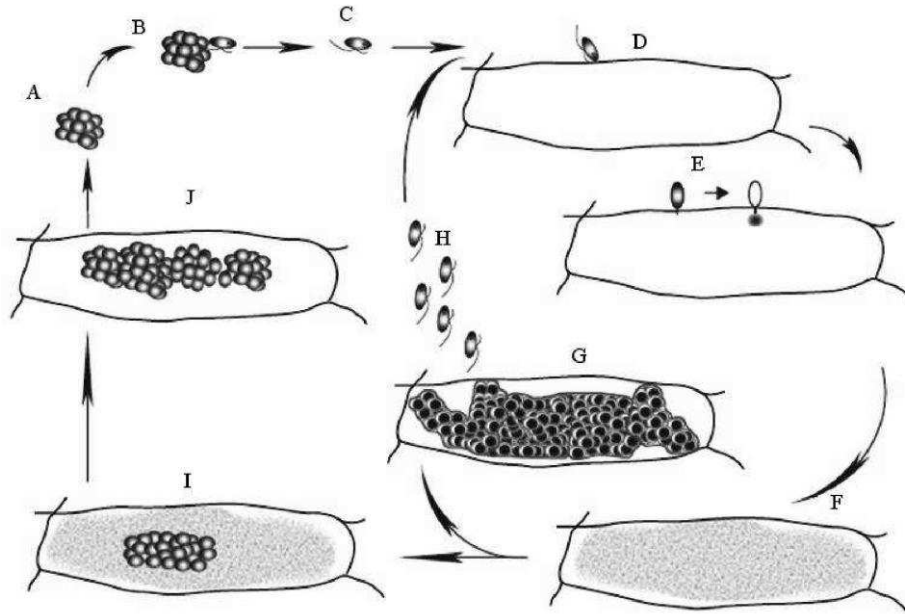


Figure 2 Representation of *P. betae* life cycle: Sporosori (A); Primary zoospore germination and movement in the rhizosphere thanks to its two whiplash flagella (B, C); Cell rootlet infection consisting in the cyst formation and cytoplasmic transfer from zoospore to the host cell (D, E); Multinucleate plasmodium formation (F); Zoosporangium evolves from sporangial plasmodium (G) and release secondary zoospore in the extracellular medium (H); Sporosori cluster (I), from sporogenic plasmodium, are released in the soil after rootlet senescence (J) (modified from Peltier et al [11]).

2.3 BNYVV host range

The natural host range of BNYVV is very narrow and limited to species from *Beta* genus, such as *B. vulgaris* and *B. macrocarpa*, or *Spinacea* genus such as *S. oleracea*. Initially thought to infect only species belonging to *Chenopodiaceae* family, relatively recently, BNYVV has been suggested to have a wider host range since together with *P. betae* it has been detected in 29 different plant species grown in naturally infested soils, including *Amaranthaceae*, *Asteraceae*, *Brassicaceae*, *Caryophyllaceae*, *Papaveraceae*, *Poaceae* and *Urticaceae* [13]. However, detection analyses have been conducted with multiplex RT-PCR on root tissues (ibid) not considering a possible contamination by viruliferous *P. betae* spores. Further experiments of BNYVV infectivity are therefore necessary to confirm and validate such important host range extension.

2.4 Rhizomania

Rhizomania is the most important transmitted soil-borne disease affecting the sugar beet (*Beta vulgaris* var. *saccharifera*). In the early seventies, BNYVV has been identified as the causal agent of the disease [14] previously described by Canova in Padan Plain (Italy) as an abnormal proliferation of lateral rootlets with consequently reduction of the tap root weight and browning of the vascular system (Figure 3) [15,16]. BNYVV infection in sugar beet is mainly restricted to the root apparatus but sometimes extend as a systemic spread in leaves, causing vein necrosis and yellowing, symptoms that were used for naming the virus [14,16].

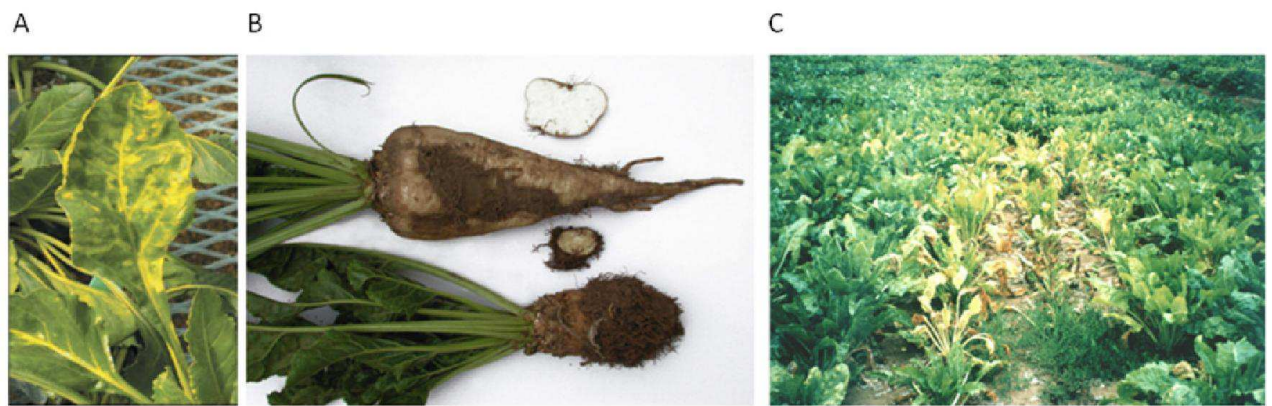


Figure 3 Rhizomania symptoms: systemic leaf necrosis and yellowing (A); diseased plant (bottom) shows abnormal proliferation of lateral rootlets with a consequent size reduction and necrosis of the tap root compared to healthy plant (top) (B); symptoms on field (C) (modified from Delbianco[17])

2.5 Genomic organization: RNA1 and RNA2 essential combination and accessory RNAs

Among the five BNYVV genomic RNAs, RNA1 is essential for the replication while RNA2 ensures the functions of encapsidation, cell-to-cell movement and RNA silencing suppression. While RNA3, RNA4 and RNA5 behave as accessory species for local infection or long distance infection in *S. oleracea* or *N. benthamiana*, these viral RNAs play important, distinct and dedicated roles in the pathogenicity and the transmission of the virus within the natural host [18,19] (Figure 4). For this reason these small genomic RNA species become accessory on laboratory hosts and could be used as viral vectors of expression in the presence of RNA1 and RNA2 helper combination [20,21].

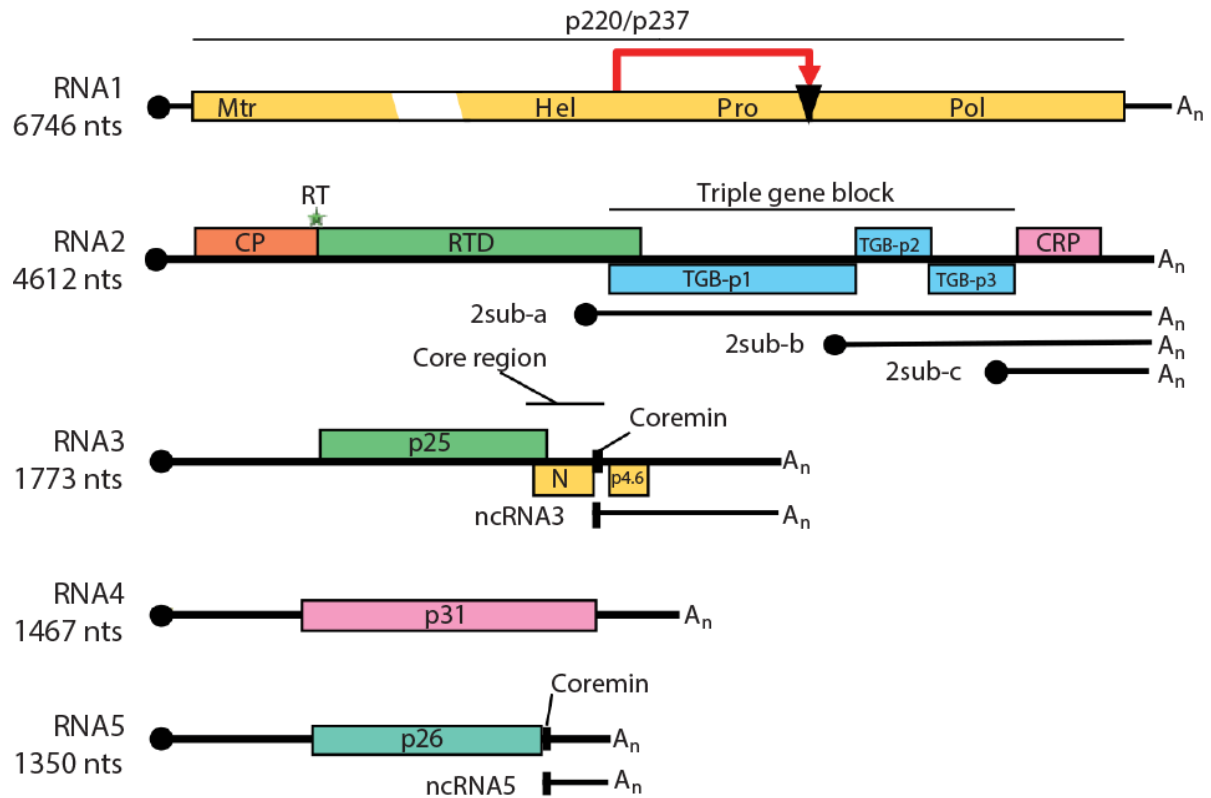


Figure 4 BNYVV genome organization and translational strategy. Genomic RNAs have a 5' end cap (black circle) and a 3' end polyA tail (A_n). RNA1 encodes a polyprotein that undergoes a self-cleavage (red arrow and black triangle). Methyltransferase (Mtr), helicase (Hel), protease (Pro) and RNA dependent RNA polymerase (Pol) are different domain identified on the polyprotein. RNA2 encodes for coat protein (CP) and for RT which expression depends on the suppressible UAG stop codon translational read trough (green star). CP and read-through domain (RTD) constitute RT protein. Via subgenomic RNA (sub) RNA2 encodes three triple gene block proteins (TGB-p1, TGB-p2, TGB-p3) and p14 a cysteine-rich protein (CRP) also known as the viral suppressor of RNA silencing. RNA3 encodes the p25 protein and possesses other two ORF which products N and p4.6 proteins were never detected. RNA4 encodes the p31 and RNA5 the p26. Noncoding (nc) RNA3 and ncRNA5 are produced by exoribonuclease activity [22]. The conserved coremin motif present in the 'core region' of RNA3 and in RNA5 is necessary for long distance movement in *Beta* species (modified from Gilmer and Ratti [2])

BNYVV RNA1 is 6,746 nts long [23] and encodes all viral factors required for the RNA replication since this RNA species is able to self replicate if transfected without the other genomic components in *Chenopodium quinoa* protoplasts [24]. RNA1 has one unique ORF containing two possible start codon (AUG₁₅₄ and AUG₄₉₆) encoding for two long polypeptides p237 and p220 [25] having both three distinct domains associated with viral replication: methyltransferase domain (MTR), NTP-binding/helicase domain (HEL) and RNA-dependent RNA-polymerase domain (RdRp) (ibid). Papain-like protease domain is located between HEL and RdRp and is responsible for the post-translational cleavage of the p237 and p220 into p150 and p66. The RNA1-encoded protein or the clivage products interact

each other, in a still unknown stoichiometric ratio, to establish a functional replicase complex associated with the endoplasmic reticulum (ER) network [25–27]. Interestingly no major endoplasmic reticulum (ER) reorganization has been observed in infected cells suggesting that production of a huge viral factory is avoided by the virus [27] probably preventing a competition for the replication machinery between essential and shorter “non essential” RNAs.

Polycistronic RNA2 is 4,612 nts long and is characterized by having different translational strategies to express five ORFs encoding six different proteins[28]. Coat protein of 21 kDa (CP) is expressed directly by genomic RNA2 starting from its AUG₁₄₅ start codon [29]. An UAG₇₀₉ stop codon is suppressed once in ten times by a read-through translation mechanism to produce the minor capsid protein p75 (RT) (ibid). Localized at one extremity of each viral particle, RT is required for the nucleation process that probably takes place in the cytoplasmic side of the outer mitochondrial membrane [30]. Furthermore, alanine scanning mutagenesis of this protein identified a C-terminal half motif (KTER) essential for the transmission of viral particles by *P. betae* vector [31].

The four other ORFs are translated from three subgenomic RNAs (sgRNAs) which sequences are collinear to the 3' half end of RNA2. These sgRNAs bring ORF2, ORF3 and ORF4, encoding TGB proteins (p42, p13 and p15), and the ORF5, required for the production of p14 closer to the 5' terminus to allow translation. P42 is translated from 2sub-a, p13 and p15 from 2sub-b while p14 from 2sub-c [32]. The p42 has a nucleic acid binding activity and sequence motifs characteristic of superfamily I DNA or RNA helicase including a P-loop ATP/GTP binding domain [33]. The p13 protein possesses a highly conserved hydrophilic motif flanked by two hydrophobic domains able to cross ER and plasma membranes while p15, translated by a ribosomal leaking scanning of p13 start codon appears mainly hydrophobic [34]. BNYVV RNAs move cell-to-cell through plasmodesmata in a CP independent manner, an hordei-like mechanism requiring only the three TGB proteins [32,35]: The p42 proteins cooperatively interact with viral RNAs thanks to their N-terminal domain and form ribonucleoprotein complexes (RNP) delivered by the integral membrane proteins p13 and p15 to plasmodesmata following the ER network [35–37]. Hordei-like TGB proteins accumulate during infection with conserved TGB1/TGB2/TGB3 ratios of approximately 100:10:1 [38] .

Such relative ratios are not determined for BNYVV but could presumably be similar since the unregulated expression of BNYVV TGB proteins inhibits cell-to-cell movement [39].

The 3' end proximal ORF5 encodes a C-4 zinc finger cysteine-rich protein of 14 kDa (p14). This protein is the viral suppressor of RNA silencing (VSR) and localizes both in the cytoplasm and the nucleolus [40]. The p14 VSR activity is mainly cytoplasmic and has been associated with reduced accumulation of secondary small interfering (si) RNAs derived from endogenous RNA-dependent RNA-polymerase 6 (RDR6) pathway [40,41]. Affecting silencing transitivity of plant RNAi, p14 counteracts the restriction of systemic spread of BNYVV genomic components, playing a crucial role in the long-distance movement of the virus [40,41].

Furthermore p14 acts synergically with a viral noncoding RNA produced from genomic RNA3 (ncRNA3) for the viral long distance movement in *B. macrocarpa* and in *Nicotiana benthamiana* infected with a BNYVV expressing an hypomorphe VSR. Together, these data suggest a link between silencing suppression and viral spread [40,41].

RNA3 is 1,774 nts long [42] and is involved in the viral long-distance movement in *Beta* species [18] as well as in the manifestation of rhizomania symptoms in *B. vulgaris* [43,44]. RNA3 contains three different ORF, encoding a protein of 25 kDa and two other proteins never detected in natural context (N and p4.6) [27]. The p25 protein localizes in the nuclear and cytoplasmic compartment independently of other viral factors [45], thanks to a N-terminal nuclear localization signal (₅₇KRIRFR₆₂) and a C-terminal nuclear export sequence (₁₆₉VYMVCLVNTV₁₇₈) [45,46]. P25 is an avirulence protein [47] and has been suggested to represent the pathogenic determinant for the rhizomania disease since transgenic *Arabidopsis thaliana* lines constitutively expressing the protein, display a root-branching phenotype, express high levels of auxin and low amounts of jasmonic acid derivatives [48]. Nuclear import and export motifs, together with a zinc finger domain, suggest that p25 could acts as a transcriptional factor. Such hypothesis has been corroborated in yeast one hybrid experiments in which the Gal4 or LexA DNA-binding domain-fusion with p25 is able to promote the transcription of the reporter genes. The domain responsible for transcription activation is constituted by p25 residues 103 to 146 [49]. Systemic spread in *Beta* species do not rely on p25 expression but on the strictly connection between “coremin” motif (located within the “core” domain) and the accumulation of ncRNA3 [18,22,50]. A 5'-3' exoribonuclease

processing of RNA3 stalls on a highly structured sequence involving the “coremin” motif and lead to the accumulation of ncRNA3 *in vivo* with a probable saturation of the exoribonuclease with consequences on the RNA silencing machinery [22,41]. As stated above, this could explain the synergy observed between the VSR and the accumulation of the ncRNA3 species [40,41].

Monocistronic RNA4 is 1,467 nts long [42] and encodes a cytosolic protein of 31 kDa [51] involved in the aggravation of foliar symptoms and, together with p75, in the vector transmission [52]. Experiments in *N. benthamiana* demonstrated a role of the p31 protein in a root specific suppression of RNA silencing [52]. Such behavior still needs to be confirmed in *Beta* species.

RNA5 is 1,350 nts long and encodes a protein of 26 kDa which functional properties resemble those of the RNA3-encoded p25 protein [53,54]. As p25, p26 protein localizes both in the cytosol and nucleus and strongly activates transcription in yeast one hybrid system [55]. Transcriptional activation domain is located in the first 17 amino acid residues of the protein and is not related to the necrosis symptoms on *C. quinoa*, suggesting a probable avirulence behavior of p26 on such host [55,56]. When present, RNA5 increases symptomatology on *B. vulgaris* roots [57] and provokes necrotic lesions on *C. quinoa* leaves. As RNA3, RNA5 possesses the coremin sequence, produces a noncoding RNA (ncRNA5) which is able to complement an absence of RNA3 required for the long distance movement on *B. macrocarpa* [58].

3. Ins and Outs of Multipartite Positive-Strand RNA Plant Viruses: Packaging versus Systemic Spread

A Review published by:

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Viruses **2016**, *8*, 228; doi:10.3390/v8080228 www.mdpi.com/journal/viruses

Review

Ins and Outs of Multipartite Positive-Strand RNA Plant Viruses: Packaging versus Systemic Spread

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Academic Editor: Eric O. Freed

Received: 31 May 2016; Accepted: 9 August 2016; Published: 18 August 2016

Abstract: Viruses possessing a non-segmented genome require a specific recognition of their nucleic acid to ensure its protection in a capsid. A similar feature exists for viruses having a segmented genome, usually consisting of viral genomic segments joined together into one viral entity. While this appears as a rule for animal viruses, the majority of segmented plant viruses package their genomic segments individually. To ensure a productive infection, all viral particles and thereby all segments have to be present in the same cell. Progression of the virus within the plant requires as well a concerted genome preservation to avoid loss of function. In this review, we will discuss the “life aspects” of chosen phytoviruses and argue for the existence of RNA-RNA interactions that drive the preservation of viral genome integrity while the virus progresses in the plant.

Keywords: phytovirus; segmented genome; genome integrity; systemic movement; RNA-RNA interaction

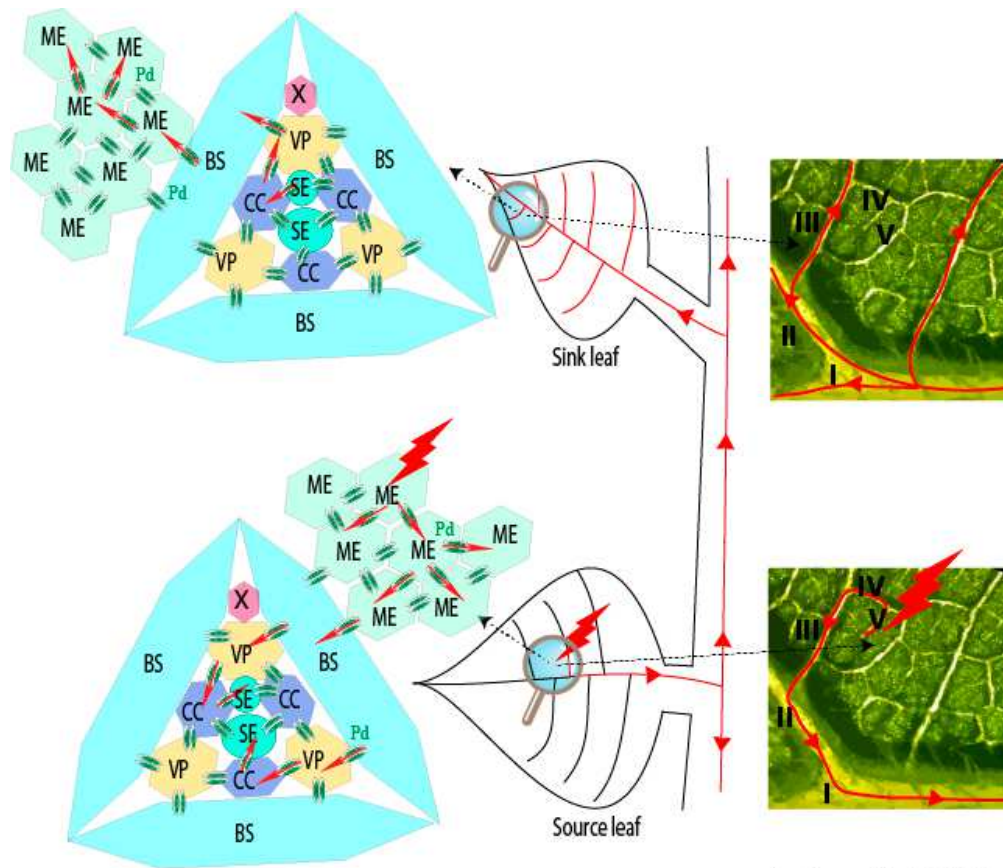
1. Introduction

Preserving genome integrity is a key challenge for any organism, and viruses with an RNA-based genome are not excluded from this basic rule. Obligate parasites that have a monopartite genome mainly face recombination events that draw viral evolution. While viruses with segmented genomes also encounter similar evolutionary traits, another constraint applies to the maintenance of genome integrity. Indeed, all genomic segments should be available within the same cell and be transmitted from one cell or one organism to another. To do so, all genomic components must be packaged within the same viral particle. This is well exemplified by *Orthomyxoviridae* members where all genomic negative-stranded ribonucleoprotein (RNP) complexes are selectively assembled together within one enveloped viral particle [1], or *Bunyaviridae* [2] where all three segments are maintained together within the envelope. Similar examples can be drawn for double-stranded RNA viruses such as *Reoviridae* or the well-known *Cystoviridae* pseudomonas phage Ø6 that possesses three genomic double-stranded (ds)RNA segments within the same viral structure [3].

Viruses that possess a positive-stranded RNA genome are found in genera that infect bacteria (e.g., *Leviviridae* enterobacteria phage Q β), animals and insects (e.g., *Picornavirales* enterovirus C—namely poliovirus—and cricket paralysis virus) and also plants (e.g., *Virgaviridae*, tobacco mosaic virus). To date, distinguishing features of (+)-strand RNA viruses that infect bacteria, animals or insects reside in the nature of the viral genome that is limited to either one single-stranded (ss)RNA molecule or two genomic RNAs packaged within a unique icosahedral capsid (e.g., *Nodaviridae*, flock house virus). However, (+)-strand RNA phytoviruses with segmented genomes range between

two to five positive-strand RNA molecules and such so-called multipartite viruses possess either an icosahedral (sometimes bacilliform) or helical shape. A large majority of these phytoviruses have distinctive features compared to the aforementioned animal (+)-strand RNA viruses, as each segment can be individually packaged as ribonucleoprotein complexes within a helical structure. Icosahedral phytoviruses with a segmented positive-sense RNA genome either package single segment species in each capsid or a combination of segments within the limits of physical size constraints. A.L.N. Rao has reviewed mechanisms driving genome packaging of spherical plant RNA viruses [4] while Solovyev and Makarov recently focused on plant viruses with helical capsids [5].

The question about the requirement for the long distance movement of the ‘virus’ of either virion (packaged RNA) or RNP complex is still open and it appears closely linked to the ability of the viral RNAs to be “packaged.” Such infection of vascular tissues is not as uniform as those of mesophyll cells where phytoviruses usually begin their journey by moving cell to cell (for review see [6]). Several boundaries constituted by the bundle sheath (BS) of V or IV class veins (for vein classification, refer to [7]) followed by the vascular parenchyma (VP) have to be passed by the viral material in order to reach companion cells (CCs) and follow the photoassimilates flow of the sieve elements (SEs). To finish the journey, the reverse route passing through class III veins to access CC and then mesophyll cells has to be pursued to start moving cell-to-cell in the distant tissues (Figure 1).



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Figure 1. Schematic representation of the initial infection (red thunderbolt) and its progression (red lines and arrows) within a source leaf of an infected plant. Infectious material passes through plasmodesmata (Pd, double green ovals) from mesophyll (ME), bundle sheath (BS), vascular parenchyma (VP) to companion cells (CC) to access sieve elements (SE) and reach the distant tissues. A reverse route occurs in the upper leaves (sink leaf) or roots (not represented). Right panels show leaf venation and illustrate viral phloem loading and unloading through minor and major veins of source and sink leaves. X, Xylem; I–V: vein classes.

Whatever the structural shape adopted for plant multipartite RNA viruses, their genomic segments are separated by distinct capsids. This situation raises concerns about the preservation of the viral genome integrity that requires all RNA segments to move from one cell to a neighbor or distant cell in the plant (Figure 1). This ensures the setup of a novel infection site where all viral genes need to be expressed. Here, up to three-decade old as well as new literature describing packaging, cell-to-cell and vascular movement of plant icosahedral and helical viruses has been reviewed to bring into the light our conceptual view of the nature of the systemic moving of viral material in the infected plant. Without addressing details about the RNA expression and replication mechanisms, we propose to distinguish between virions acting as storage of infectious material, ready for transmission, and moving material constituted of RNP complexes containing all RNA segments of the multipartite genome. These RNP complexes involving RNA-RNA interactions act as drivers for the “in planta” viral cycle and appear as the adequate entities for genome preservation, particularly in the long-distance journey of the virus. While this concept appears “insignificant” for monopartite RNA viruses, multipartite viruses need to preserve the expression of their entire genome in the targeted cells. After the description of chosen monopartite phytoviruses, we review and discuss the situation of multipartite RNA viruses and focus on their need to preserve the expression of their entire genome in the targeted cells. A model for benyviruses has been illustrated to present our hypotheses.

2. Lessons from Turnip Crinkle Virus, Satellite Tobacco Mosaic Virus, Groundnut Rosette Virus and Tobacco Mosaic Virus: Monopartite RNA Genomes

For some viral species, icosahedral shells are able to assemble in virus-like particles where the genome, usually dsDNA, is incorporated using energy-dependent nanomachines, extensively studied for bacteriophages T4, P22 or ϕ 29. The latter uses a non-coding RNA hexamer for DNA entry. Recent insights about the genomic encapsidation of the positive-strand RNA genome of bacteriophages MS2 revealed the importance of coat protein (CP) dimer interactions, with an estimated number of 60 hairpin RNA structures distributed around the RNA genome (packaging signals), leading to induced-fit RNA-viral protein interactions rather than electrostatic interactions. In this sense, genomic MS2 RNA constitutes a scaffold for assembly initiated or terminated by the assembly protein, following a Hamiltonian path [8–10].

2.1. Positive-Strand RNA Packaging into Icosahedral Units

2.1.1. Autonomous Turnip Crinkle Virus

Turnip crinkle virus (TCV) from the *Tombusviridae* family is structured by an association of 180 copies of a 38 kDa CP. Its crystallographic structure resembles the tomato bushy stunt virus. TCV viral particles can be dissociated at elevated pH where RNA is attached to six CP subunits that permit reassembly in vitro [11]. Mapping protein-RNA interactions revealed the interaction between CP and two RNA domains. A 15 base-pair (bp) long hairpin structure has been identified within the replicase gene and a 28 nucleotide (nt) bulged hairpin loop is present within a 186 nt essential element within the CP coding sequence. Viral encapsidation was also shown to be dependent on the length of the viral RNA [12], suggesting the requirement of a separation between 5' (replicase gene) and 3' (CP gene) CP-interacting sequences, suggesting a bipartite packaging signal for this monopartite RNA viral genome. TCV CP is dispensable for the viral cell-to-cell movement but essential for the systemic spread [13] as this protein acts as a suppressor of RNA silencing [14,15].

2.1.2. Helper Virus Replication-Dependent Satellite Tobacco Mosaic Virus

Satellite tobacco mosaic virus (STMV) is an icosahedral $T = 1$ virus with a small genomic RNA. Atomic structure revealed 30 stem-loop RNA structures, each associated with a two-fold symmetry axis [16]. Interestingly, the RNA secondary structure of STMV analyzed in solution [17] does not correspond to the structure predicted in the $T = 1$ capsid. This discrepancy indicates a selection of the RNA genome into an icosahedral structure following a Hamiltonian pathway as described for MS2

bacteriophage [10]. The existence of multiple RNA folding structures of the genome could account for distinct functions tightly regulating viral protein expression and regulated by viral proteins. This satellite virus requires a helper virus (HV) for its replication. Interestingly, HV replicating STMV belong to helical viruses such as tobacco mosaic virus (TMV) [18]. A systemic movement of both the satellite virus spherical particle and HV helical entities would require a mechanism able to recognize both structural entities. However, drawing hypotheses about possible interactions between satellite RNA and HV RNA could explain a satellite RNA transport by an HV RNP moving complex.

2.1.3. Umbraviruses Replicate Autonomously but Are Packaged in *Trans*

Groundnut rosette virus (GRV) belongs to the *Umbravirus* genus and does not form conventional virions. Indeed, this species does not encode coat proteins. GRV possesses a monopartite genome encoding proteins necessary for its autonomous replication and movement within the plant. Its genome encapsidation occurs during mixed infection with an HV that belongs to the *Luteoviridae* family, specifically transmitted by aphids and only in the presence of a satellite RNA required for the selective encapsidation of GRV [19]. In a host plant, GRV is able to replicate on its own and infect the entire host while the helper luteovirus remains restricted to the phloem. GRV movement is provided by the expression of the open reading frame (ORF) 3 product and recruits fibrillarin to ensure the formation of an RNP complex essential for its systemic spread [20]. This example illustrates the systemic movement of a viral entity without viral shell formation.

2.2. Positive-Strand RNA Packaging into Helical Units: Lessons from Tobacco Mosaic Virus

The reconstruction of TMV from purified CP and viral RNA [21] set up the fundamental basis for understanding rod-shaped and flexuous helicoidal viruses that has been recently reviewed elsewhere [5]. Upon the recognition of an RNA signature on the viral genome, CP nucleates and cooperatively recruits further copies of structural proteins to cover the entire genome with a first coverage of the 5' domain followed by the protection of the 3' terminus. Conversely, uncoating of TMV is thought to occur in the cell by a partial disassembly of the CP subunits at the 5' termini of viral RNA (Ω region) leading to ribosomal scanning and uncoating while the first ORFs are translated. The encoded proteins produce the replicase complex that recognizes the 3' partially uncoated domain and starts the synthesis of the viral complementary strand and simultaneously removes remaining CP-associated subunits. This ensures a proper recognition of internal promoters required for the subgenomic RNA synthesis and left-ended terminal promoter required for positive-strand synthesis. While subgenomic RNAs (sgRNAs) are produced, expression of the movement protein (MP), required for the efficient transport of viral material from the infected cell to neighboring cells, and CP expression finalize the progression of the viral cycle. TMV CP assembles spontaneously without any cellular factors. TMV genome recognition and encapsidation require both the formation of a 34 to 38 CP subunit disk (namely protohelix or two-ring disk) and the presence of an origin of assembly (OA) on the viral RNA. This leads to the rapid encapsidation (~6 min) of the ~6.4 kb RNA with 2130 copies of the CP subunits. TMV-OA is constituted by three stem loops, with stem loop 1 playing an essential function for the initiation of nucleation, together with a repetition of G residues every three nucleotides within an unpaired region [22]. Importantly, this property led to the engineering of a viral Lego[®] for nanotechnologies. The combination of genetically modified CP subunits, sometimes together with wild-type CP (both expressed in *Escherichia coli*) with RNAs containing TMV-OA, led to the construction of nanostructures displaying new chemical reactivity, owing to their usage as nanomaterials [23].

Reverse genetics using a full-length infectious clone of TMV demonstrated that the CP is not required for cell-to-cell movement but is needed for the efficient systemic infection of hosts such as *Nicotiana benthamiana* (*N. benthamiana*) [24]. Hence, TMV particles would thereby constitute stable mechanically transmissible entities between sessile hosts whereas RNP complexes containing the CP could represent the moving material within the host. This is illustrated by the description of TMV RNPs isolated from infected *N. tabacum* cv. Samsun plants [25]. Such RNPs differ from virions by their

higher buoyant density and contain TMV genomic RNA, polypeptides of 17.5 kDa (TMV CP), 31 kDa (possibly TMV MP), 37 kDa and 39 kDa. Purified RNAs from these RNPs retained the ability to be encapsidated in vitro [25].

The progression of TMV infection within mesophyll cells is well characterized and occurs thanks to the transport of uncoated viral RNAs and MP RNP complex. However, little is known about the nature of the infectious material that permits viral vascular movement. Disruption of the OA by point mutations that preserve functional CP abolishes TMV systemic movement, suggesting that virions represent the long-distance entities [26]. Nevertheless, experimental results outlined below suggest that the hypothesis of TMV virion as the moving material may have to be reconsidered.

As stated above, in the absence of CP synthesis, TMV is able to move cell-to-cell to access VP but fails to enter CC, suggesting a requirement of the CP to cross the VP/CC boundary [27]. The TMV CP seems to play a central role in the passage of the infectious material through vascular tissues. However, the formation of viral particles does not appear to be a prerequisite because several mutants (insertion or deletion) that affect the RNA binding region of the CP are still able to move long distance, albeit with sometimes delayed kinetics [28]. The crossing of each specific boundary between different cell types seems to require different strategies that could involve dedicated viral factors.

Masked strain M-TMV shows a delayed systemic spread as compared to the virulent strain U1-TMV. However, comparable replication levels and cell-to-cell movement efficiency in mesophyll cells is described for the inoculated leaves [29]. The delayed systemic movement behavior is attributed to an eight-amino acid mutation in the 126 kDa protein, formerly acting as an RNA silencing suppressor, and hence in the 183 kDa replication protein. Immunohistochemical analysis of class V veins of infected leaves revealed a deficient viral accumulation in the VP and, consequently CC cells, for viruses carrying the p126 mutation [30]. The different behavior of the two strains is maintained in class III veins and mesophyll tissues of non-inoculated leaves even if the number of infected CCs is comparable, suggesting that wild-type p126 is required for BS/VP and CC/VP boundary crossings [30]. It is not known if p126 acts alone or as part of a vRNP complex [30]; however, viral replication complexes constituted of p126, MP and viral RNA are reported to move between cells [31].

Although RNA stabilization by encapsidation may be a prerequisite for TMV to enter VP and CCs, it is not necessary in the SE where no RNase activity was detected in exudates [32–34]. Furthermore, the absence of translation could reinforce the hypothesis of a viral RNP complex as the form of transport, because its correct conformation should not be disrupted by the scanning activity of ribosomes. Replacement of the TMV CP by GRV Orf3 leads to systemic movement of the recombinant viral RNA without the formation of virions [35]. The Orf3 product interacts with TMV RNA to form helical-structured filamentous RNP complexes lacking the uniform rod-shaped viral particles [36].

Other indirect experiments permit speculation about the possibility of MP to be included as a constituent of the viral entity that moves long distance. In tobacco plants with a reduced synthesis of pectin methylesterase in vascular tissue, a cell-wall enzyme which interacts with MP [37], TMV is able to load in the SE but cannot be unloaded in non-inoculated leaves [38]. Beyond the CP, the MP is required for TMV cell-to-cell and long-distance movement that could involve two independent mechanisms. Gain and loss-of-function experiments support such a hypothesis. TMV does not systemically infect vanilla orchid. The related *Tobamovirus odontoglossum* ringspot virus protein (ORSV) is able to infect such a host but is unable to move long distance in tobacco [39]. The replacement of TMV MP by ORSV MP permits chimeric TMV to systemically infect vanilla orchid and restrains the infection to local lesions in tobacco plants [39]. The host-range determinant of ORSV resides in the eleven C-terminal amino acids of the MP. The deletion of this domain restores the ability of the chimera to provoke a systemic infection on tobacco and prevents the spread in orchids [39].

3. Multipartite RNA Genomes

3.1. Positive-Strand RNA Packaging into Icosahedral Units

3.1.1. Dianthovirus: Bipartite Genome

Red clover necrotic mosaic virus (RCNMV) belongs to the *Dianthovirus* genus and *Tombusviridae* family. This icosahedral virus possesses a bipartite positive-sense single-stranded RNA genome. RNA1 codes for the proteins involved in replication and also for CP subunits through a sgRNA [40–44]. RNA2 is monocistronic and codes for the MP, a multifunctional protein implicated in suppression of RNA silencing, in cell-to-cell and long-distance movement of the virus [45–47]. Although RNA1 replicates alone, it is not able to produce the sgRNA or CP subunits in the absence of RNA2 [48–51]. A kissing interaction has been found between RNA2 and RNA1. This 8-base pairing occurs between the trans-activating TA element, present in the RNA2 MP ORF and the TA-binding site (TABS) present in sgRNA promoter on the RNA1. The heterodimerization is essential for the production of sgRNA [51], whose production is regulated by RNA2. This interaction is also required for the RNA1-RNA2 packaging [48], because no OA is present on RNA1. Besides these biological roles, the kissing interaction within the OA allowed the engineering of virus-like particles able to efficiently encapsidate nanoparticles or Quantum Dots [52]. Therefore, the particularity of RCNMV is, on the one hand, the encapsidation of the RNA heterodimer in the same particle and, on the other hand, the packaging of multiple copies of RNA2 that could fit in tailored viral shells [53].

While the CP is not required for cell-to-cell movement, it is considered necessary for the systemic spread of RCNMV [54]. However, some experiments performed in *N. benthamiana* revealed that RNA1 and RNA2 could move long distance in the absence of the CP and thus without the formation of virions [42]. Although this property appears to depend on the host plant and temperature, it does illustrate again that systemic movement as an RNP complex is possible. Whatever the precise mechanism of its systemic movement, this bipartite viral genome provides direct evidence for the regulation of the viral cycle via the RNA-RNA interaction of distinct viral RNA species.

3.1.2. Brome Mosaic Virus: Tripartite Genome

All three components of the tripartite genome of brome mosaic virus (BMV) (~8.2 kbp) are essential for viral replication and for systemic infection of plants. The RNAs are encapsidated separately in 28 nm particles with $T = 3$ quasi-icosahedral symmetry [55]. RNA1 and 2 are packaged separately, whereas RNA3 is co-packaged with sgRNA (namely RNA4) into a third particle [4]. A ~200 nt conserved 3' tRNA-like structure (TLS) with determinants for tyrosylation is present on all genomic RNAs and acts as a nucleating element (NE) of CP subunits. This NE is required for the packaging of viral RNA3 and RNA4 [56] but not for RNA1 and 2 packaging [57]. Conserved 3' untranslated regions (UTRs) appeared to contain additional elements promoting encapsidation and distribution of the RNAs in three indistinguishable particles. The RNA3 encapsidation is dependent on a 187 nt long packaging element (PE) that resides within the MP coding sequence [58]. Besides, RNA3 and 4 packaging was found to be dependent on the replication and on a possible interaction between RNA3 PE and RNA4 NE domains [59]. In wheat germ extracts, pre-swollen particles render viral RNAs available for direct protein synthesis [60], suggesting a co-translational disassembly that has been observed for other spherical viruses such as alfalfa mosaic virus, cowpea chlorotic mottle virus and southern bean mosaic virus [61]. During the course of the viral replication cycle, viral RNAs and proteins allow new viral particles to accumulate. This morphogenesis takes place around replication factories: the co-localization of BMV replicase, CP and viral RNAs could thus control packaging specificity [62,63]. Viral cell-to-cell movement is dependent on the expression of the 3a movement protein, whereas CP is dispensable in some natural or MP-mutated BMV isolates [64,65].

Packaging was thought to play an important role in long-distance movement of the virus in the plant. However, using *Agrobacterium tumefaciens*-mediated transient expression of BMV genomic RNAs in leaves of *N. benthamiana*, Gopinath and Kao demonstrated that viral RNAs can also move long

distance independently, without the presence of the viral CP [66], probably forming RNP complexes with cellular factors. When produced alone, BMV RNA3 is able to move long distance without the assistance of any viral proteins, while RNA1 and RNA2 both need the MP for systemic movement [66]. This allowed the reconstruction of the entire functional genome in distant tissues of the plant, a requisite for the successful infection of the host. Taken together, these data suggest that the preservation of genome integrity during long-distance movement appears to be mediated by means of a complex in which BMV RNA3 together with MP could play a fundamental role. This led to the hypothesis that viral RNAs interact, in the same cell or in the same environment such as vascular tissues, during the transit of RNA/RNP species. Another aspect of a systemic movement mechanism can be drawn as a result of this experiment. One could argue that in plant, interconnected cells could exchange viral proteins involved in the amplification or in the movement of the viral RNAs that could reach distant cells. In this situation, the proteins or the RNAs will then act in *trans*. To access distant cells, *trans* acting proteins or viral messenger RNAs will be needed in huge amount. Taking these requirements into consideration, only segmented DNA viruses—such as geminiviruses or nanoviruses (circular ssDNA genome) that express mRNA thanks to their stabilized replicative dsDNA segments—can fulfill such a constitutive expression. These viral DNAs ensure a continuous expression of proteins in different tissues, and viral products could move from one cell to another as proteins, mRNAs or both. In the case of BMV described above, the use of agroinfection allowed the continuous production of infectious viral RNAs from cDNAs. This situation recapitulates what could happen for segmented DNA phytoviruses described above.

Recent advances using RNA sequencing (RNAseq) analyses demonstrated the role of TLS structures in the systemic transport of mRNAs containing such structural motifs [67]. The presence of such TLS on BMV RNAs (and TMV as well) may explain why these RNAs could move on their own. Some multipartite RNA viruses such as the benyviruses described below do not possess 3' TLS and thus will require the presence of a dedicated highly structured motif within each segment to fulfill the movement of each genomic RNA. However, one should take into account that RNA amplification of multipartite RNA viruses depends at least on the expression of the replication machinery and a viral suppressor of RNA silencing. Segregated segments will not be autonomous for amplification and their expression will be transient and not sufficient to promote the production of viral protein or to allow a stabilization of yet non-replicated RNA segments. Therefore, these RNAs will face the RNA decay or could induce cell death if they are expressed. Furthermore, if we assume the movement of viral proteins between communicating cells, this will require the presence of a sequence signal for each protein species for their intercellular transport. A study quantifying the degree of symplastic continuity between plant cells revealed that mobility of non-targeted proteins is strongly dependent on their molecular weight, on the developmental stages and the growth conditions [68]. Thus, if these hypotheses could not be completely excluded, they are difficult to conceive for plant multipartite RNA viruses, particularly for those possessing a helical shape.

3.2. Positive-Strand RNA Packaging into Helical Particle Units

If TLS motifs and/or signal peptides could drive the systemic movement of RNP complexes, the hypothesis of an RNP involving RNA-RNA network nucleating genomic segments and driven by few viral proteins appears easier to draw. To highlight our thoughts and findings, we present our views applied to the benyvirus biology and then extend our approach to pomoviruses.

3.2.1. Benyviruses: Up to Five Genomic RNAs

The *Benyviridae* family is composed of the *Benyvirus* genus of four viral species, namely, beet necrotic yellow vein virus (BNYVV), beet soil-borne mosaic virus (BSBMV), burdock mottle virus and rice stripe necrosis virus, the latter two species having a bipartite genome [69]. Benyviruses are rod-shaped virions of variable length with a constant diameter ranging from 18 to 20 nm. The particle length depends on the genomic RNA individually packaged. Among this family, BNYVV is the most studied species together with BSBMV. These two viruses share a similar genome organization, segment

number, host range and transmission vector. Serologically distant, one species is able to amplify and package genomic RNA segments of the other in laboratory conditions [69]. This indicates the presence of conserved promoter structural elements at the extremities of the genomic RNAs, the ability of subgenomic promoter recognition by the replicase, as well as a possibility of RNA-RNA interactions allowing RNP complexes' formation and function.

The OA for viral particle morphogenesis has only been studied on the RNA3 and RNA4 species. Preliminary experiments indicate the presence of *cis* acting sequences located in the first 300 and the last 70 nt of the RNA3 species [70]. Since the non coding RNA 3 (former sgRNA3) sequence coterminous with RNA3 is not encapsidated, the OA sequence was presumed to reside within the 5' UTR of RNA3 [70]. Using a collection of deletion mutants, an encapsidation signal was identified between nt 181–207 [71,72]. The absence of sequence conservation between this RNA OA and other viral RNAs suggests that this domain could be involved in a particular and transient folding required for the packaging. Moreover, nothing is yet known about the possible existence of a bipartite motif that could involve the 3' conserved UTRs since the RNA3-derived replicon vector (Rep3, constituted by the 5' and 3' UTRs of RNA3) is encapsidated [70,72]. The recognition of the viral OA by structural proteins differs from the one described for tobamoviruses (e.g., TMV). To ensure a correct encapsidation process, the minor structural protein needs to be expressed by a read-through bypass of the coat protein stop codon [73]. This 75 kDa coat protein-readthrough (CP-RT) protein is recovered from purified viral particles and has been visualized by transmission electron microscopy (TEM) after immunolabelling [74]. Mutants expressing the CP alone or missing the N-terminal part of the RT domain are deficient in packaging [75,76]. These data suggest a recognition of the viral OAs by the CP-RT domain followed by a cooperative recruitment of the CP subunit to ensure proper packaging [76]. If it exists, the specific subcellular localization of virion assembly is unknown. However, on the one hand, the encapsidation could be coupled to the replication process and thus be associated with the endoplasmic reticulum (ER) vicinity as the replication seems to occur on the ER (Figure 2, step 2) [77]. On the other hand, this packaging could also be transiently associated to mitochondria as virions surrounding the organelle structures have been observed [78]. Further work conducted on the CP-RT protein allowed the identification of a mitochondrial targeting sequence and a transmembrane domain on the minor structural protein [79].

In local host species such as *Chenopodium quinoa*, BNYVV produces local lesions within which each RNA species accumulates and encapsidates individually (Figure 2, step 1) [80]. Local lesion formation requires at least the presence of RNA1 and RNA2 to fulfill the housekeeping functions of replication, cell-to-cell movement and viral suppression of RNA silencing. The coat protein is dispensable for local infection [81].

In systemic hosts such as *Beta* species or *N. benthamiana*, local infection starts as in *Chenopodium quinoa* and then reaches the vasculature of the plant leading to the possible dissemination of the viral material in the entire plant as described in Figure 1. The differential accumulation of virion species in primary infected cells [80] argues against a dissemination of viral particles. On the one hand, taking into account the existence of an unidentified receptor for the virus, there is likelihood to saturate this entry gate with particles containing small genomic RNA species unable to replicate on their own. On the other hand, in a model lacking a receptor for the viral entry, even if still non-estimated, the probability for each segment to enter the same distant cell appears low. Similarly, non-orchestrated dissemination of a viral genomic segment to randomly distant cells will end up with the same conclusion: a low probability to reconstruct the entire viral genome. Our interrogations about the nature of moving material started with the following observations on BNYVV. All viral particles are structurally identical and only differ by their size, and all four (sometimes five) viral genomic RNAs are required to fulfill the entire cycle on the *Beta* species hosts [82–84]. However, RNA1 and RNA2 are sufficient for *N. benthamiana* systemic infection thanks to a replication machinery provided, as stated before, by RNA1 and to structural proteins together with the triple gene block (TGB) movement proteins (TGB1, -2 and -3) and post-transcriptional gene silencing suppressor (viral suppressors of RNA silencing, VSR) provided by RNA2 species [85,86] (Figure 2). In this host, RNA3

is dispensable, but when present it moves long distance and accentuates symptom severity [87]. Interestingly, in the presence of RNA1 + RNA2, the RNA3-derived viral expression vector Rep3 does not move long distance in *N. benthamiana* host. As Rep3 is encapsidated, we ruled out the thesis of virions as moving material and we formulated the hypothesis about the existence of RNA-RNA interactions allowing RNA segments to interact with one another to ensure a stabilization of an RNP complex for the long-distance movement of the viral genome in the plant. Such an RNP complex contains CP as this structural protein is required for systemic spread [81]. A reinvestigation of the properties of RT mutants' behavior described previously [76] confirmed our thoughts. Indeed, in this article some BNYVV mutants unable to form virions are still able to infect plants systemically. Thus, the minimal mobile RNP complex requires RNA1 and RNA2-interacting species to ensure the expression of housekeeping genes. Because RNA2 drives the production of subgenomic RNAs collinearly with its 3' extremity [88], we postulated that the interaction domain consists of a region present on RNA2 but not included in the sgRNAs sequence, lest 3' RNA2 collinear sequences interfere with genomic RNA interaction. Therefore, we focused our attention on the first 2000 nt and identified a sequence able to interact with RNA1 species using electromobility shift assay performed on in vitro transcripts. A similar approach performed on RNA3 species revealed the existence of more than one domain involved in the interaction with RNA2 (Dall'Ara et al., in preparation). To confirm our approach and share our findings, we transposed our hypothesis onto potato mop top virus, a tripartite helical virus that does not require structural proteins for its long-distance movement. These findings are discussed below in regard to mutants described in the literature.

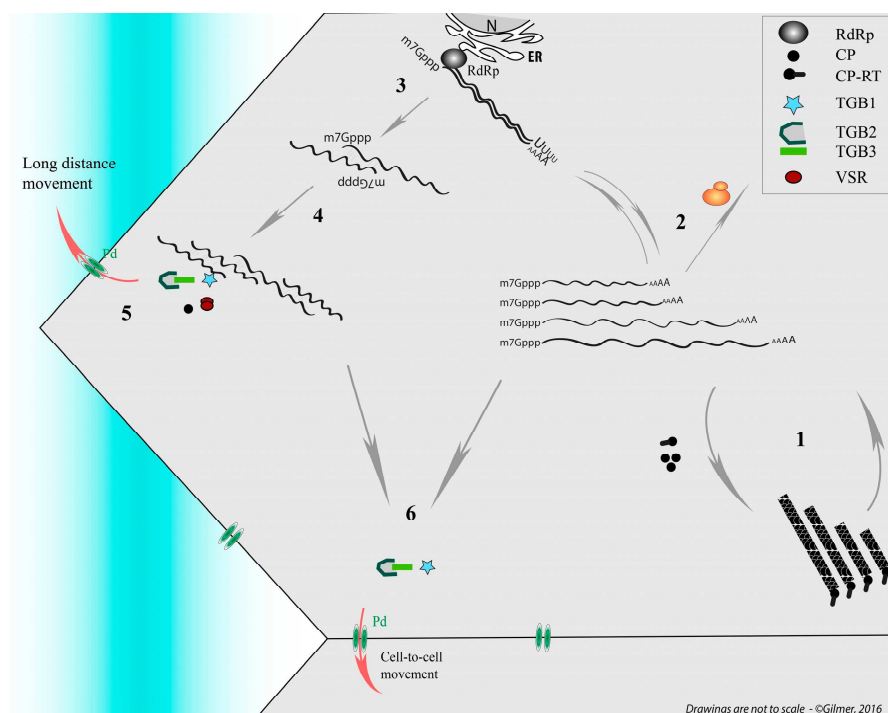


Figure 2. Simplified representation of replication cycle and movement of Beet necrotic yellow vein virus (BNYVV) used as model. The four genomic RNAs of the segmented helical virus (1) are expressed and amplified (2). On this scheme the RNA-RNA interaction network between genomic segments (3 and 4), stabilized by viral proteins, allows the preservation of genome integrity during long-distance movement (5) in the sieve elements (blue shaded). In particular the presence of the coat protein (CP) and the viral suppressors of RNA silencing (VSR) is necessary for their systemic movement. Vice versa the genomic RNAs can move cell to cell without the need of the CP expression but require triple gene block (TGB) movement proteins (6). ER: endoplasmic reticulum; CP-RT: coat protein-readthrough; N: Nucleus; RdRp: RNA-dependent RNA polymerase.

3.2.2. Potato Mop Top Virus: Tripartite, but Two Is Enough

Potato mop top virus (PMTV) belongs to the *Pomovirus* genus together with beet soil-borne virus, beet virus Q, and broad bean necrosis virus. PMTV is a helical rod-shaped virus with a diameter of 18–20 nm and variable lengths (~120 to ~290 nm) according to the genomic ssRNA being packaged [89]. The particles possess a fragile and partially uncoiled extremity [90]. The viral genome is segmented on three positive-sense RNAs. RNA1 allows the expression of two proteins involved in viral replication thanks to a read-through mechanism. RNA2 encodes for major (CP) and minor (CP-RT) structural proteins, the later produced by ribosomal read-through of the CP Amber stop codon. RNA3 possesses four different ORFs that encode for the triple gene block movement proteins (TGB1, TGB2 and TGB3) and one cysteine-rich protein of 8 kDa [89]. As described for BNYVV, minor structural protein is detected at one extremity of some particles. CP-RT is involved in the transmission by the *Spongospora subterranean* vector, because spontaneous deletions occur in isolates with disrupted transmission, particularly after serial mechanical inoculation [91,92]. However, this minor structural protein is dispensable for viral morphogenesis because deletions of the RT domain, or mutations that prevent read-through from occurring, accumulate viral particles [93]. The encapsidation of viral genomic RNAs is also dispensable for systemic spread because “no-protein” and “CP-RT only” RNA2 mutants move [93]. This particularity explains the systemic infection of *N. benthamiana* using a combination of transcripts corresponding to RNA1 and RNA3 [94] and thus demonstrates that PMTV long-distance movement occurs as an RNP complex. The RNA-binding domain constituted by the 84 N-terminal residues of the TGB1 movement protein ensures PTMV RNP complex long-distance movement and appears dispensable for cell-to-cell movement [95]. This domain ensures TGB1 nucleolar localization and its association to microtubules that could allow the recruitment of a cellular factor necessary for the vasculature entry of the RNPs [95]. This renders “PMTVΔRNA2” comparable to GRV, which uses ORF3 protein to recruit fibrillarin to form viral RNP complexes able to move long distance [96] (see Section 2.1.3).

With the reinterpretation of the literature describing the systemic movement of PMTV RNA2 deletion mutants [93], we pointed out an interesting property for the “CP-only” mutant. This viral RNA2 mutant is unable to move long distance in the presence of RNA1 and RNA3. A similar behavior appears for deletion mutants within the C-terminal part of the CP-RT sequence, leading the authors to conclude that a 140 amino acid CP-RT domain is specifically required for the systemic movement of RNA2. However, abolition of CP synthesis in these mutants restored the RNA2 species’ long-distance transport. Consequently, we postulate on the existence of a sequence acting as a “riboswitch” that could be regulated either by a CP interaction or by refolding during ribosomal translation. Such alternative structures could allow or prevent genomic RNA-RNA network interactions, required for long-distance transport or its recruitment as messenger RNA, respectively.

Taking into consideration our preliminary data accumulated on BNYVV, we checked if similar RNA-RNA interactions could exist, using the IntaRNA program (Freiburg RNA Tools) [97,98] and looking for heterologous RNA-RNA interactions between PMTV genomic RNAs (Sw isolate). We took into consideration the sequences that were preserved in natural and artificial RNA2 deletion mutants that were still able to move long distance [93,99]. We found a 20 nt long sequence (5′-²⁷⁶⁰UAGGGAAGAUGUCAGGUAAG²⁷⁷⁹-3′), located within the CP-RT C-terminal coding sequence that could possibly interact with a sequence (5′-²²⁴⁴CUUGCCUGACGUCGAUCCUG²²⁶³-3′) present in the RNA1 replicase sequence with a 2 nt bulge (underlined). In a model where genome integrity needs to be preserved during the systemic movement by the association of all genomic RNAs in a RNP complex, such 20 nt base pairing, with a predicted variation of the Gibbs free energy of −16.35 Kcal/mol, could participate in an RNA2-RNA1 specific interaction. We did not extend our computational analyses to the RNA3-RNA2 or RNA3-RNA1 interaction domains. However, taking into account the systemic infection mediated by RNA1 and RNA3, such domains should exist [100].

The advantage of a regulatory network involving RNA-RNA interactions eliminates the need for a viral protein dedicated to associate RNA species with a correct stoichiometry. At the beginning of a cell infection, translation and replication events are predominantly monopolizing viral RNAs

that switch between unstructured and structured forms. Thereby, RNA-RNA interactions could take place in *trans* and will guarantee the formation of RNP complexes dedicated to the long-distance movement thanks to the movement proteins produced via subgenomic RNAs. Late in the infection, the accumulation of structural protein would negatively regulate the RNA-RNA interaction, leading to the encapsidation of genomic RNA species ready for their acquisition by the vector thanks to the minor structural protein recognition [92]. As for BNYVV, experiments need to be performed to verify the existence of a “riboswitch” in order to validate or discard these hypotheses.

4. Multipartite Positive-Strand RNA Genomes Have to Preserve Their Genome Integrity

As previously mentioned, to maintain their viral genome as a functional unit, segmented positive-strand viruses require the presence of at least one of each viral RNA segment in the target cell. Such conditions require a high multiplicity of infection (MOI), especially for viruses with four or more genomic entities as demonstrated by theoretical studies [101]. Because the infection of different plant cell types and tissues does not occur as a uniform process, a multipartite phytovirus has to pass through constraints and to sustain other biological costs in order to maintain its genome integrity.

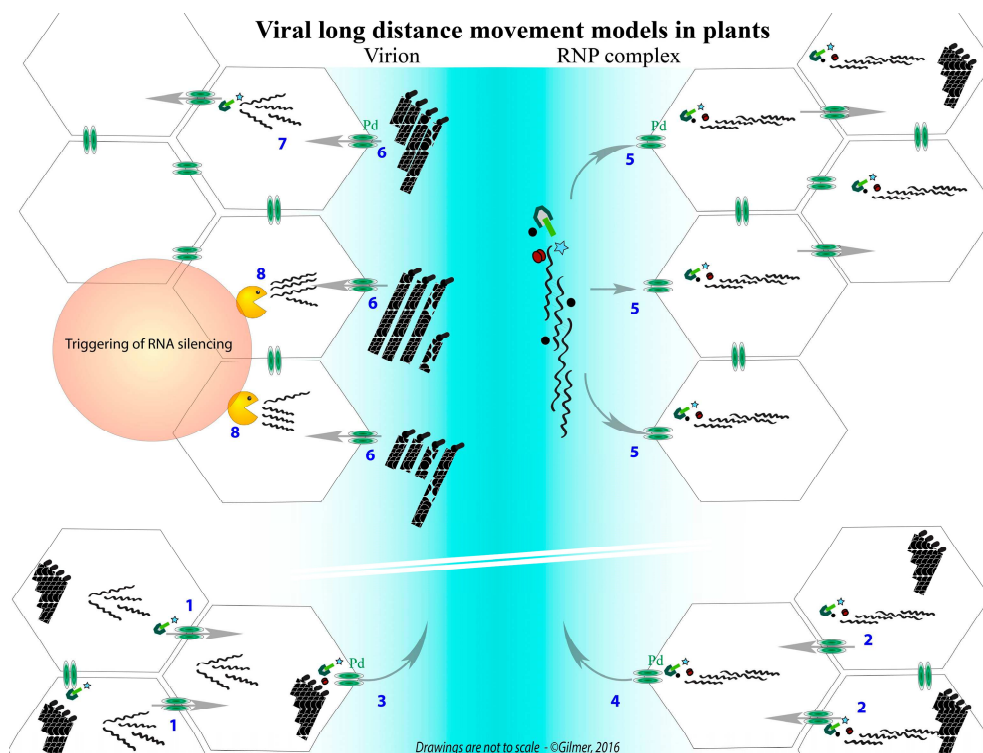


Figure 3. Confrontation of two models describing the long-distance movement of multipartite RNA viruses using the BNYVV as an example. The bottom of the graphic represents the progression of an initial infection of mesophyll cells, wherein the infection progresses thanks to the individual cell-to-cell movement of viral RNAs, driven by TGB movement proteins (1) or as ribonucleoprotein (RNP) complexes involving RNA-RNA network between genomic segments (2). In the sieve elements (blue), the virion (3) or the RNP complexes (4) are transported through plasmodesmata thanks to the viral proteins. RNP complexes, driving the export of the viral genomic RNA network, could provide each distant cell with an entire and fully functional genome (5). Virions (or single viral RNAs, not presented) are transported in the sieve elements and are randomly distributed in the distant cells (6). Only the combination providing one of each viral particle (or RNA) is able to initiate an infection that preserves the genome integrity (7). The particles (or RNA) entering cells without the entire genome are either deficient for replication (not shown) or unable to move from cell to cell and express the VSR, leading to the trigger of RNA silencing (8).

Viral cell-to-cell movement ensures the progression of the infection via plasmodesmata, the connection channels between plant cells (Figure 3, steps 1 and 2). In the phloem (a circulation fluid that provides nutrients to all parts of the plant), fluidic mechanic rules dominate. These physical constraints tend to separate small and large viral particles with two tendencies: the first will reduce the velocity of large entities in the fluid, due to increased contacts with the phloem capillary system, whereas small entities will be less affected. Because the phloem is not just a “tube” but consists of vascular elements (sieve elements) connected with companion cells (see Figure 1), viral entities are subject to a “gel filtration” separation. These two effects increase the risk of a loss of one viral component during the journey, compromising the integrity of the viral genome and, therefore, moderate and reduce the speed of systemic movement and infection that should occur in each “visited cell”.

However, the viral genome must contend with RNA decay mechanisms whereby RNA species are translated or cleaved by the silencing machinery (Figure 3, step 8). Therefore, having its genomic components physically separated would increase the chance of essential viral segments being degraded, thus leading to a defective viral entity. Natural infections show that this is not the case, favoring our idea of a collective transport of genomic RNA species (Figure 3, steps 4 and 5). Hence, RNA-RNA interactions are not the only key elements. Various experiments indicate the involvement of structural protein(s), probably in the stabilization of the interactions, as well as non-structural proteins, such as movement proteins or viral suppressors of RNA silencing that counteract the cellular defenses in the distant cells.

5. Concluding Remarks: Lessons from Superinfection Exclusion Reports

We have reviewed published literature about distinct virus packaging and long-distance movement focusing on some data and arguments in favor of a viral transport as RNP complexes rather than virions. Describing BMV long-distance movement, it appears that the RNA3 species could as well be involved in RNA-RNA interactions with other viral species to nucleate the formation of a RNP complex as postulated for BNYVV and PMTV RNA1-RNA2 species. While one RNA species modulates the recruitment of other species, no information is yet available about the stoichiometry of the genomic components in the moving RNP complex. In order to safeguard genomic integrity, it could be postulated that more than one RNA species should be present to ensure, on the one hand, the expression of essential protein(s) in the distant cell and, on the other hand, the presence of replication-ready genomic components.

A notable example of preservation of viral genomic integrity is the superinfection exclusion (SIE) that exists among some phytoviruses and prevents some co-infections from occurring. Described for T-even bacteriophages, SIE consists on the inhibition of viral genome entry in the cytoplasm of an infected cell thanks to the expression of viral proteins [102]. In eukaryotes, viral mechanisms are also described for viruses that tend to deplete viral receptors from the infected cell as described for the human immunodeficiency virus (HIV) CD4 receptor internalization and proteolysis thanks to the expression of the Vpu protein [103]. A limited co-infection of distant tissues has been described in plants inoculated with two recombinant monopartite potyvirus variants (Tobacco etch virus) thanks to the use of fluorescent reporter proteins, suggesting as well the establishment of a limited access to the cell, with one variant excluding the other [104].

A similar observation was made in sugar beet infected with both BNYVV and BSBMV (two benyviruses). The infection of the plant with one virus limits the possible infection of the same plant with the second virus, if inoculated a few days later [105], suggesting a “SIE-like” mechanism for benyviruses. Using viral reporter RNAs from BSBMV and BNYVV RNA3 replicons, both packaged in *trans* during their amplification by BNYVV RNA1 + RNA2, we previously demonstrated that within local lesions, reporter RNA species exclude one another, leading to sectorized lesions as shown in Figure 4 [106]. If such a process occurs on viral particles rather than on viral RNAs, then there will be a high probability for an exclusion of small entities (such as particles containing RNA3 or RNA4) in cells where RNA1 and RNA2 already started to be expressed and replicated. This property bolsters our hypothesis that specific RNA-RNA interactions preserve viral genome integrity during the viral

journey within an infected plant. While some plant species susceptible to local infection could resist the systemic spread of these multipartite viruses, it could be assumed that some cellular RNA could compete by interacting with the domains involved in this RNA-RNA network formation, but this is another story . . .

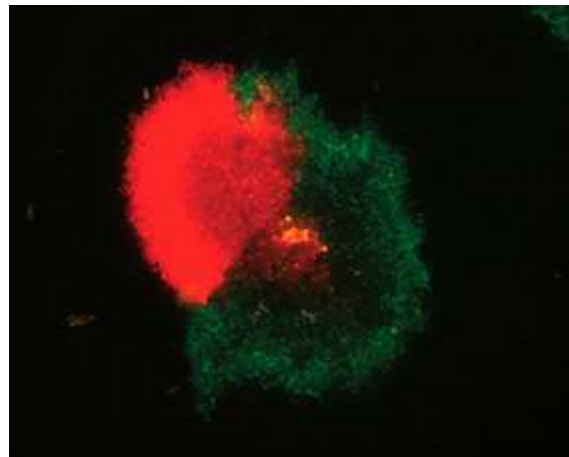


Figure 4. Fluorescence profile of a *C. quinoa* local lesion observed 7 days post infection after rub-inoculation of BNYVV RNA1 and RNA2 supplemented with a mixture of two replicons derived from BSBMV and BNYVV RNA3s, expressing monomeric red fluorescent protein (mRFP) and enhanced green fluorescent protein (EGFP), respectively. This image illustrates the existence of an exclusion mechanism for similar but distinct RNA species during the progression of the infection, with both replicons being able to be encapsidated by BNYVV CP (details are described in Ratti et al., 2009 [104]).

Acknowledgments: The authors are grateful to current and past members of their laboratories for constructive discussions and would like to acknowledge Todd Blevins for his help in language editing and the three reviewers for their constructive comments. Mattia Dall'Ara's work was supported by the Italian ministry PhD grant (048794-0000712558) and by Marco Polo and Da Vinci programs of mobility contribution, funded by the University of Bologna and the Italo-French University of Turin, respectively. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Conflicts of Interest: All authors report no conflicts of interest.

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4. Aim of the study

The aim of this study is to investigate fundamental aspects of *Benyviridae* lifestyle related to its multipartite structure. I proposed to evaluate and validate some aspects of a proposed model involving RNA/RNA interactions regulating the viral cycle and a conservation of genomic integrity during the viral spread in the host. In this model, a specific network of RNA/RNA interactions is suspected to determine the recognition and mobilization of each genomic RNA in a modular RNP complex that represents the mobile infectious unit for BNYVV spread in the plant [59].

In Chapter I, I explored one of the main benefits of having a split genome: the capacity for BNYVV to adapt to different infection conditions by the differential accumulation of its genomic segments. For this purpose, I optimized and validated a dual step Digital droplets (dd) RT-PCR protocol to verify if BNYVV stabilizes the ratio between its genomic RNAs in set-point genomic formulas. These experiments were conducted in different infected hosts (*C. quinoa*, *S. oleracea* and *B. macrocarpa*) and organs (leaves and roots), comparing total and encapsidated genomic RNAs, the latter corresponding to the transmissible form of the virus.

To date, the viral movement across the plant is described as an uncoordinated trafficking of particles or RNP complexes [60]. However, the differential accumulation of BNYVV vRNAs within the infected tissues could require unsustainable costs to preserve viral genomic integrity, ensuring in each infected cell the presence of a complete set of viral segments. This lead to the formulation of two opposed hypotheses:

1. BNYVV segments may not necessarily be present “all together” in each infected cells and RNA replication and stabilization are ensured by the trafficking of viral protein that takes advantage of the symplastic continuity.
2. A specific mechanism guarantees the coordinated movement of each segment as infective units.

To assess one or the other hypothesis, in Chapter II, I reported the experiments performed to test for a simultaneous presence of the four genomic BNYVV RNAs within each infected cell. For this purpose, I optimized a protocol of Real-Time RT-PCR on single protoplast isolated from systemically infected *S. oleracea*.

Finally, in Chapter III, I searched for the existence of RNA1 and RNA2 interactions that would explain the long distance movement of these essential RNAs. Indeed, RNA1 and RNA2 are sufficient for productive infection in the entire plant and therefore represent a minimal efficient RNP complex stabilized by their interaction. *In silico* and *in vitro* experiments have been exploited to establish if any interaction occurs and determine the domains in the interactions. The biological relevance of the interactions has been tested by reverse genetic experiments.

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Chapter I

Differential accumulation of BNYVV genomic RNAs during host infection

1. Introduction: benefits for multipartitism

Genomic segmentation of the hereditary material into multiple segments is a common feature for cellular organisms. Despite the heterogeneous distribution of genome types and hosts such as bacteria, animals, plants and fungi, segmentation feature is found in about 13% of all viral genera where genomes are split in two or more segments [1]. Virus infecting animal, bacteria and fungi prevalently co-package their complete set of genomic segments in a single virion or enveloped entity (described as segmented viruses), while plant viruses predominantly adopt the multipartite strategy, separating genomic segments into independent particles [1]. Furthermore 13 out of 20 plant viral families possess species with multipartite genomic organization making multipartitism a noticeable characteristic of plant viruses (ibid). This brought scientists to investigate the possible benefits for multipartite viruses and host specialization. Having small sized segments rather than one large genomic nucleic acid could represent the main advantage since replication of shorter genomes is, in theory, faster and present a higher fidelity rate [2,3]. However, competition experiments in cell culture between monopartite and “spontaneous bipartite form” of *foot and mouth disease virus* [4] evidenced fitness advantages for the segmented variant. This fitness was linked to the higher particle thermal stability rather than a faster kinetics of RNA synthesis. Moreover, a sufficiently high multiplicity of infection (MOI) in transmission was seen as a requirement for persistence [5]. Split genomes in complementary modules could also confer to co-infecting similar viruses, a reassortment capacity, that led to the selection of chimerical progeny with potential fitness advantages [6]. Segment exchanges represents also supplementary ways to counteract the effect of deleterious mutations [7].

If the above mentioned benefits are still under argumentation [8] and referred as well to segmented viruses, two recent studies on multipartite viruses have shown that, during host infection, relative frequencies of encapsidated genomic fragments converge to a set point genomic formulas (SGF) [9,10]. The distinct particles accumulate differentially, with some segments more abundant than others. The resulted ratio seems to be adaptive to the infected host suggesting that partition have evolved to regulate gene expression in different contexts. Viruses selected for these studies were the nanoviridae *faba bean necrotic stunt virus* (FBNSV) [9] and the bromoviridae *alfalfa mosaic virus* (AMV) [10]. Both viruses are transmitted by animal vector (aphid) like 60% of multipartite virus genera [1], but represent

two different model of multipartitism. With eight circular single stranded DNAs (ssDNAs), FBNSV has the largest number of genomic segments among multipartite plant viruses but such genome type is common only within 9% of multipartite genera [1] (Table 1). On the contrary, AMV possesses a tripartite positive sense single stranded RNA (ssRNA (+)) genome; an organization conserved within 74% of plant multipartite genera (ibid). However, bromoviridae genome organization is characterized by the distribution of essential housekeeping genes among all three segments and has no equivalent with other multipartite plant viruses except for *Hordeivirus* and *Pomovirus* (*Virgaviridae*) [1]. Interestingly, *Potato mop top virus*, the type member of Pomoviruses, is able to move cell to cell and long distance in *Nicotiana benthamiana* plants in the absence of the genomic RNA segment encoding structural proteins (major and minor CP) [11]. Multipartite plant viruses with a ssRNA(+) genome mainly present the segregation of housekeeping genes in two essential segments and, when present, extra segments carry so-called “unessential” genes linked to host or vector related functions usually needed to optimize the infection [1]. The *beet necrotic yellow vein virus* (BNYVV), which belongs to the *Benyviridae* family, is a representative member of this group of viruses and has the highest number of genomic RNAs organized in two essential segments and up to three accessory RNAs (Figure 4 in general introduction). RNA1 encodes the viral replicase, while RNA2 directs the synthesis of the major and minor capsid proteins (CP and p75) of the rod-shaped particles. Thanks to the synthesis of sub-genomic RNAs, the proteins required for the cell-to-cell movement (triple gene block, TGB) and the suppression of host posttranscriptional gene silencing (p14) are produced. The RNA3 species is involved in the long-distance movement and encodes the pathogenic determinant of the Rhizomania disease on sugar beet (p25) while RNA4 is involved, together with the encapsidation process, in the efficiency of virus transmission by the soil-borne vector *Polymyxa betae*. When present, the RNA5 is responsible of the symptoms worsening [12].

Family (multipartite genera/total)	Genome type	Capsid	Segments
Nanoviridae (3/3)	Circular ssDNA	Icosahedral	6_8
Geminiviridae (1/9)	Circular ssDNA	Twinned icosahedral	2 or 1 and satellite
Luteoviridae (1/3)	ssRNA (+)	Icosahedral	2
Secoviridae (6/9)	ssRNA (+)	Icosahedral	2
Tombusviridae (2/16)	ssRNA (+)	Icosahedral	2
Benyviridae (1/1)	ssRNA (+)	rod-shaped	2,4_5
Virgaviridae (6/7)	ssRNA (+)	rod-shaped	2_3
Bromoviridae (6/6)	ssRNA (+)	Icosahedral and bacilliform	3
Closteroviridae (1/3)	ssRNA (+)	filamentous	2
Potyviridae (5/8)	ssRNA (+)	filamentous	2
Ourmiavirus, Idaeovirus, Cilevirus	ssRNA (+)	Icosahedral and bacilliform	2_3
Ophioviridae (1/1)	ssRNA(-)	filamentous	3_4
Rhabdoviridae (2/18)	ssRNA(-)	bullet-shaped and bacilliform	2
Tenuivirus	ssRNA(-)	rod-shaped	4_6
Partitiviridae (5/5)	dsRNA	Icosahedral	2_3

Table 1: List of multipartite phytoviruses among viral families. The number of multipartite genera within total genera members is indicated as well as the nature of the genome, the viral morphology and the number of segments. *Benyviridae* family regroups bipartite species or species having four or five genomic RNAs. List has been drawn up with information taken from Lucía-Sanz and Manrubia [1].

Study of the gene copy number variation in a host dependent manner is a newborn aspect for the biology of multipartite viruses and has been investigated for BNYVV. The relative abundance of BNYVV RNAs has been calculated in different plant organs (leaves and roots) of hosts of different genera (*Chenopodium quinoa*, *Spinacea oleracea* and *Beta macrocarpa*) verifying if and how the virus stabilizes the ratio between its genomic segments in a host specific genomic formula (GF). Besides having a different genomic organization and capsid structure when compared to AMV and FBNSV (Table 1), BNYVV possesses different biological proprieties since it is a soil-borne virus transmitted by protozoa. AMV and FBNSV are aphid transmitted viruses and during aphid feeding, these viruses are guaranteed for important viral loads in sieve elements, thanks to the high vector population density and repeated inoculations events. This ensures a maintenance of a viral reservoir and a high MOI required to initiate cell to cell movement in the host from different companion cells (CCs) [8]. Conversely, BNYVV initial infection occurs after protozoan transmission at a singular cell level. The cytoplasmic fusion between a target rootlet cell and the viruliferous zoospore [13] theoretically permits viral transmission from vector to host with reduced levels of MOI. From

such kind of initial infection, the viral amplification takes place and gains adjacent cells before vascular spread of the infectious material in distant tissues. As stated above, GF could be dependent on host and organ and has never been studied for *Benyviridae* family.

In order to calculate the relative accumulation of BNYVV genomic segments in leaves and roots of *C. quinoa*, *S. oleracea* and *B. macrocarpa* a protocol of dual step Droplets digital (dd) RT-PCR has been optimized and validated.

2. Droplet digital (dd) PCR

Digital droplet (dd) PCR is a recently developed innovative technology that allows a precise quantification of target nucleic acids in a sample [14]. Using the same operating principle of conventional PCR methods of specific DNA target amplification, ddPCR differs from standard quantitative (q) PCR for the quantification method of nucleic acids. qPCR analyzes the amplification reaction in real time, following an increase of a fluorescence signal associated to the number of amplicons produced after each PCR cycle. Using standards and calibration curves, qPCR permits the determination of “gene” copy numbers. Similarly, ddPCR uses fluorescence to measure amplicon accumulations, but does not rely on standard curves. PCR reactions are analyzed at end-point within up to 20,000 of 1 nl water-in-oil droplet partitions acting as separated reactions. Therefore in theory, single nucleic acid molecules are dispersed in droplets following a Poisson distribution. The presence of a target nucleic acid gives rise to a single positive amplification event confined in the droplet (high amplitude fluorescence droplet). The ratio between positive and total events is used to determine exactly the copy number of target molecules in a sample (Figure 1). Raw results are statistically corrected after Poisson distribution analyses that calculate the probability to have one or more molecule target in each positive droplet. Compared to qPCR, ddPCR present obvious advantages and is a reliable method for the absolute quantification of DNA targets in a sample. The direct and absolute counting of target DNA copies per input sample makes dispensable the use of standard curves or endogenous controls. Furthermore, end-point analyses render the method less susceptible to inhibitors that impact qPCR amplification curve by increasing the threshold cycle (CT) [15]. Finally, ddPCR sensitivity is increased for low target DNA quantities represented by a lower coefficient of variation compared to qPCR [16,17].

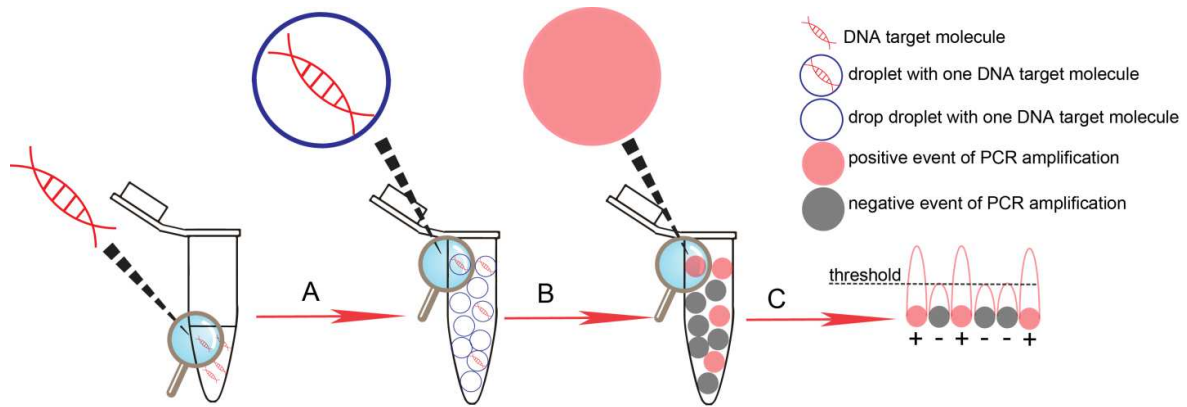


Figure 1 Schematic representation of ddPCR workflow. Sample DNA together with ddPCR reaction mix is partitioned by a droplet Generator (QX200™ Bio-Rad) into ~20,000 droplets (A). Template PCR amplification takes place in all droplets through a standard thermocycler (B). Droplet end-point fluorescence is detected in two channels (FAM or HEX) using a droplet reader (QX200™ Bio-Rad) (C). Raw data are analyzed using QuantaSoft software (Bio-Rad) using thresholds lines set manually according to fluorescence amplitude of negative controls. The software assigns a binomial value (positive or negative) for each droplet fluorescence amplitude. Ratio between positive and total droplets and statistical correction according to Poisson distribution, give the number of DNA molecules in the sample.

3. Validation of dual step ddRT-PCR protocol for the absolute quantification of BNYVV genomic RNAs

One of the main constrain of using retro-transcriptase (RT) reaction for a RNA quantification assay is the optimization of the synthesis of a single copy of cDNA for each target RNA molecule. Complex RNA secondary structure or insufficient level of downstream RNase H activity could compromise the equimolar RNA/cDNA conversion leading to misestimate the RNA template copy number. RNA targets and reagent mix partitioning in different droplets makes such aspects negligible in one step ddRT-PCR where each reaction event effectively assigns one unequivocal fluorescent amplitude for each molecule target. However, since RNA retro-transcription must precede cDNA droplet partitioning to prevent the conversion and the amplification of BNYVV viral complementary RNAs issued from replication intermediates, optimization and validation of the first step of a dual step ddRT-PCR is mandatory. For this reason, standard BNYVV B-type genomic RNAs have been produced and quantified.

3.1 BNYVV Standard production

Standard B type BNYVV RNAs have been produced *in vitro* by run-off transcription of the full length linearized T7 cDNA clone pB214 (RNA2), pB35 (RNA3) and pB45 (RNA4) as

described previously [18,19]. Since pB15 (RNA1) [19] run-off transcription produces significant amount of incomplete RNAs due to premature T7 polymerase stops, the last 2,991 nucleotides, have been amplified using 3755F and 6746R specific RNA1 primer (table 2) and GoTaq® Long PCR Master Mix (Promega) and cloned in pGEM®-T Easy Vector (Promega). The resulting *Hind*III-linearized plasmid has been *in vitro* transcribed. All enzymatic reactions have been carried out according to manufacturer's protocol. After DNA removal with RQ1 RNase-Free DNase (Promega), RNAs have been purified by phenol/chloroform extraction and NH₄OAc/EtOH precipitation prior analysis by agarose gel electrophoresis and quantification by Qubit™ 3.0 fluorometer (Thermo Scientific). Molecular concentration of each transcript has been determined using online calculator tool (www.end-memo.com/bio/dnacopynum.php) and four standard serial dilutions (4×10^4 ; 2×10^4 ; 2×10^3 ; 2×10^2 molecules) have been prepared. The combination of all transcripts were mixed with 1ng of a total RNAs extracted from *S. oleracea*, *B. macrocarpa* or *C. quinoa* healthy plants.

RNA	PRIMERS	TAQ-MAN PROBES
1	3755F(CGGAAGAAGCTCGAAAGAAGG) 6746R (TTTTTTTTTTTTTATATCAATATACTG)	
1	3946F (TGGTTTCACAAGGAGATGTCGTT) 4024R (TCTGCACAATCAAAGGCATCA)	3944 FAM-TTTTGGACATAGCACGTGTGGAAAACGATA
2	662F (TGGACCCGGGATAAATTTGA) 734R (CGGGTGGACTGGTTCTACCTT)	653 HEX-ACCGGTTCAAATTACCATGGACACCTGTT
3	54F (ATATGTGAGGACGCTAGCCTGTT) 172R (TGAAACGATGGAGTCACTATGCTT)	84 FAM- CTGACCGACCAAATCCAAGCGAGCTTAAT
4	415F (TCCTCCTTTGATACGTCATGAAGA) 490R (CAATGGGCCAATCTCAATCC)	445 HEX-TGATTGTACTGCTAGGATGGTGCA

Table 2 Primers and probes used to amplify and detect BNYVV RNAs. Primers used to clone the 3' portion of BNYVV RNA1 in pGEM®-T Easy Vector (3755F and 6746R) and used for ddRT-PCR experiments are included.

3.2 Comparison between one step and dual step ddRT-PCR for the absolute quantification of standards

To quantify BNYVV genomic RNAs, primer and TaqMan probes (labeled with FAM and HEX fluorophores, Table 2) were designed using Primer Express Software v2.0 (Applied Biosystems) avoiding targeting of subgenomic RNA2 and noncoding RNA3 species. Primer specificity was successfully validated by performing dual step ddRT-PCR assays using standard dilutions with or without the specific BNYVV RNA targets as templates. 2 µl of

each standard solution template were mixed with 3 µl of the following reverse transcriptase mixture:

- 1 µl M-MLV 5X Reverse Transcriptase Reaction Buffer (Promega)
- 0.3 µl dNTPs (10 mM each)
- 0.2 µl M-MLV RT (200U/µl) (Promega)
- 0.5 µl BNYVV Reverse Primer (20 µM)
- 1 µl DNase/RNase free water

Reverse transcription reaction was carried out at 42°C for 1 h prior to the enzyme inactivation at 90°C for 5 min. To 5 µl of each reverse transcriptase reaction, 15 µl of the following ddPCR mixture was added:

- 10 µl 2x ddPCR Supermix for Probes (Bio-Rad)
- 0.5 µl BNYVV Forward Primer (20 µM)
- 1 µl BNYVV FAM or HEX probe (4 µM)
- 3.5 µl DNase/RNase free water

The ddPCR reactions were carried out after droplet generation. Thermal cycling conditions are described in Table 3.

Step	Cycles	Temperature	Time
Polymerase activation	1	95° C	10 min
Denaturation	40	95° C	30 s
Annealing/Extension		62° C	1 min
Enzyme inactivation	1	95° C	10 min
Holding	1	4°C	∞

Table 3 Thermal cycling conditions used for one step ddRT-PCR reactions. Ramp rate was adjusted to 2°C/sec

Accuracy of dual step ddRT-PCR reactions was tested comparing copy numbers resulting from previous assays, to copy numbers obtained by one step ddRT-PCR using the same standard templates. The latter reaction was performed by mixing 2 µl of each standard solution template with 18 µl of the following ddRT-PCR mixture:

- 5 µl 4x One-Step RT-ddPCR Advanced kit for Probes (Bio-Rad)
- 2 µl Reverse transcriptase 20 U/µl (Bio-Rad)
- 1 µl DTT 300 mM

- 0.5 µl BNYVV Forward Primer (20 µM)
- 0.5 µl BNYVV Reverse Primer (20 µM)
- 1 µl BNYVV FAM or HEX probe (4 µM)
- 8 µl DNase/RNase free water

Thermal cycling conditions are described in Table 4.

Step	Cycles	Temperature	Time
Reverse transcription	1	42° C	30 min
Polymerase activation	1	95° C	10 min
Denaturation	40	95° C	30 s
Annealing/Extension		62° C	1 min
Enzyme inactivation	1	95° C	10 min
Holding	1	4°C	∞

Table 4 Thermal cycling conditions of one step ddRT-PCR. Ramp rate is adjusted to 2°C/sec

Data analysis was interpolated in two dimensions scatter plots for each BNYVV RNA having in ordinate axis one step ddRT-PCR and in abscissa axis dual step ddRT-PCR absolute quantifications of RNA standards serial dilutions quantified by Qubit™ 3.0 . As shown in figure 2, best fit lines were linear ($R^2 > 0.99$) over standard range covering three order of magnitude with slopes ranging from 0.952 to 0.980 indicating a low error rate when one step ddRT-PCR absolute RNA quantification is compared with that of dual step ddRT-PCR. Relevant copy number underestimations (RNA1 and RNA2) comparing to Qubit quantification have been associated to the RNA degradation rate of “non-certified” standard used. Taken together these data indicate that retro-transcriptase events in dual step ddRT-PCR gave equimolar RNA/cDNA conversion for BNYVV RNAs.

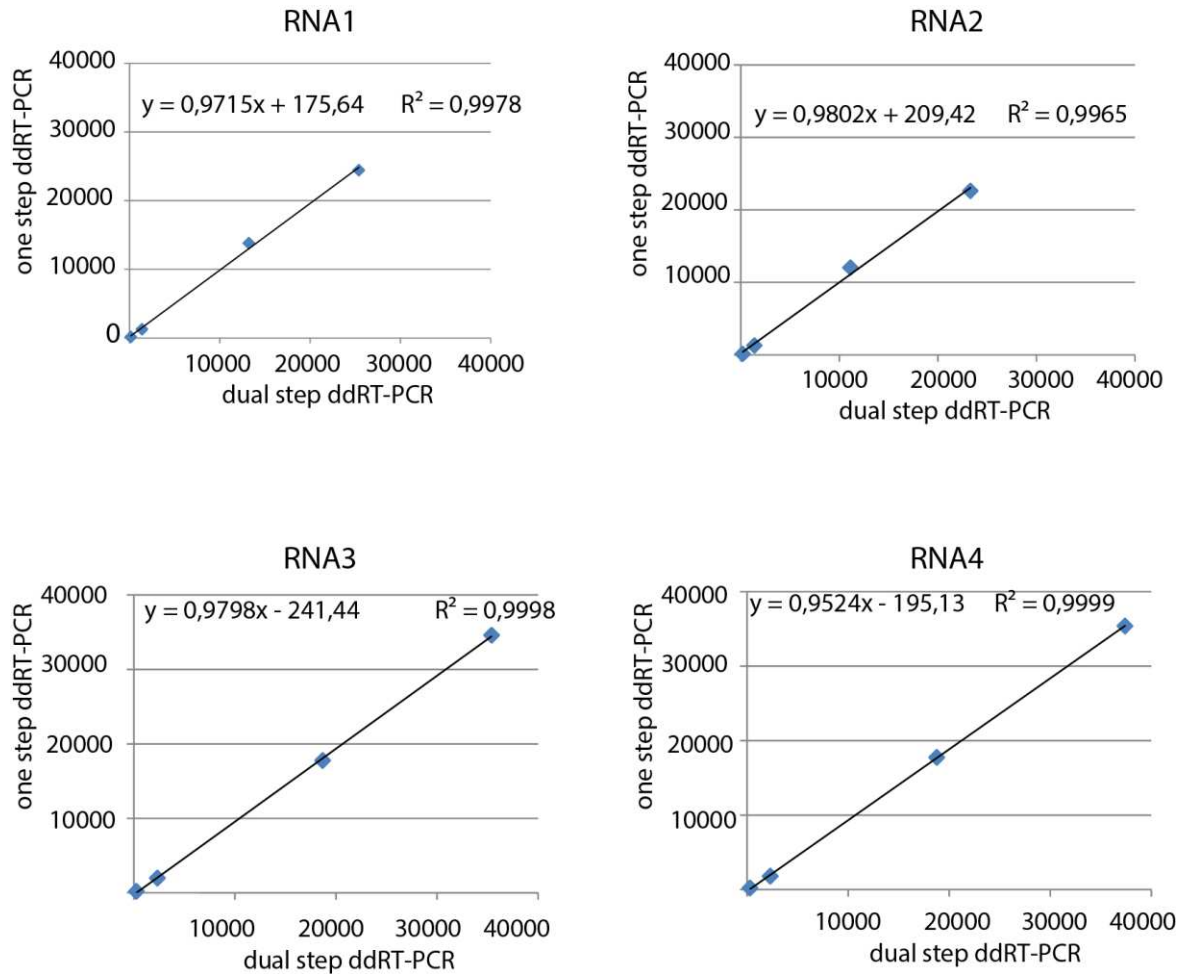


Figure 2 Scatter plots having in ordinate axis one step ddRT-PCR and in abscissa dual step ddRT-PCR absolute RNA quantifications of standard serial dilutions quantified by Qubit™ 3.0. For each BNYVV RNAs, absolute quantification scatter plot is reported as well as best fit lines and R^2 values.

3.3 Optimization of dual step ddRT-PCR for the quantification of BNYVV RNA1 to 4

Duplex dual step ddRT-PCR reactions for the RNA1 and RNA2 or RNA3 and RNA4 simultaneous absolute quantifications have been optimized. The following mixtures of retro-transcriptase and ddPCR reactions have been conducted using the same thermal cycling condition described for single dual step ddRT-PCR:

Retro-transcriptase mixture:

- 1 μ l M-MLV 5X Reverse Transcriptase Reaction Buffer (Promega)
- 0.3 μ l dNTPs (10 mM each)
- 0.2 μ l M-MLV RT (200U/ μ l) (Promega)
- 0.5 μ l BNYVV1 or BNYVV3 Reverse Primer (20 μ M)

- 0.5 µl BNYVV2 or BNYVV4 Reverse Primer (20µM)
- 0.5 µl DNase/RNase free water
- 2 µl standard solution template

ddPCR mixture:

- 10 µl 2x ddPCR Supermix for Probes (Bio-Rad)
- 0.5 µl BNYVV1 or BNYVV3 Forward Primer (20µM)
- 0.5 µl BNYVV2 or BNYVV4 Forward Primer (20µM)
- 1 µl BNYVV1 or BNYVV3 FAM probe (4µM)
- 1 µl BNYVV2 or BNYVV4 HEX probe (4µM)
- 2 µl DNase/RNase free water

Recorded FAM and HEX fluorescence amplitudes of corresponding probes have been reported in a scatter plot where negative and positive droplets are distributed into four distinct groups: negative, FAM-positive, HEX-positive and positive droplets for both channels FAM and HEX. Results obtained from duplex RNA1 and RNA2 reaction shown clearly distinct and analyzable groups, while a non specific HEX signal from RNA3 FAM probe, prevents discrimination between the negative droplets and HEX positive droplets in RNA3 and RNA4 duplex reaction (Figure 3). For this reason absolute quantification of BNYVV RNA3 and RNA4 have been carried out using two independent reaction of dual step ddRT-PCR allowing a clear differentiation between negative and FAM or HEX positive droplets (Figure 4).

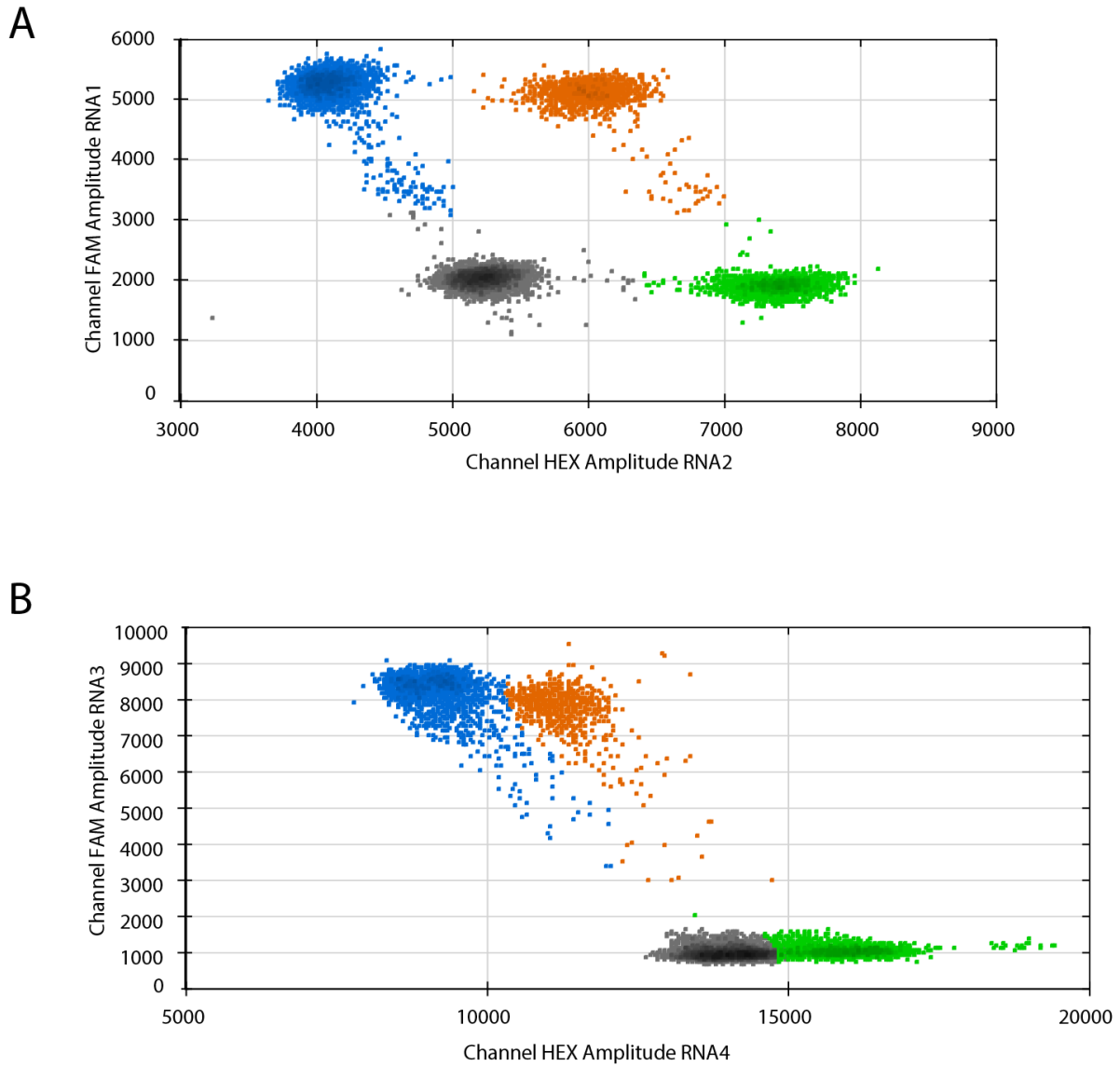


Figure 3 Duplex dual step dd-RT-PCR scatter plots for the simultaneous quantification of BNYVV RNA1 and RNA2 (A) or RNA3 and RNA4 (B). Ordinate and abscissa axis report FAM and HEX amplitude signal respectively. Each dot represents one droplets reaction and colors define the kind of positivity assigned. Black dots represent negative reactions, blue dots positive reaction for RNA targeted by FAM probes, green dots positive reaction for RNA targeted by HEX probes and orange dots are positive reactions for both target RNAs. Duplex RNA1 and RNA2 reaction dots forms four distinct and clearly analyzable groups, while unspecific HEX signals from RNA3 FAM probe, prevent discrimination between the negative droplets and HEX positive droplets in RNA3 and RNA4 duplex reaction.

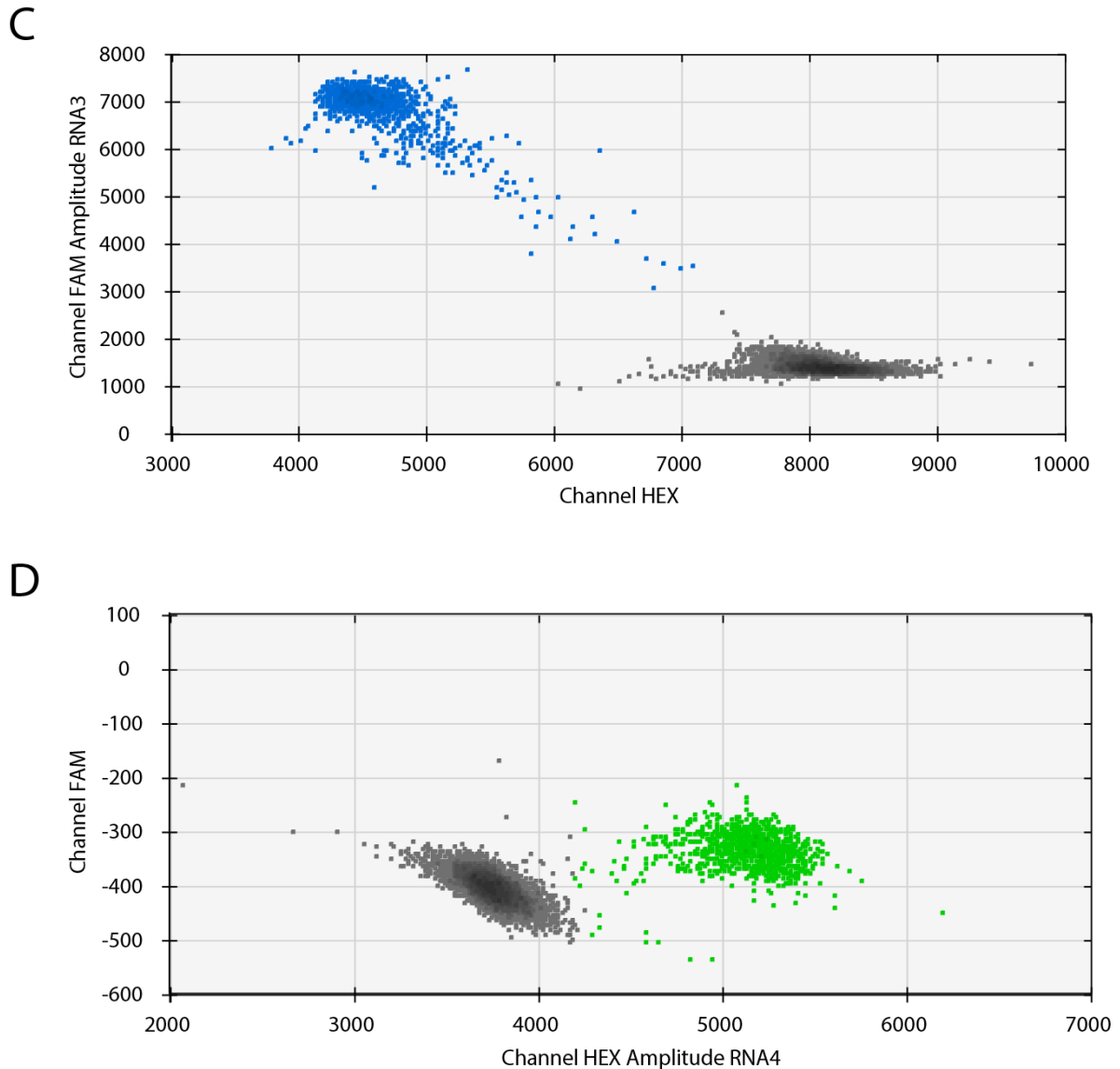


Figure 4 Dual step dd-RTPCR scatter plots for the quantification of BNYVV RNA3 (A) and RNA4 (B). Dots representing positive amplification reaction of RNA3 (blue) and RNA4 (green) cluster in groups sufficiently distinguishable from groups of negative droplets (black dots).

3.4 Host species and virus inoculation

B. macrocarpa, *S. oleracea* cv. Viking and *C. quinoa* have been chosen as experimental host. *B. macrocarpa* and *S. oleracea* have been infected by agro-infiltration with four agrobacteria, each containing one of the four plasmid allowing *in vivo* expression of infectious B type BNYVV RNA as described previously [20]. Mixture of bacterial suspension adjusted to $OD_{600} = 0.3$ in MA buffer (10 mM $MgCl_2$, 200 mM acetosyringone) have been syringe-inoculated on two true leaves of *B. macrocarpa* and *S. oleracea* seedlings. *C. quinoa* is not a

susceptible host for agro- inoculation [21] , therefore adult plant leaves were rub-inoculated with sap issued from a 3 day-old post inoculation (d.p.i.) agro-infected leaf of *S. oleracea*.

3.5 Total and encapsidated RNA extraction and preparation of samples for dual step ddRT-PCR

Two leaf discs (1 cm Ø) or root tissues (200 mg) have been collected from four infected *B. macrocarpa* and *S. oleracea* plants while four single local lesions have been selected from *C. quinoa* leaves. In particular, leaves and roots samples have been collected 3 weeks p.i. from symptomatic systemic *B. macrocarpa* and *S. oleracea* and 7 d.p.i. from *C. quinoa* inoculated leaves. Samples have been homogenized in 600 µl of cold TM buffer (Tris 100mM, MgCl₂ 10mM, pH 7.5) and total RNA has been immediately purified by phenol/chloroform extraction and NaOAc/EtOH precipitation from half of the homogenate. Nuclease protected RNAs corresponding to encapsidated viral RNA has been purified following the Jupin et al. protocol [22], by extracting and precipitating the remaining homogenate after 1h of incubation at 37°C to allow vacuolar nuclease digestion of unprotected RNAs. Pellets have been resuspended in 50 µl of DNase/RNase free water and purified RNAs quantified with Qubit™ 3.0 fluorometer (Thermo Scientific). Dilutions were adjusted to analyze ~ 2 ng of *B. macrocarpa* and *S. oleracea* RNAs and ~ 0.4 ng of *C. quinoa* RNAs.

4. Results

RNA copy number for each segment has been converted into relative frequency by dividing its value with the sum of all quantified genomic RNAs of the sample (Figure 5). BNYVV GF in the different hosts and organs has been determined by dividing the mean of relative frequency for each segment by the mean of relative frequency of the less abundant RNA (RNA2). GF values have been rounded to the nearest tenth. One-way analysis of variance (ANOVA) has been used to compare the mean of frequency values of genomic RNAs within categories such as host type, organ type (leaves or roots) for each kind of RNA extraction protocol (total or encapsidated). Differences between segment abundances were considered significant when P values were <0.05 (table 5). Furthermore, significant homogeneous group of segment accumulation have been considered using Tukey HSD pair tests (Figure 5) while significant difference in the frequency of the segments between the two types of RNA extraction have been evaluated using Student's t-test.

	LEAVES/LESIONS		ROOTS	
	Total RNA	Encapsidated RNA	Total RNA	Encapsidated RNA
<i>B. macrocarpa</i>	$F_{3,12} = 21.219;$ $P = 4.3 * e^{-5}$	$F_{3,12} = 45.613;$ $P = 7.8 * e^{-7}$	$F_{3,12} = 44.170;$ $P = 9.26 * e^{-7}$	$F_{3,12} = 5.000;$ $P = 0.18$
<i>S. oleracea</i>	$F_{3,12} = 44.773;$ $P = 8,06e^{-7}$	$F_{3,12} = 17,180;$ $P = 1,02 * 10^{-4}$	$F_{3,12} = 17,612;$ $P = 1 * 10^{-4}$	$F_{3,12} = 39.985;$ $P = 1,59e^{-6}$
<i>C. quinoa</i>	$F_{3,12} = 101.876;$ $P = 8,38e^{-9}$	$F_{3,12} = 134,139;$ $P = 1,70 * e^{-9}$		

Table 5: one-way ANOVA analyses were performed to estimate the significant difference between relative frequency within total or encapsidated RNAs from *B. macrocarpa*, or *S. oleracea* leaves and roots and total or encapsidated RNA from *C. quinoa* local lesions. One Way ANOVA were determined by SPSS 15.0 software. F(x,y) correspond to the Fisher distribution where x and y refer to the degree of freedom between group and within group respectively.

As described for FBNSV and AMV [9,10] some genomic segments of the BNYVV appear less abundant than others independently from the organ and the host analyzed. RNA1 and RNA2 represent in most cases a homogeneous group with a mean of relative frequencies that range from ~0.04 to ~0.18 and from ~0.02 to ~0.1 respectively. RNA3 and RNA4 appear, in general, more abundant with mean of relative frequencies ranging from ~0.21 to ~0.57 and from ~0.21 to ~0.65 respectively. The significant variability observed between RNA3 and RNA4 accumulation is noticeable. These RNA species cluster in heterogeneous or homogeneous groups depending on the host, the organ analyzed and the RNA extraction (Figure 5). Significant difference between relative abundances of the same RNA in total and encapsidated RNA extraction have been founded in *S. oleracea* (RNA1 and RNA2 in leaves and RNA3 and RNA4 in roots) and in local lesions of *C. quinoa* (RNA1) but not in *B. macrocarpa* infected plants (Figure 5).

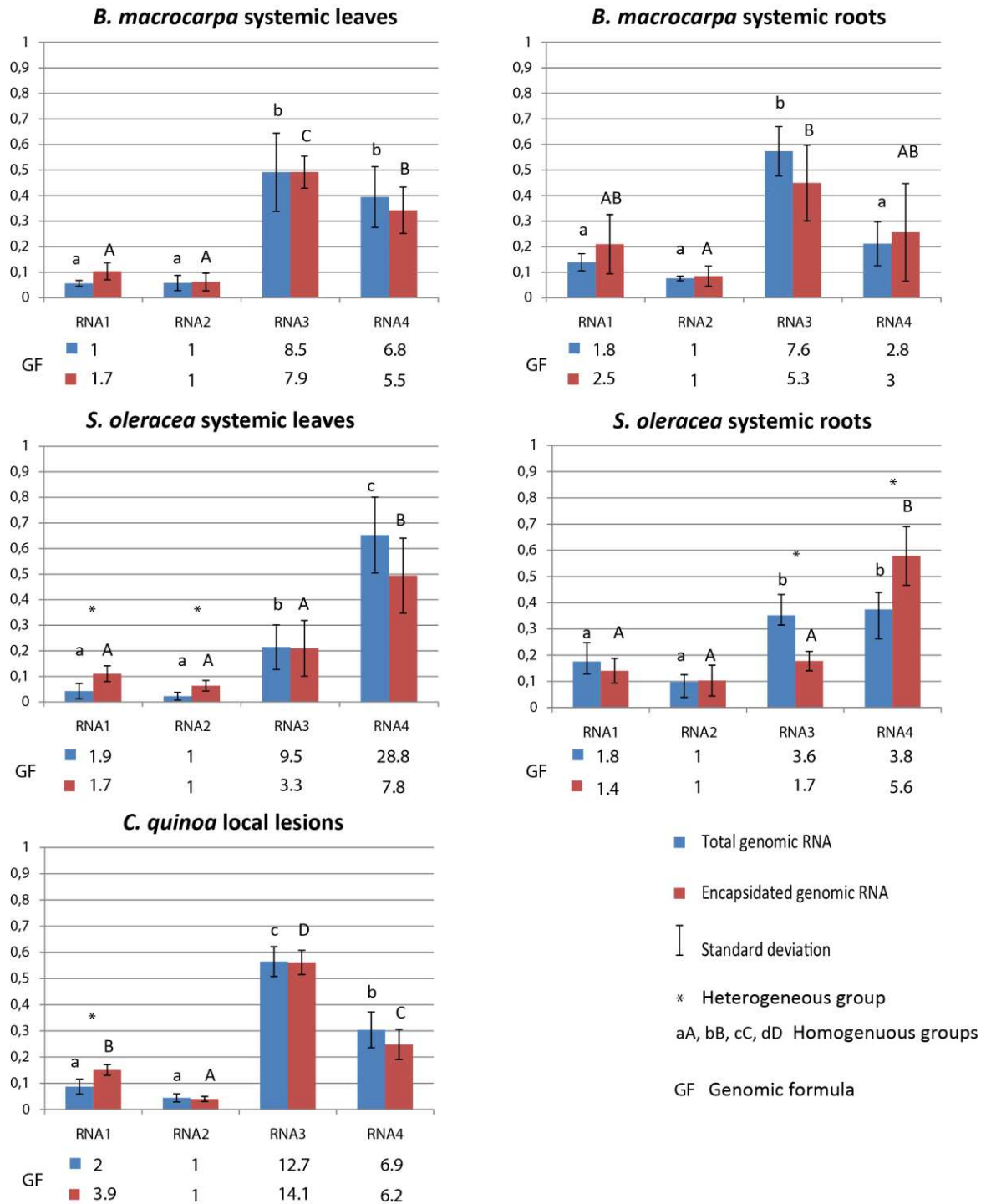


Figure 5 BNYVV genomic RNA relative frequencies evaluated in *B. macrocarpa* or *S. oleracea* leaves and roots or *C. quinoa* local lesions. Blue histograms represent the mean of relative frequencies calculated for total RNA extraction while red histograms represent the mean of frequencies for encapsidated genomic RNA. Bars represent standard deviation. Groups of homogeneity assessed by Tukey HSD pair tests are indicated by letter while significant differences between frequencies of the same RNA in total or encapsidated RNA extractions are indicated with asterisks and have been evaluated with Student's t-test. The genomic formula, calculated by dividing the mean of relative frequency for each segment by the mean of relative frequency of the less abundant RNA (RNA2) is reported under each histogram. One Way ANOVA and Student's t-test were calculated using SPSS 15.0 software

Student's t-test analyses were used to evaluate the variation of the mean of relative frequencies of each RNA species between leaves and roots of *B. macrocarpa* and *S. oleracea* (Figure 6). Interestingly, in both systemic hosts, significant differences were observed for total RNA species accumulation between leaves and roots while no significant variation were noticed for encapsidated RNA abundance between the two organs.

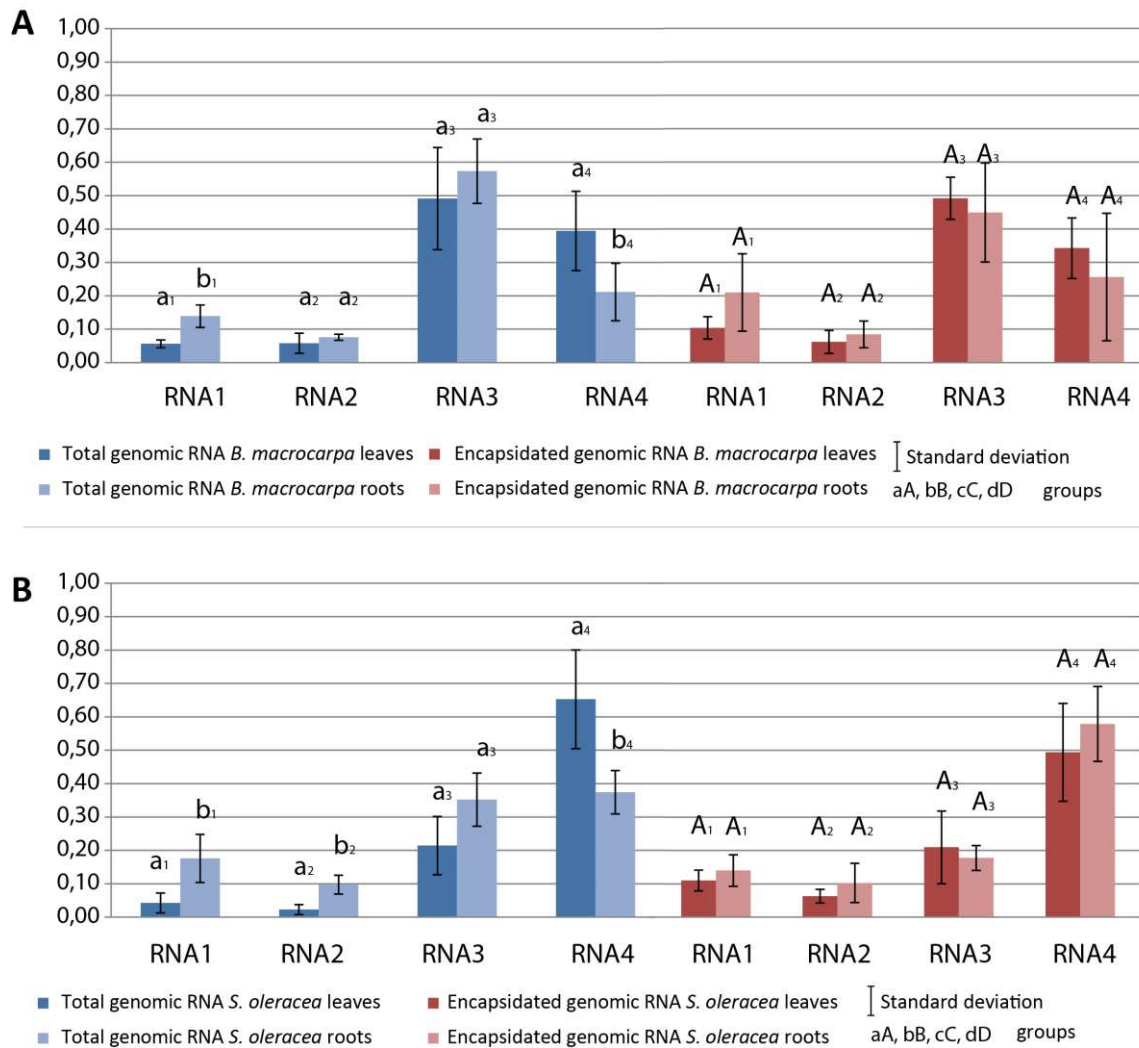


Figure 6 RNAs relative frequencies differ within different organs of the host. Comparison of the means of relative frequencies for each BNYVV RNAs calculated in systemically infected *B. macrocarpa* (A) or *S. oleracea* (B) leaves and roots. Significant differences in segment accumulation are indicated by letters and were assessed by Student's t-test. Error bars represent standard deviation. Student's t-test was determined using the SPSS 15.0 software.

Finally, Student's t-test and ANOVA one-way analysis have been applied to verify host type influence on the variation observed for each RNA in roots and leaves (Figure 7). In *B. macrocarpa* and *S. oleracea* roots, RNA variation appears significant for RNA3 and RNA4

for both total and encapsidated RNA extractions. For leaves of the three hosts, independently of the RNA extraction protocol, RNA3 and RNA4 species appear to accumulate differentially in *S. oleracea* compared to *B. macrocarpa* and *C. quinoa* where each genomic RNA is represented by two homogenous groups of mean of relative frequencies (Figure 7).

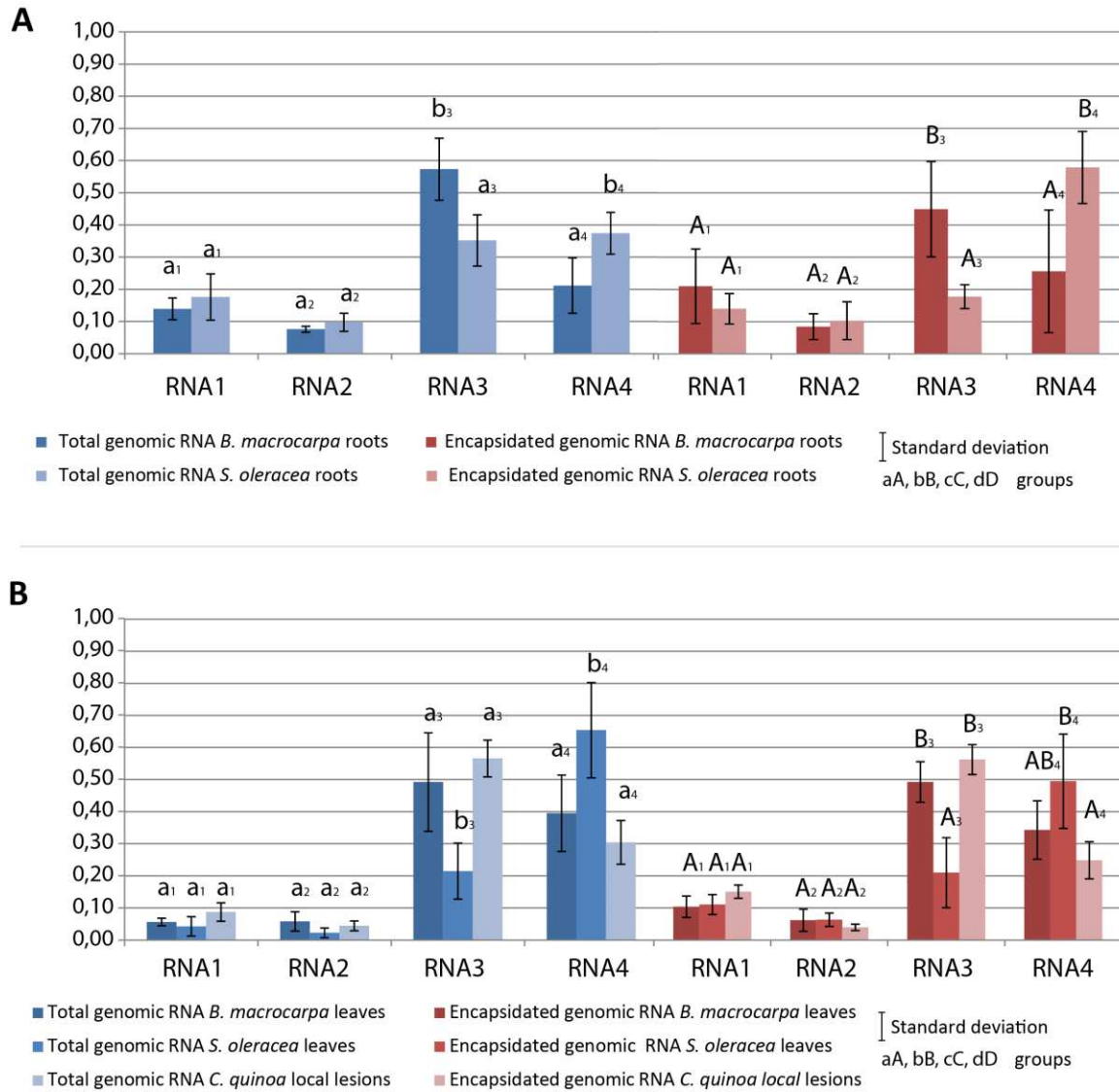


Figure 7 RNAs relative frequencies differ from one host to the other (A) Comparison of the calculated mean of relative frequencies of each BNYVV RNA in *B. macrocarpa* and *S. oleracea* roots. Significant differences in segment accumulation are indicated by letters and were assessed by Student's t-test. (B) The similar approach was applied for BNYVV RNA present in *B. macrocarpa*, *S. oleracea* and *C. quinoa* leaves. Groups of homogeneity indicated with letter have been assessed by Tukey HSD pair tests. Error bars indicate standard deviation. One Way ANOVA and Student's t-test were determined using the SPSS 15.0 software.

6. Discussion

The accumulation of BNYVV genomic RNAs has been compared in three hosts infected by the BNYVV. Host plant *C. quinoa* restricts BNYVV infection within the inoculated areas while *B. macrocarpa* and *S. oleracea* allow systemic BNYVV infection. However, while RNA3 is mandatory for BNYVV systemic infection in *B. macrocarpa*, the sole presence of RNA1 and RNA2 is sufficient for the virus to move long distance in *S. oleracea* [23]. It has been suggested that multipartite viruses adapt to different host or conditions by changing gene expression thanks to a gene copy number variation [9]. Such strategy has also been associated to monopartite viruses such as *Vaccinia virus* that responds to selection pressures experiments with the rapid amplification and duplication of K3L gene with a consequent expansion of the genome length [24]. Adaptive gene copy number variation is a common feature of prokaryote and animal parasites, other than of viruses. Among insect, *Acyrtosiphon pisum* forms genetically distinct host-associated populations on multiple legume species thanks to copy number variation of up to 434 genes [25]. Furthermore resistance genes are commonly amplified by many bacteria as an adaptive response to antibiotic treatment [26,27]

If the variation of genomic segments relative abundance is a way for the rapid adaptation of the virus, the orchestration of gene expression often requires the maintenance of conserved stoichiometric ratios between particular genomic segments. AMV genomic formula is characterized by having fixed RNA1 and RNA2 stoichiometric coefficients (~1:3), and variable RNA3 coefficients that range from ~2 in *Nicotiana benthamiana* to ~16 in *Cucurbita pepo* [10]. In total RNA extractions, BNYVV RNA1 and RNA2 relative frequencies are low and comparable, and their stoichiometric rapport is mostly fixed to ~2:1. Conversely, RNA3 and RNA4 are more abundant and their ratio varies upon the host and the organ. RNA3 was found ~13 fold more abundant than RNA2 in *C. quinoa* local lesions whereas RNA4 accumulated ~29 time more than RNA2 in *S. oleracea* leaves.

Such enhanced amplification of accessory RNAs represents an interesting aspect of gene copy number influence on gene expression regulation. BNYVV RNA3 species encodes the p25 protein, a pathogenic determinant described as an avirulence gene triggering resistance mechanisms [28,29]. The p25 protein expression is likely regulated during the different viral cycle steps in order to prevent its early expression and a resulting premature cell death. As

soon as viral counter defenses are set, p25 expression could start to favor the viral infection progression. Regulation of p25 expression could be dependent on a mechanism that requires a viral effector to trans-activate RNA3 translation. This protein is efficiently expressed in the viral context of systemic host infections as well as *C. quinoa* local lesions and poorly detected when translated *in vitro* from full length RNA3 or ectopically expressed in yeast or plant using Pol-II promoter driven expression vectors [30,31]. One could imagine a putative riboswitch motif present within the 450 nt-long 5' UTR of the RNA3 that allows such fine-tuned regulated p25 expression. Indeed, vectors producing 5' UTR truncated RNA3 allow the efficient p25 protein production [30,31]. Similarly, the 380 nt-long 5' UTR present on BNYVV RNA4 may as well contain such *cis* elements allowing a regulated p31 expression [30]. For these reasons a massive replication of ancillary RNAs is not correlated to the over expression of the encoded proteins but could be implicated in a mechanism that counteracts plant defense system. Increased amount of RNA3 and RNA4 could also lead to a saturation of the RNA silencing machinery protecting RNA1 and RNA2 from a degradation and consequently insuring the expression of the housekeeping genes. Such hypothesis should be corroborated by a quantitative analysis of BNYVV derived small interfering (si) RNAs during the infection expecting a prevalence of species derived from RNA3 and RNA4 rather than RNA1 and RNA2.

Quantification analyses of BNYVV genomic RNAs accumulation have been conducted choosing fixed BNYVV infection conditions: the inoculum consisted on identical concentrations agro-clones and sampling has been performed on symptomatic tissues likely representing late stages of viral infection. However, the initial conditions of the infection may not constitute a determinant variable for the calculation of the genomic formula since the relative frequency variations in the inoculum do not produce significant variation in the analyzed tissues for AMV and FBNSV segments [9,10]. Conversely, quantifications performed on symptomatic tissues could average different snapshots of viral cycles steps, each characterized by specific gene expression and, probably, by specific relative frequencies between genomic RNAs. To explore how genomic formula changes during the infection, kinetic experiments of virus production in transfected protoplasts will be carried out. This will allow the study of the progression of the GF along a viral cycle initiated in a homogenous population of single cells, performing the renowned "single burst experiment" [32].

Comparative analyses of total or encapsidated BNYVV RNAs relative frequencies revealed that a correlation does not always occur in infected tissue of *C. quinoa* and *S. oleracea*. Such behavior has also been reported for AMV in *N. benthamiana* and was explained as an existing competition between RNAs during the encapsidation process [10]. When we compare the BNYVV RNAs relative frequencies in leaves and roots of *B. macrocarpa* or *S. oleracea* we observe that even without a clear correlation between segment relative frequencies within total RNAs, encapsidated RNA species accumulate as a host specific manner conserved in both organs. We could therefore speculate that the equilibrium between the replicative and expressed RNAs and the vector-transmitted encapsidated RNA species is regulated independently of the total amount of viral RNA species.

If on the one hand BNYVV RNAs encapsidation drives the viral transmission from host to vector and vice versa, on the other hand, during the host colonization, its biological importance is reduced and limited to the stabilization of genomic RNAs and to the shutdown of viral gene expression [30]. Moreover, non encapsidated viral RNAs represent the active form of the virus that drive viral expression and move within the host [33]. Knowing how such non encapsidated RNAs constitute the moving genomic formula and how it is stabilized is essential to understand how the BNYVV and perhaps other multipartite plus-strand RNA viruses orchestrate viral infections. However, as no correlation between relative frequencies calculated in total and encapsidated RNA extractions was drawn, it appears difficult to determine the stoichiometric ratio between BNYVV non encapsidated RNAs. An optimization of the analysis is therefore required to distinguish between encapsidated and non encapsidated RNAs. One possibility would consist on the addition of purified virions of the BNYVV-closely related *beet soil borne mosaic virus* [21] in the homogenization TM buffer as a nuclease resistant reference genome. This will allow the normalization of total and encapsidated RNA extractions and then deduce the copy number of non encapsidated RNAs by a simple subtraction.

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Chapter II

BNYVV genome integrity within infected cells

1. Introduction: a link between viral movement and multipartitism

Described as obligate intracellular pathogens, viruses require, always and without any exception, to enter and replicate within an infected cell. The way viruses propagate from one cell to another seems to be dependent on the type of infected multicellular host and on the virus itself. Animal viruses spread by two distinct but not exclusive models (Figure 1), both requiring the capsid formation and expulsion from the cell after budding, exocytosis or cell lysis. Dissemination of viral particles in the extracellular space allows the long distance spread of the virus in the infected host and an easier transmission to new hosts [1]. Besides dissemination, cell to cell transmission takes place when viral particles are retained on the outside surface of the plasma membrane (PM) and their passage in new cells is promoted by existing or *de novo* formed cell contacts. Neurotropic viruses take advantage of the microtubule-mediated transport to spread from one neuron to another through synaptic connections [2]. Instead, retroviruses induce uninfected cells to form virological synapses or filopodial bridges allowing a stable link with the infected cells [3,4]. T cells that usually don't engage prolonged interaction to each other, could be physically connected by membrane nanotubes to allow *human immunodeficiency virus* type 1 (HIV-1) to be transferred from infected to uninfected cells in a receptor-dependent manner and with a cell surface movement [5]. However, in some rare cases the virus can move cell to cell within vesicles through cytoplasmic connections such as bridging conduits that allow HIV-1 trafficking between macrophages following the endoplasmic reticulum (ER) and Golgi network [6]. It has been proposed that *Influenza A virus* (IAV) uses similar intercellular connections to transport viral ribonucleoprotein complexes (vRNPs) that contain polymerase as infectious viral machinery rather than virions [7].

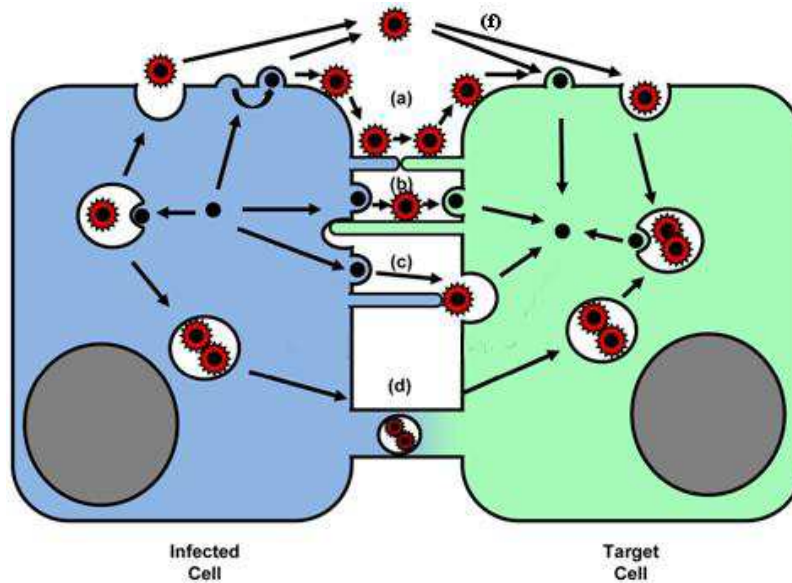


Figure 1 Schematic representation of viral movement in animal hosts requiring as prerequisite virion formation: virus can move as free particles released in extracellular matrix (f) or exploiting existing (a) or de novo formed intercellular communication for cell to cell transmission (b, c, d). Retrovirus can remain associated with the surface of the infected cell and spread thanks long filopodial bridges that connect target to donor cells (b); *Vaccinia virus* polymerizes actin to propel itself from infected to target cell [8] (c). Lastly, in some rare circumstance, animal viruses can induce the formation of cytoplasmic connections that are used as conduits for the transfer of virion enveloped by vesicles (d). Figure is adapted from Zhong et al. [1]

If animal viruses rarely promote cytoplasmic connection formations, it appears a prerequisite for phytoviruses. Indeed, the main morphological feature of plant tissues is the cell holding and delimitation within rigid and thick cell wall that renders the direct access to the PM surface impossible for macromolecules and viruses. Phytoviruses must be retained in the inner side of the PM (symplast) to be replicated and to spread cell to cell. Plants possess intercellular junctions, namely plasmodesmata (Pds) that directly interconnect the cytoplasms of adjacent cells. This provides a whole-body macromolecular transport network called symplastic continuity [9]. Functionally comparable to animal cell gap junction, the simplest form of Pd is composed essentially by membrane and spaces [10] that form a linear channel delimited by PM and containing an axial membranous tubule called desmotubule, derived from the ER union. The region between desmotubule and PM is called cytoplasmic sleeve and presents structurally organized cytoskeleton components, together with other structural and non-structural proteins required for trafficking functions [11]. Soluble environments (cytoplasmic sleeve and ER lumen) and membrane environments (PM and ER) are both exploited as paths for molecular trafficking. This trafficking is controlled by Pd size exclusion limit (SEL), which is regulated by the highly dynamic callose (β -1.3-glucan) deposition in the

cell wall. This callose deposition surrounds and constricts the neck region of the pore [11]. Aimed to defines the standard SEL, experimental studies demonstrate that GFP (26kDa), 2XGFP (52kDa) and, less efficiently, 3XGFP (78kDa) soluble proteins can diffuse through most tissues of the plants especially in early stage of embryogenesis [12]. However, with tissue development, branches and cavities formation modifies Pd permeabilities and creates symplastic domains with different types of connectivity between cells. This restricts GFP diffusion in younger sink tissues and makes the protein movement between vascular boundaries difficult [13]. To use Pd as gates for viral cell to cell movement within the symplast, phytoviruses have to increase SEL thanks the production of viral movement proteins (MPs) that adjust Pd structure to let virions or vRNP complexes transport, allowing the propagation of the infection [11]. This symplastic route is maintained even during the long-distance movement of macromolecules and therefore viral entities. Once gained the companion cells (CCs), viral entities are loaded in the sieve element (SE) conduits of source tissues, passively transported within the source-to-sink flow of photoassimilates and then unloaded into sink tissues [14].

With some rare exceptions (Bidensoviruses [15] and *Guaico Culex virus* infecting silkworm mosquitoes [16], respectively) multipartite viruses mostly infect plants and fungi. They represent about 30% - 40% of phytovirus genera and families [17]. Such viral strategy could be explained by the virus retention in the symplastic continuity, rather than virions expulsion and entry from one cell to another. The separated genomic components would then act as one unique genome segregated all along the interconnected cells. If such a situation exists, there is still no answer on the way it is accomplished. The results presented in this chapter suggest that for BNYVV, as it is modeled to date [18], an uncoordinated trafficking of viral genomic material through Pds does not guarantee the complete set of genomic segments in each infected cell. Indeed, the differential accumulation of each BNYVV RNA measured within the infected tissues drastically increases both the multiplicity of infection (MOI) and the biological cost, in terms of viral load, required to preserve BNYVV genomic integrity. This lead to the formulation of two opposed hypotheses illustrated in figure 2:

1. BNYVV segments may not necessarily be present “all together” in every infected cell. The RNA replication and stabilization are therefore ensured by the exchange of viral proteins through the symplastic continuity.

2. A specific mechanism guarantees the coordinated movement of each segment as infective collective units.

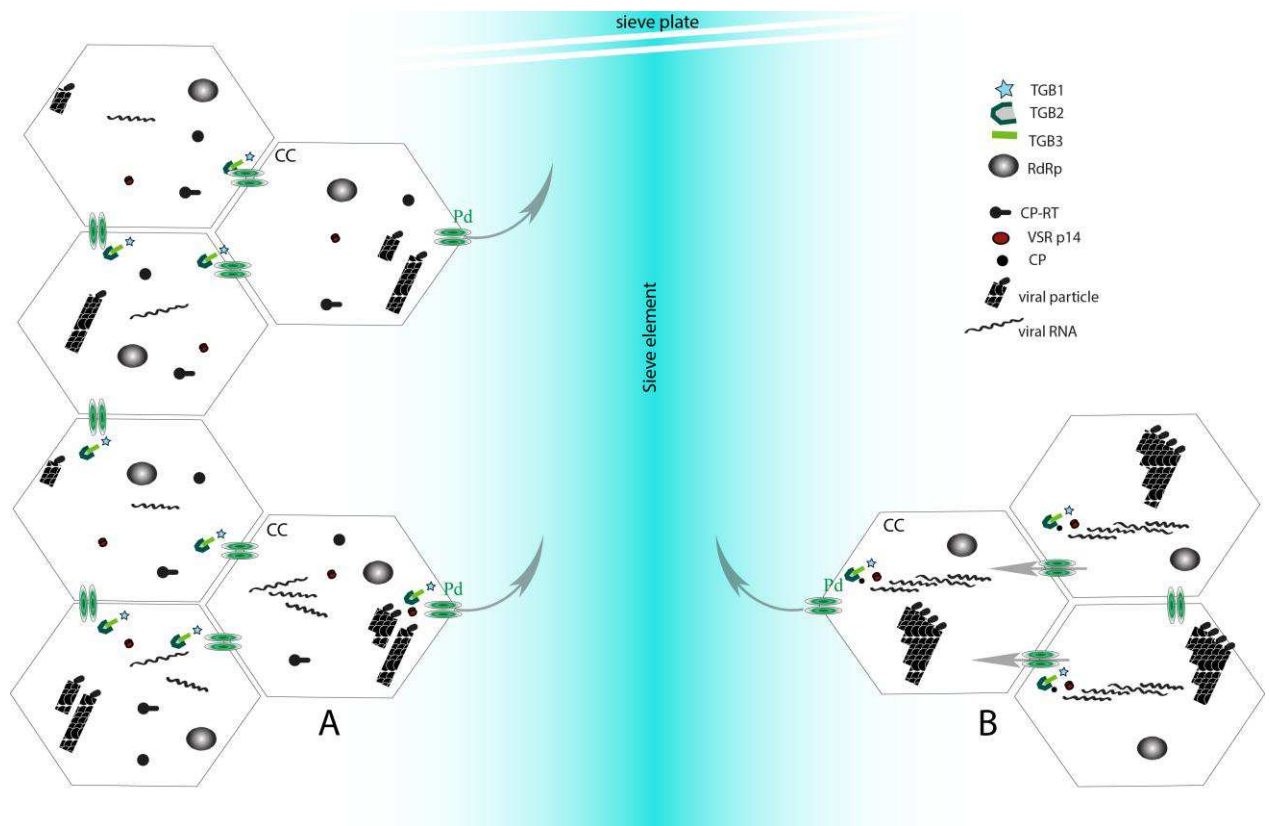


Figure 2 Simplified representation of the two hypotheses formulated for BNYVV genomic preservation during plant colonization. In panel A genomic segments, as particles or free RNAs, are distributed randomly in neighbored cells. Replication and stabilization of the four vRNAs are ensured by the free symplastic trafficking of house-keeping viral factors such as replicase (RdRp), coat protein (CP), coat protein-readthrough (CP-RT), viral suppressor of RNA silencing (VSR) and TGB proteins involved in the SEL increasing and in the free diffusion of high molecular weight proteins through plasmodesmata (Pd). In this model Sieve elements (Blue shaded) are considered as reservoir of viral RNAs and particles stochastically loaded from companion cells (CC). In panel B collective units keep together the different genomic elements and permit their co-presence in each infected cell and their coordinated movement through Pds and Sieve elements. Collective mobile units are here represented as both vRNP complexes and particle aggregates. Figure is adapted from Dall'Ara et al. [19]

As it has been demonstrated performing leaf rub-inoculation of GFP tagged viruses in permissive host, BNYVV is able to move cell to cell and long distance infecting all plant tissues, demonstrating an extensive tissue tropism [20]. For this reason, to gain experimental data to favor one or the other hypothesis, I choose to characterize the viral content of individual cells that have been infected after long distance movement of BNYVV. To perform such analyses, I isolated cells from systemically infected tissues and searched for the presence of viral RNA species. Therefore, I had to develop a multiplex RT-qPCR protocol on single

protoplast isolated from mesophyll of systemically infected *Spinacia oleracea* with the aim to test the co-presence of the four genomic BNYVV RNAs within each infected cell.

2. Preparation of *S. oleracea* protoplast and fixation

In the present work, the optimized protocol for individual protoplast isolation and selection from mesophyll BNYVV infected *S. oleracea* leaves does not require specialized expertise or instrumentation and reduces vRNA cross contamination. Indeed, viral titer per cell can be in general extremely high [21] and cross contamination of genomic material between infected cells is an eventuality that has to be taken into consideration to avoid the generation of false positives by RT-qPCR.

Young spinach seedlings (cv. Viking), with two true leaves, were infected by sap mechanical inoculation, using, as source of inoculum, frozen leaf tissues of systemically BNYVV-infected *S. oleracea* plants. Inoculated plants were grown in the green house at 24/20°C and 16/8h (days/night) and 4 weeks later an equal amount of young leaves from symptomatic (infected) and healthy (non inoculated plants), were mixed together for protoplast preparation. Four grams of sterilized and scarified leaf lamina were treated to isolate protoplasts according to Veidt et al. protocol [22] revised in the maceration step. In particular, after overnight maceration at 24°C under dark condition, in a medium (pH 5.6) containing Mannitol 0.6M, MES 5mM, CaCl₂ 10mM, Macerozyme R10 0.4% and Cellulase R10 0.7%, protoplasts were collected by two passages of low centrifugation (88 RCF), suspended in MMC buffer (Mannitol 0.6M, MES 5mM, CaCl₂ 10mM, pH 5.6) and adjusted to a concentration of 1 cell/10µl before 15 min incubation step in fixing solution (MMC buffer, glutaraldehyde 0.5%). After two washing steps, protoplasts were decontaminated from viral particles or RNAs retained on the cell external surface by 10 min incubation in MMC buffer, containing Trypsin 0.1%, RNase A 0.025% and MgCl₂ 50mM. Trypsin and RNase A were then removed from the cell suspension by three washing steps and protoplast concentration was adjusted to 1 cell/10µl in MMC.

3. Individual protoplast isolation and triplex RT-qPCR

Single protoplast isolations for the RT-qPCR based analysis was performed by free hand pipetting under light microscope: 100 µl of protoplast suspension was placed on glass slides

and, under magnification of 40X, individual cells were collected by pipetting volumes of 4µl. Sampling accuracy was increased by the use of acrylamide gel tips (ART 20 GEL, Barrier Tip) and by controlling the protoplast shape, under 80X microscope magnification, after selection and loading in new glass slides. Selected and checked protoplasts or MMC buffer depleted from protoplast (as negative control) were then loaded in PCR tube and used as template for a triplex RT-qPCR reaction targeting two different BNYVV RNAs and Rubisco Large subunit (RbcL). In each tube 16µl of the following RT-qPCR mixture were added:

- 10µl Promega GoTaq® Probe qPCR Master Mix (2X)
- 0.5µl Promega M-MLV RT (4 unit/µl)
- 0.5µl RNA1 or RNA3 Forward primer (20µM)
- 0.5µl RNA1 or RNA3 Reverse primer (20µM)
- 0.5µl RNA1 or RNA3 FAM probe (4µM)
- 0.5µl RNA2 or RNA4 Forward primer (20µM)
- 0.5µl RNA2 or RNA4 Reverse primer (20µM)
- 0.5µl RNA2 or RNA4 HEX probe (4µM)
- 0.5µl RbcL Forward primer (20µM)
- 0.5µl RbcL Reverse primer (20µM)
- 0.5µl TWEEN 20 (8%)

Primers and probes sequences as well as amplicon sizes are given in table 4 (annexes).

Tubes were vigorously vortexed and briefly centrifuged before RT-qPCR reactions described in Table 1.

Step	Cycles	Temperature	Time
Reverse transcription	1	42° C	30 min
Polymerase activation	1	90° C	10 min
Denaturation	45	90° C	30 s
Annealing/Extension		60° C	1 min

Table 1 RT-PCR cycling parameters

The RT-qPCR of BNYVV RNAs was followed in Real-Time using TaqMan probes with different fluorophores (FAM and HEX) while the presence of the endogenous control RbcL was confirmed after amplification (end-point) in agarose gel (Figure 3). Four different reactions were performed to detect the presence of RNA1 with RNA2 (RNA1/RNA2), RNA2

with RNA3 (RNA2/RNA3), RNA1 with RNA4 (RNA1/RNA4) and RNA3 with RNA4 (RNA3/RNA4) (Figure 3). To discriminate the background from real amplification signals, for each reaction, threshold lines of different targets were set manually above the base line derived from the delta Rn against PCR cycle plot. Basically, two population of curves occurred in each reaction distinguishing positive from negative samples. To produce a reference pattern for collected protoplasts analyses, 500 ng of total RNAs extracted from *S. oleracea* infected tissues (qualitative positive control) was used (Figure 3). Fourteen up to sixteen protoplasts have been tested for each of the four reactions described above and the presence of each RNA target has been evaluated using Ct values as qualitative data (Table 2 and 3). The percentages of BNYVV positive protoplasts were ~ 50% for RNA1/RNA2 and for RNA2/RNA3, ~31% for RNA1/RNA4 and 42% for RNA3/RNA4 reactions. Such results are in agreement with the initial mixing of infected and not infected tissues during the protoplast preparation. Furthermore, infected protoplasts (determined by RT-qPCR analyses) have been associated with a co-amplification of the analyzed BNYVV RNAs with an exception for RNA4 that has been sometimes found without its co-analyzed genomic RNAs. Since RNA1 is always co-detected with RNA2 and RNA2 is co-detected with RNA3, I determined that RNA4 has been found alone with an incidence of 20% (Table 2 samples 42 & 52). For all co-amplification, the endogenous control RbcL was confirmed and no amplification was observed in the absence of cell.

Sample	Detector	Target	Ct	Sample	Detector	Target	Ct
1	FAM	RNA1	Undet.	17	FAM	RNA3	30.61
	HEX	RNA2	Undet.		HEX	RNA2	31.72
2	FAM	RNA1	Undet.	18	FAM	RNA3	36.88
	HEX	RNA2	Undet.		HEX	RNA2	42.83
3	FAM	RNA1	Undet.	19	FAM	RNA3	Undet.
	HEX	RNA2	Undet.		HEX	RNA2	Undet.
4	FAM	RNA1	Undet.	20	FAM	RNA3	Undet.
	HEX	RNA2	Undet.		HEX	RNA2	Undet.
5	FAM	RNA1	30.9	21	FAM	RNA3	Undet.
	HEX	RNA2	32.28		HEX	RNA2	Undet.
6	FAM	RNA1	36.06	22	FAM	RNA3	Undet.
	HEX	RNA2	42.62		HEX	RNA2	Undet.
7	FAM	RNA1	30.88	23	FAM	RNA3	Undet.
	HEX	RNA2	33.02		HEX	RNA2	Undet.
8	FAM	RNA1	33.87	24	FAM	RNA3	Undet.
	HEX	RNA2	38.16		HEX	RNA2	Undet.
9	FAM	RNA1	31.49	25	FAM	RNA3	31.22
	HEX	RNA2	34.01		HEX	RNA2	31.74
10	FAM	RNA1	Undet.	26	FAM	RNA3	31.00
	HEX	RNA2	Undet.		HEX	RNA2	33.92
11	FAM	RNA1	32.00	27	FAM	RNA3	37.73
	HEX	RNA2	33.09		HEX	RNA2	43.6
12	FAM	RNA1	Undet.	28	FAM	RNA3	29.55
	HEX	RNA2	Undet.		HEX	RNA2	31.04
13	FAM	RNA1	35.04	29	FAM	RNA3	32.83
	HEX	RNA2	39.35		HEX	RNA2	35.4
14	FAM	RNA1	Undet.	30	FAM	RNA3	Undet.
	HEX	RNA2	Undet.		HEX	RNA2	Undet.
15	FAM	RNA1	33.48	31	FAM	RNA3	Undet.
	HEX	RNA2	36.5		HEX	RNA2	Undet.
16	FAM	RNA1	Undet.	32	FAM	RNA3	32.08
	HEX	RNA2	Undet.		HEX	RNA2	34.2

Table 2 Real time Cts for RNA1/RNA2 and RNA2/RNA3 reactions

Sample	Detector	Target	Ct	Sample	Detector	Target	Ct
33	FAM	RNA1	Undet.	49	FAM	RNA3	38.47
	HEX	RNA4	Undet.		HEX	RNA4	31.89
34	FAM	RNA1	33.21	50	FAM	RNA3	Undet.
	HEX	RNA4	33.74		HEX	RNA4	Undet.
35	FAM	RNA1	30.76	51	FAM	RNA3	Undet.
	HEX	RNA4	30.39		HEX	RNA4	Undet.
36	FAM	RNA1	Undet.	52	FAM	RNA3	Undet.
	HEX	RNA4	Undet.		HEX	RNA4	42.16
37	FAM	RNA1	Undet.	53	FAM	RNA3	Undet.
	HEX	RNA4	Undet.		HEX	RNA4	Undet.
38	FAM	RNA1	Undet.	54	FAM	RNA3	Undet.
	HEX	RNA4	Undet.		HEX	RNA4	Undet.
39	FAM	RNA1	Undet.	55	FAM	RNA3	34.78
	HEX	RNA4	Undet.		HEX	RNA4	34.04
40	FAM	RNA1	41.13	56	FAM	RNA3	Undet.
	HEX	RNA4	38.53		HEX	RNA4	Undet.
41	FAM	RNA1	Undet.	57	FAM	RNA3	36.49
	HEX	RNA4	Undet.		HEX	RNA4	38.15
42	FAM	RNA1	Undet.	58	FAM	RNA3	Undet.
	HEX	RNA4	35.14		HEX	RNA4	Undet.
43	FAM	RNA1	Undet.	59	FAM	RNA3	Undet.
	HEX	RNA4	Undet.		HEX	RNA4	Undet.
44	FAM	RNA1	40.87	60	FAM	RNA3	29.68
	HEX	RNA4	37.32		HEX	RNA4	30.69
45	FAM	RNA1	Undet.				
	HEX	RNA4	Undet.				
46	FAM	RNA1	Undet.				
	HEX	RNA4	Undet.				
47	FAM	RNA1	Undet.				
	HEX	RNA4	Undet.				
48	FAM	RNA1	Undet.				
	HEX	RNA4	Undet.				

Table 3 Real time Cts for RNA1/RNA4 and RNA3/RNA4 reactions

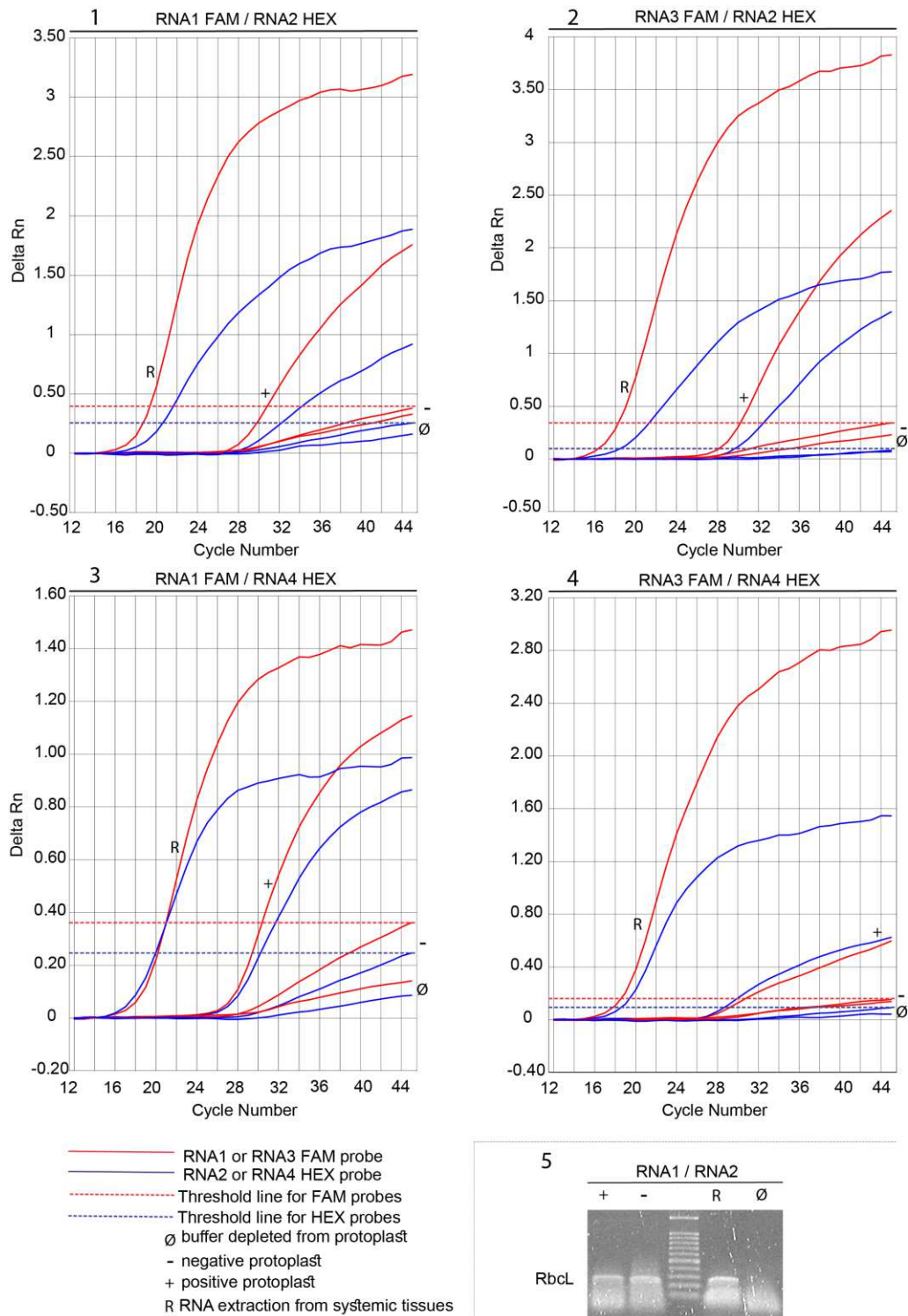


Figure 3 Profile examples of the co-detection of genomic BNYVV RNA pairs in isolated protoplasts. Real-Time PCR plots (1, 2, 3, 4) represent target amplification in the four different reactions: co-amplification of RNA1/RNA2 (1), RNA2/RNA3 (2), RNA1/RNA4 (3) and RNA3/RNA4 (4). The amplification issued from positive control is present in each plot (R), positive protoplast (+), negative protoplast (-) and of MMC buffer depleted from protoplast (Ø) (negative control). Dashed lines set above negative control signals indicate threshold for FAM and HEX signals. End-point reactions are loaded in agarose gel to verify RT-PCR amplification of RbclL endogenous control (5).

4. Discussion

S. oleracea is a permissive host that allows BNYVV systemic infection even in the absence of RNA3 and RNA4 [23]. The obtained results demonstrate that the virus maintains its genomic integrity in each infected cell even if the dispensability of ancillary RNAs could lead to the conclusion that the virus is prone to lose one of its unnecessary RNAs during its spread from cell to cell in the host. On the one hand RNA1 and RNA2 are intrinsically coupled, since the expression of the TGB and VSR proteins depend on the production of subgenomic RNA2 by the RNA1-encoded replicase; on the other hand RNA3 and RNA4 can be lost in systemic tissues where they appear unessential, as described for BNYVV laboratory isolates on *Chenopodium quinoa*, *Tetragonia expansa* and *Nicotiana benthamiana* [24–26]. However, this was mainly explained as an effect of deletions compromising the RNA abilities to be replicated rather than spontaneous segregation of defective BNYVV genomes. From the results obtained in this study, we can conclude that in every RNA1 and RNA2 infected cells both RNA3 and RNA4 are present. This confirms the necessity for BNYVV to lower the MOI deriving from its genomic formula favoring cell entry of the genomic segments as collective infective units. In such scenario, the “significant” incidence of RNA4 segregating alone can be interpreted as a result of its relative abundance. In leaves of *S. oleracea*, RNA4 was found with a relative frequency average value of 0.65 (Chapter 1) meaning that its spread from the infected cell appears more stochastic than regulated. However, in roots of the same analyzed plants, relative frequency value lowers of 0.37 (approximately), suggesting the requirement of a coordinated movement for this abundant RNA4 species.

Moreover, the particular biology of BNYVV reinforces the hypothesis of having the full set of genomic RNAs in each infected cell. Indeed, to naturally extend the infection in new hosts the virus is transmitted by the soil-borne protozoa vector *Polymyxa betae*: avirulifer zoospores transfer their cytoplasmic content into the rootlets infected cells. The multinucleate sporangial plasmodium form matures into zoosporangium and eventually in sporosori [27]. Cytoplasmic sharing between infected host and spore occurs at the level of a single infected cell that consequently must possess all genomic set of particles.

If on the one hand BNYVV genomic integrity within infected cells suggests the formation of mobile collective infective units, on the other hand further experiments are required to

directly demonstrate their existence. Sieve elements are plant conduits where viruses rapidly move long distance but do not replicate. For this reason vascular elements should be particularly enriched with mobile BNYVV entities. A non-invasive extraction from such tissues could provide the material for mobile BNYVV entity characterization. Nanofluidic technology as digital droplets (dd)RT-PCR can be used to demonstrate the existence of viral entities that transport the complete set of BNYVV genomic RNAs. Similarly to a Real-Time multiplex reaction on single protoplasts, the phloem exudate partitioning in 1 nl droplets allows to link the co-detection of different genomic targets in the same reaction event to their association in a molecular complex. Furthermore, comparing results obtained from protocols of one-step and two-step ddRT-PCR reaction it will be possible to calculate the genomic formula characterizing such complexes (Figure 4).

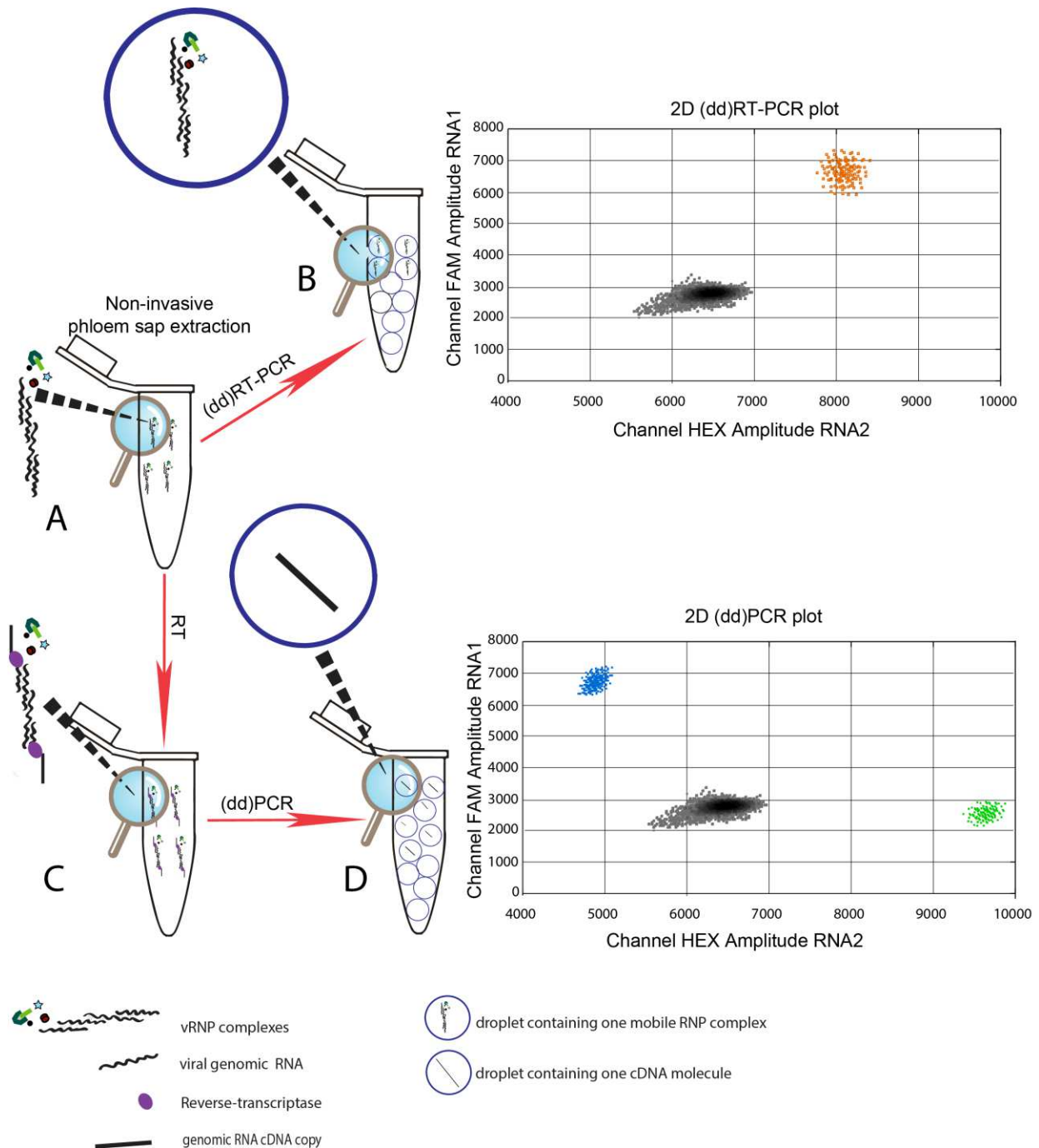


Figure 4 Simplified representation of the work flow to demonstrate the existence of viral entities that transport the complete set of BNYVV genomic RNAs. From phloem sap extraction enriched of BNYVV mobile collective units (A), here represent as vRNP complexes, both ddRT-PCR one step (B) and duplex dual step (C & D) will be performed. With ddRT-PCR one step, after partitioning, vRNP complexes are singularly segregated in droplets (B). As result of the end-point amplification, represented in the two dimension HEX and FAM amplitude plot, each positive droplet will have signals deriving from both probes (here RNA1-FAM and RNA2-HEX). Contrarily, with a step of reverse-transcription prior to partitioning (C), single cDNA copies of genomic RNAs will segregate in droplets (D). If phloem sap extraction was diluted properly, as result of ddPCR amplification FAM and HEX signals will not merge. The copy number of the two RNAs calculated in dual step ddRT-PCR divided by the number of co-amplifications in one step ddRT-PCR will give the genomic RNAs composition and ratio of the BNYVV mobile infective units.

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5. Annexes

Primers	Taq-man TAMRA probes	Amplicon length
BNYVV1 F(TGGTTTCACAAGGAGATGTCGTT) BNYVV1 R (TCTGCACAATCAAAGGCATCA)	FAM-TTTTGGACATAGCACGTGTGGAAAACGATA	78
BNYVV2 F(TGGACCCGGGATAAATTTGA) BNYVV2 R(CGGGTGGACTGGTTCTACCTT)	HEX-ACCGGTTCAAATTACCATGGACACCTGTT	72
BNYVV3 F(ATATGTGAGGACGCTAGCCTGTT) BNYVV3 R(TGAAACGATGGAGTCACTATGCTT)	FAM-CTGACCGACCAAATCCAAGCGAGCTTAAT	118
BNYVV4 F(TCCTCCTTTGATACGTCATGAAGA) BNYVV4 4R(CAATGGGCCAATCTCAATCC)	HEX-TGATTGTACTGCTAGGATGGTGCA	75
Rbcl F(GGTAACGTATTTGGGTTCAAAGC) Rbcl R(CATCTTTGGTAAAATCAAGTCCACC)		244

Table 4 Primers, probes and relative amplicons used for triplex RT-qPCR

Chapter III

A specific heterologous RNA/RNA
interaction is involved in the BNYVV
infectivity

1. Introduction: regulated assembly of collective infectious units

Maintenance of genome integrity to prevent loss of genetic information is a vital requisite for viruses as well as for any kind of organism. If genomic corruption by recombination events, inaccurate replication, attack by nucleases or other host modifying enzymes [1] are excluded, monopartite viruses fulfill easily the “one genome one infectious unit” paradigm [2] since their genome is constituted by one unique codifying unit. Conversely, segmented and multipartite viruses have to develop strategies to bring the different genomic entities together in collective units especially during the transmission from one cell to another within the same host or between hosts [3]. Such strategies are well described for segmented virus and are finalized to assure in each mature virion the complete set of genomic segment. The tripartite dsRNA genome of phage $\phi 6$ is packaged through a NTP dependent mechanism involving the controlled and sequential recognition of Small (S), Medium (M) and Large (L) different plus strand transcripts by preformed dodecahedral pro-capsid particle or inner core [4,5] (Figure 1). The inner core recognizes each different segments from their specific 5' end packaging domains. Furthermore, sequential recognition depends on conformational changes that take place whenever previous type of ssRNA has been packaged [4,5]. After packaging of the last (L) ssRNA, the inner core changes its conformation to allow minus strands syntheses [4,5]. From dodecahedral, the particles become spherical [4,5]. Specific recognition between viral proteins and genomic RNAs is not the sole determinant for packaging specificity of segmented virus. Recent studies demonstrate that for some segmented animal virus, belonging to *Reoviridae* and *Orthomyxoviridae* family, the specific and coordinated segment packaging is determined by RNA/RNA interaction networks [6–8]. Such model was deeply investigated for Influenza A virus (IAV) together with its important genetic reassortment processes [9]. IAV genome consists of eight single stranded negative sense RNAs (vRNAs) organized in individual ribonucleoprotein complexes (vRNPs) that show a conserved tridimensional supramolecular organization inside the viral particle. This organization is characterized by the disposition of seven vRNPs around one central vRNP corresponding to vRNA1 (7+1 pattern) [7,10]. This supramolecular complex formation depends on a selective packaging mechanism implying direct RNA/RNA interactions [9]. The resulted network of interactions between viral RNAs appears to be strain specific and plays an important function in the regulation of

genomic reassortment events. Only IAV strains with a compatible network of interaction can generate reassortant progenies [9] (Figure 2).

Genomic RNAs of multipartite viruses do not co-package in one mature virion but are segregated in different and independent particles [3]. Such aspect discriminate segmented and multipartite viruses that should have evolve alternative strategies of selective co-packaging in order to produce collective infectious units able to preserve genome integrity during the host colonization. The multipartite *Beet necrotic yellow vein virus* (BNYVV) spreads in the plant as RNP complexes that require TGB proteins for cell to cell movement and CP for its systemic infection [3,11,12]. Segment encapsidation appears dispensable for the long distance movement of the virus [3,13]. No BNYVV protein has been identified as able to specifically bind and discriminate one genomic RNA from another [14]. We expected BNYVV collective infection unit to depend on network of RNA/RNA interaction that could insure the presence of at least one of each genomic RNAs [3]. This hypothesis recalls the demonstration of RNA-RNA interactions required for IAV segments packaging. My objective was to experimentally determine the existence of such RNA/RNA interactions between genomic RNA1 and RNA2, the minimal infectious unit of BNYVV. Indeed, within certain hosts, RNA1 and RNA2 are sufficient for the systemic infection of the plant. Thus, the minimal efficient RNP complex should involve RNA1 and RNA2 interaction. For this reason, the existence of specific heterologous interactions between these two RNAs has been evaluated as well as the implication for such interactions in the BNYVV viral cycle.

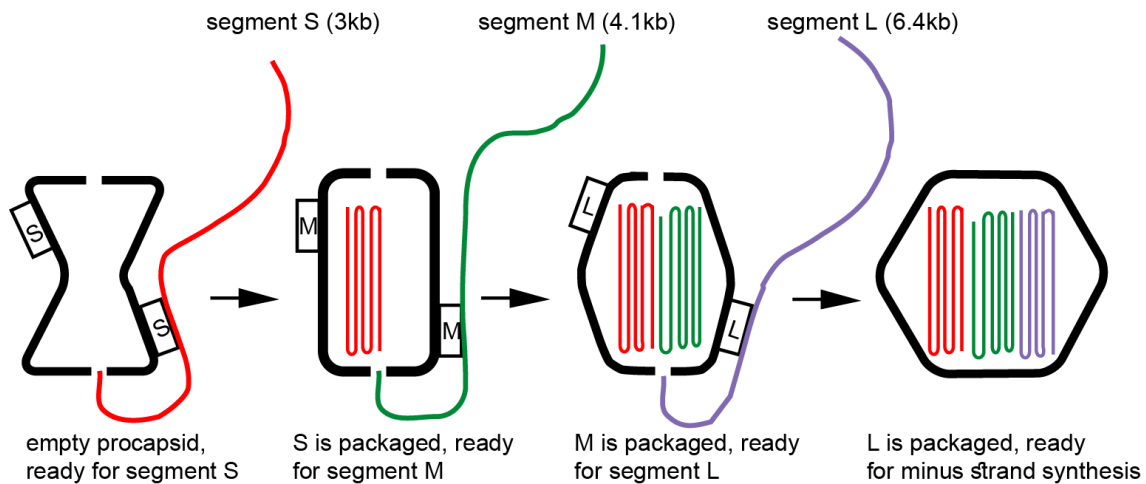


Figure 1. Packaging model proposed for the $\phi 6$ bacteriophage. The inner core interacts specifically with S segment. After S packaging, a M binding site appears on the inner core and similarly, after M packaging L binding site allows the entry of the largest segment When all three segment are packaged minus strand synthesis starts. (modified from Mindich [4]). This model could fit with a specific S entry bringing M segment and then L within the capsid thanks to RNA/RNA interactions.

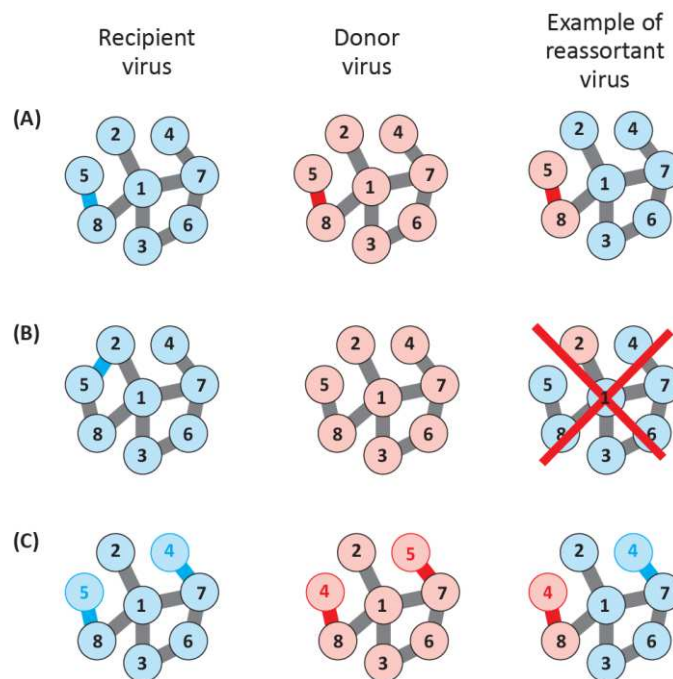


Figure 2 Model of IAV RNA/RNA interactions and its implications on genetic reassortment. (A) example of compatible exchanges of vRNAs: vRNA5 and vRNA8 interact through different (incompatible) sequences (blue and red lines) in the two parental viruses while vRNA1 and vRNA8 interaction is compatible. The two parental viruses have the same RNA/RNA network of interaction. Co-segregation of vRNA5 and vRNA8 from donor virus to recipient virus is possible and results in a new functional reassortant virus. (B) vRNA2 and vRNA5 interaction exists in the recipient virus but not in the donor virus. This might prevent the segregation vRNA2 from donor to recipient virus. (C) In recipient virus vRNA8 interacts with vRNA1 and with vRNA5 while in donor virus vRNA8 interacts with vRNA1 and vRNA4. Segregation of vRNA8 together with vRNA4 from donor virus to recipient virus is possible but lead to the formation of a defective virus for vRNA5 (modified from Gerber et al. [9]).

2. Material and methods

2.1 Plasmids for Electro Mobility Shift Assay (EMSA)

RNA1b, RNA1c, RNA1d, RNA1e and RNA2d partial cDNA clones (Table 1) have been obtained by cloning of PCR products, integrating T7 RNA polymerase promoter, into pUC19-based vectors. PCR reactions have been conducted using Phusion High-Fidelity PCR Master Mix (Thermo Scientific), BNYVV (B type) RNA1 (pB15) and RNA2 (pB214) full length cDNA clones [12] as templates and DNA oligonucleotide (ODN) primers described in Table 2. Amplicons have been cloned into pUC19 using *EcoRI* and *HindIII* restriction sites, after digestion and ligation by, respectively, FastDigest restriction and T4 DNA Ligase enzymes (Thermo Scientific). Recombinant plasmids have been linearized by *HindIII* restriction enzyme before *in vitro* transcription. All enzymatic reactions have been carried out according to manufacturer's protocol.

Partial clones	First nucleotide	Last nucleotide	Restriction site for linearization
RNA1a	1	1424	<i>AgeI</i>
RNA1b	1367	2455	<i>HindIII</i>
RNA1c	2455	3801	<i>HindIII</i>
RNA1d	3655	4748	<i>HindIII</i>
RNA1e	4748	6746	<i>HindIII</i>
RNA2a	1	2715	<i>SalI</i>
RNA2b	1	1113	<i>MluI</i>
RNA2c	1	668	<i>NcoI</i>
RNA2d	1155	2789	<i>HindIII</i>
RNA2e	1155	2065	<i>PstI</i>

Table 1 List of partial cDNA clones used to produce *in vitro* transcripts for EMSA. The first and the last nucleotide of BNYVV sequence produced is indicated for each clone as well as the restriction enzyme used for linearization before run-off transcription.

RNA1a, RNA2b, RNA2c and RNA2e clones derives from previously reported plasmids whose sequences, downstream T7 promoter, have been shortened by linearization using alternative restriction enzymes. In particular, RNA1a has been produced from pB15 linearized with *AgeI*. RNA2a, RNA2b and RNA2c have been produced using the partial cDNA clone pB218 [15] linearized with *SalI*, *MluI* and *NcoI* restriction enzymes, respectively. RNA2e derives from RNA2d. *PstI* plasmid linearization has been followed by an exonuclease digestion of 3' end protrusion by T4 DNA polymerase (Thermo Scientific). All enzymatic reactions were carried out according manufacturer's protocol.

Clone	Primer pair	Sequence
RNA1b	EcoRI T7 RNA1 1367 F	TT GAATTC TAATACGACTCACTATAGGG ₁₃₆₇ GTGAAG ATGTTGGTTAC
	HindIII RNA1 2566 R	AA AAGCTT ₂₅₆₆ CCGCCCCGCACTCTAC
RNA1c	EcoRI T7 RNA1 2455 F	TT GAATTC TAATACGACTCACTATAGGG ₂₄₅₅ CCTGCTGACACTTTAC
	HindIII RNA1 3801 R	AA AAGCTT ₃₈₀₁ CCCAAGGTACATCCCAAC
RNA1d	EcoRI T7 RNA1 3655 F	TT GAATTC TAATACGACTCACTATAGGG ₃₆₅₅ CTGGTAGCTTTATCTCG
	HindIII RNA1 4783 R	TT AAGCTT ₄₇₈₃ CAGTCTTCCCCCTGCC
RNA1e	EcoRI T7 RNA1 4748 F	TT GAATTC TAATACGACTCACTATAGGG ₄₇₄₈ ACGATGATTTTGTG
	HindIII RNA1 6746 R	GCC AAGCTT TTTTTTTTTTTTTTTTTT ₆₇₄₆ ATATCAATATACTGA
Rna2d	EcoRI T7 RNA2 1155 F	TT GAATTC TAATACGACTCACTATAGGG ₁₁₅₅ TGTTAGTTTGGGAACC
	HindIII RNA2 2789 R	TT AAGCTT ₂₇₈₉ TAACCCCTTGCGCCGATC

Table 2 Primers used for RNA1 and RNA2 partial cDNA cloning into pUC19

2.2 Native or partially denaturing EMSA of *in vitro* co-transcribed transcripts

Couples of linearized plasmid templates were co-transcribed *in vitro* at 37°C for 3 h, using RiboMAX™ Large Scale RNA Production System-T7 (Promega) according to the following protocol:

- 0.5 µl linearized plasmid 1 (250 ng)
- 0.5 µl linearized plasmid 2 (250 ng)
- 2 µl Buffer 5X
- 3 µl rNTP (25 mM each)
- 0.25 µl RNase inhibitor (40U/µl) (RNasin Promega)
- 0.5 µl DTT (100 mM)
- 1 µl T7 RNA polymerase (20U/µl)
- 2.25 µl DNase/RNase free water

DNA templates were then removed adding 1µl of RQ1 RNase-Free DNase (1U/µl) (Promega) and incubating the reaction for 30 min at 37°C. Two µL of blue loading solution (50% glycerol, bromophenol blue) were added to the transcription reaction products prior analysis on 0.8% native (TBM: Tris 25 mM; boric acid 22.25 mM; MgCl₂ 50 µM; Ethidium bromide 0.5 µg/ml) or partially denaturing Mg²⁺-chelating (TBE: Tris 45 mM; boric acid 45mM; EDTA 1.25 mM; Ethidium bromide 0.5 µg/ml) agarose gel electrophoresis conducted at 150 V for 4 h at 4°C. After imaging the gel under UV exposure, RNAs were transferred from agarose gels to Hybond-NX membranes overnight by capillarity in 20X SSC buffer. RNAs were UV cross-linked and membranes were pre-hybridized at 60°C for at least one hour in Perfect Hyb Plus™ hybridization buffer (Sigma-Aldrich). Hybridizations with ³²P labeled

ODNs were carried out overnight at 42°C. Membranes were washed for 15 min at 50°C in 2XSSC 2% SDS buffer. Transfer of the γ -phosphate from ^{32}P γ labeled ATP to the 5'-OH of ODN probes (10-30 pmol) (Table 3) was performed by T4 Polynucleotide Kinase according to manufacturer's protocol (Thermo Scientific). Unincorporated radiolabeled ATP was removed by gel filtration using G-25 Sepharose columns. Radiolabeled RNAs and related band-shifts were detected by autoradiography.

RNA	ODN	Sequence
RNA1	3739 R	³⁷³⁹ TGCCTTTCAAAACAGCACC
RNA2	334 R	³³⁴ GTCAGTCCAACCCGAGCCC
RNA2	B3	¹⁸⁸ TGTGACATATCCTTCCATGT
RNA2	F3	⁶⁴⁶ ATTATCCCGGGTCCACATT

Table 3 Complementary ODNs used to probe RNA1 and RNA2 partial clones in EMSAs

2.3 Site directed mutagenesis of BNYVV full length cDNA clones

Site directed mutagenesis of full length RNA1 and RNA2 cDNA clones have been carried out using primer listed in table 4 using the one step quick change strategy described by Liu et al.[16].

RNA	Primer pair	Sequence
RNA1	Mut RNA1 F	⁴³⁵³ GTCCGGT <u>GACA</u> CAAGTAAGAAACC
	Mut RNA1 R	⁴³⁷⁶ GGTTTCTTACT <u>GTTGTC</u> ACCGGAC
RNA2	Mut RNA2 F	GG ₁ AAATTCTAACT <u>GTTGTC</u> ACCATTG
	Mut RNA2 R	³⁸ GGTGAAATTCTATTCAATGGT <u>GACA</u> CAAGT

Table 4 ODNs used for site directed mutagenesis of RNA1 and RNA2 cDNA clones. Point mutations are underlined.

Briefly, PCR reactions have been conducted using Phusion High-Fidelity PCR Master Mix (Thermo Scientific) and pB15 or pB214 plasmids as templates. *In vivo* methylated templates were removed by *DpnI* digestion (FastDigest Thermo Scientific) and amplicons were purified by phenol/chloroform extraction and NaCl/EtOH precipitation. All enzymatic reactions have been carried out according to manufacturer's protocol. Purified PCR products have been used to transform Sure2 (RNA1 mut) and MC1022 (RNA2 mut) (Stratagene) electro-competent *Escherichia coli* cells.

2.4 Construction of RNA5 derived replicons carrying RNA1 or RNA2 interaction domains

RNA5 derived replicons (Rep5) [17] carrying RNA1 and RNA2 interaction domains in their natural context (Rep5 RNA1 I.D. and Rep5 RNA2 I.D.), have been used to conduct *in vivo*

experiments with the aim to disrupt RNA1/RNA2 interactions. The first 400 nts of pB214 or the mutagenized clone as well as the RNA1 interaction domain (present between nts 4,143 and 4,520 of pB15) and the mutagenized version have been amplified by PCR using Phusion High-Fidelity PCR Master Mix (Thermo Scientific) and primers, flanked with *Bam*HI (Table 5). *Bam*HI-digested amplicons were introduced into *Bgl*II linearized Rep5-cDNA clone and ligated using their compatible cohesive ends. After transformation, insert orientation was verified by PCR. Prior to *in vitro* transcription, replicons Rep5 RNA1 I.D, Rep5 RNA1 I.D. mut, Rep5 RNA2 I.D and Rep5 RNA2 I.D. mut were linearized by *Hind* III digestion.

RNA	ODN	Sequence
RNA1	RNA1 <i>Bam</i> HI 4143 F	AA GGATCC ₄₁₄₃ GTCTCCATCAACTTGG
RNA1	RNA1 <i>Bam</i> HI 4520 R	AA GGATCC ₄₅₂₀ CACGATTGCAAGCTC
RNA2	RNA2 <i>Bam</i> HI I 1 F	AA GGATCC ₁ AAATTCTAACTATTATCTCC
RNA2	RNA2 <i>Bam</i> HI 1mut F	AA GGATCC ₁ AAATTCTAACT <u>TTGTC</u> ACC
RNA2	RNA2 <i>Bam</i> HI 400 R	AA GGATCC ₄₀₀ ACACAAGTGACCATAC

Table 5 ODNs used for the obtention of RNA5-derived cDNA replicons carrying RNA1 or RNA2 interaction domains. RNA2 point mutations are underlined

2.5 *In vitro* transcription, infection of *Chenopodium quinoa* protoplasts or plants and Northern blotting (high molecular weight RNAs)

Linearized full-length Benyviridae clones pB15, pB214, RNA1 mut, RNA2 mut, *Beet soil-borne mosaic virus* (BSBMV) RNA2 [18] as well as linearized Rep5 replicons carrying wild type or mutated BNYVV RNA1 or RNA2 interaction domains, served for *in vitro* run-off transcription as previously described [12,17,18]. Electroporation of *C. quinoa* protoplasts and mechanical infection of *C. quinoa* plants have been conducted as previously described [19,20]. Viral RNAs have been extracted using “polysome buffer” protocol [21] while Northern blot RNA analyses have been conducted as previously described [11,22,23].

3. Results

3.1 EMSAs of *in vitro* co-transcription of partial RNA1 and RNA2 cDNA clones

In vitro co-transcription of RNA1 and RNA2 full length or partial T7 cDNA clones has been followed by electrophoresis into native and Mg²⁺-chelating agarose gels. Homo and hetero RNA duplexes have been observed both by ethidium bromide staining and hybridization using specific radiolabeled ODN probes after RNA transfer. In the described conditions no

significant band-shift has been observed with full length RNA1 and RNA2 (data not shown) probably due to an absence of cellular or viral factors that could help and stabilized RNA/RNA interactions in the natural context. The use of RNA1d and RNA2a partial clones (Figure 3 (A)) highlighted one important hetero duplex retrieved as well in semi-denaturing conditions where Mg^{2+} -stabilization of RNA structures is reduced (Figure 3 (1, I, 2, II)). Such interaction appears specific since no band shift was observed when Luciferase mRNA was co-transcribed with RNA2a (data not shown). Interestingly, using specific probes, I also evidenced RNA1c and RNA1e homo-dimerization indicating that BNYVV RNAs self-interact. If the biological significance of such homo-interaction has been not investigated in this chapter, it could suggest that BNYVV RNP supramolecular assembly can involve more than one genomic RNA molecule of the same species, rendering the stoichiometry determination difficult. The complete sequences of RNA1 and the domain of RNA2 ranging from its 5' to last residue preceding the first nucleotide of subgenomic RNA2 have been tested. This follows our proposed model of supramolecular assembly of genomic RNAs where shorter RNA species should not compete.

EMSA of products obtained co-transcribing RNA1d with partial sequences of RNA2a (RNA2b, RNA2c, RNA2d and RNA2e) (Figure 4 (A)) reveals that the RNA2 domain involved in the RNA1d/RNA2a interaction is located within its first 668 nts (Figure 4 (1, 2)). Interestingly, two RNA2 self-interaction domains, located within the first 668 and between nucleotides 1155 and 2065, have been detected. In particular, RNA2e self-interaction does not appear as an alternative to an hetero-dimer formation since both complexes are present after co-transcription of RNA1d/RNA2c.

To localize more precisely the region involved in the RNA1d/RNA2c interaction, we introduced into the co-transcription mixes of five complementary ODNs (10 pmol each). These ODNs represent putative competitors covering the first 668 bases of RNA2 (table 6). In these condition, no RNA/RNA interaction was detected in the presence of A1 - A5 ODNs mixes, covering the first 116 nucleotides (Figure 5 (1, 2)). Finally, using single ODN (A1 to A5) as competitor, the RNA2 interaction domain has been localized within the first 20 nts. Indeed, the use of ODN A1 alone prevent RNA1d/RNA2b hetero-dimer formation (Figure 6 (2, 3)).

In order to identify the domain of RNA1d sequence putatively able to interact with the first 20 nts of RNA2, the IntaRNA program (Freiburg RNA Tools) has been used for *in silico* analysis [24,25]. A maximum score has been found and corresponds to a 17 nts long sequence located in the coding region of RNA1, between nucleotides 4357 and 4373. This suspected RNA1/RNA2 interaction consists therefore in Watson-Crick base pairing of 17 nucleotides with a bulge due to two unpaired bases (Figure 7 panel I). BLAST analyses revealed a high conservation of both motifs within BNYVV RNA1 and RNA2 published sequences of different strains: 100% nucleotide conservation was found for RNA2 whereas one nucleotide mismatch exist for a RNA1 isolate that results in a A-U to G-U base pair substitution in the extended duplex dimer. However, one Japanese isolate (GenBank accession number: KX665536.1) presents a supplemental mismatch in the RNA1 domain sequence and no compensatory sequence was found in this isolate RNA2 domain.

Synonymous and compensatory mutations based on *three nucleotide changes* (Figure 7, panel I) were designed on ODNs and EMSA experiments. For this purpose, I used competitor oligonucleotides covering interactions domains and carrying or not the indicated mutations. Competitor ODNs A1, G1 and H1 were able to prevent the RNA1/RNA2 interactions as seen for A1 ODN previously (Figure 6). However, ODN A1 mut and H1 mut, were not able to disrupt the interaction between wild type RNAs (Figure 7, panel II) (Table6).

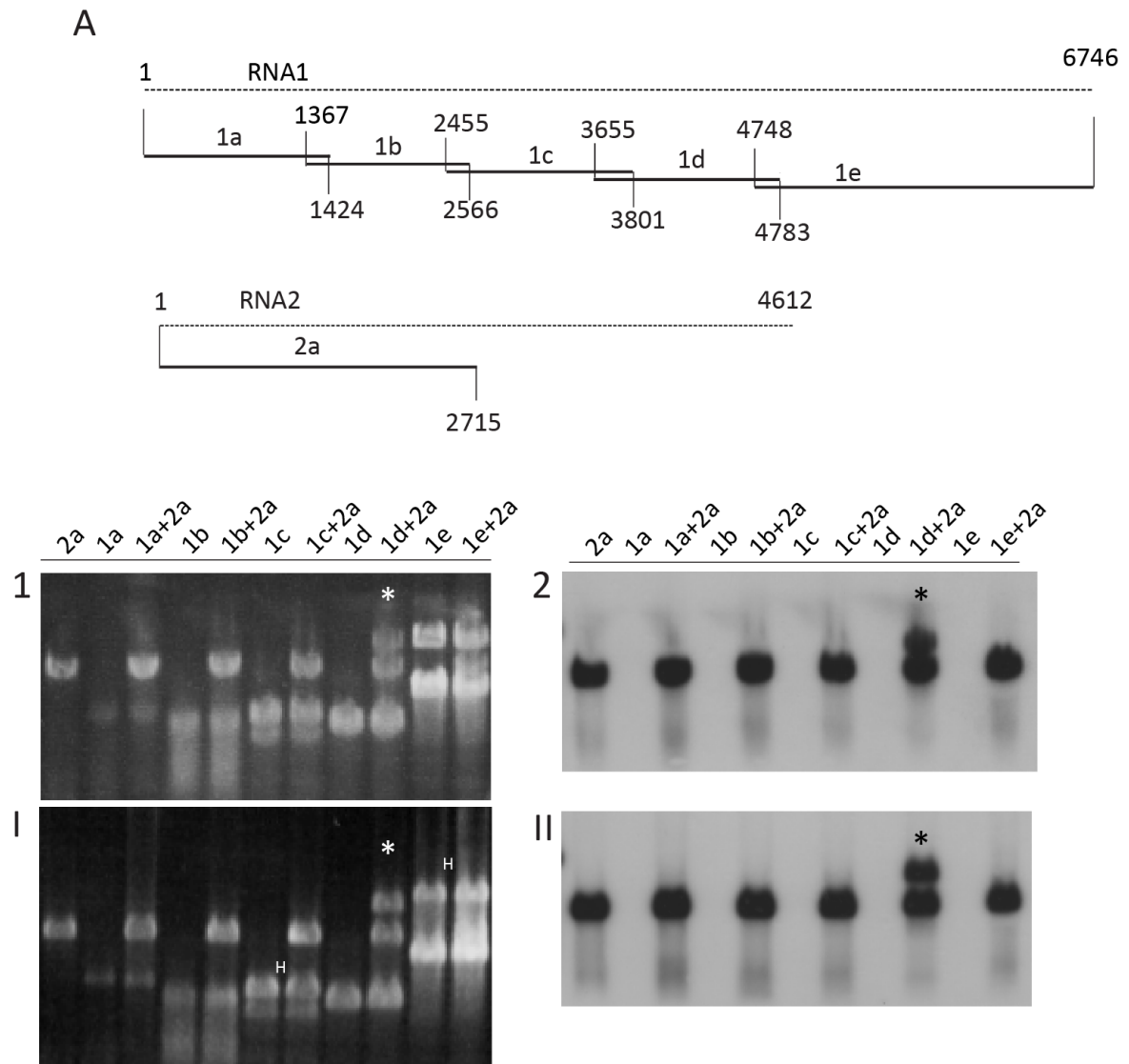


Figure 3 EMSA I: (A) representation of the sequences covered by RNA1a, b, c, d, e and RNA2a partial clones used for qualitative analysis of RNA/RNA interaction by native agarose gel (1) or semi-denaturing TBE agarose gel (I). To improve the resolution, RNAs were transferred onto membranes (2 and II) and RNA2 revealed using radio-labeled ODNs 334R (Table 3). Asterisks indicate hetero-dimer formations while H indicates RNA self interaction.

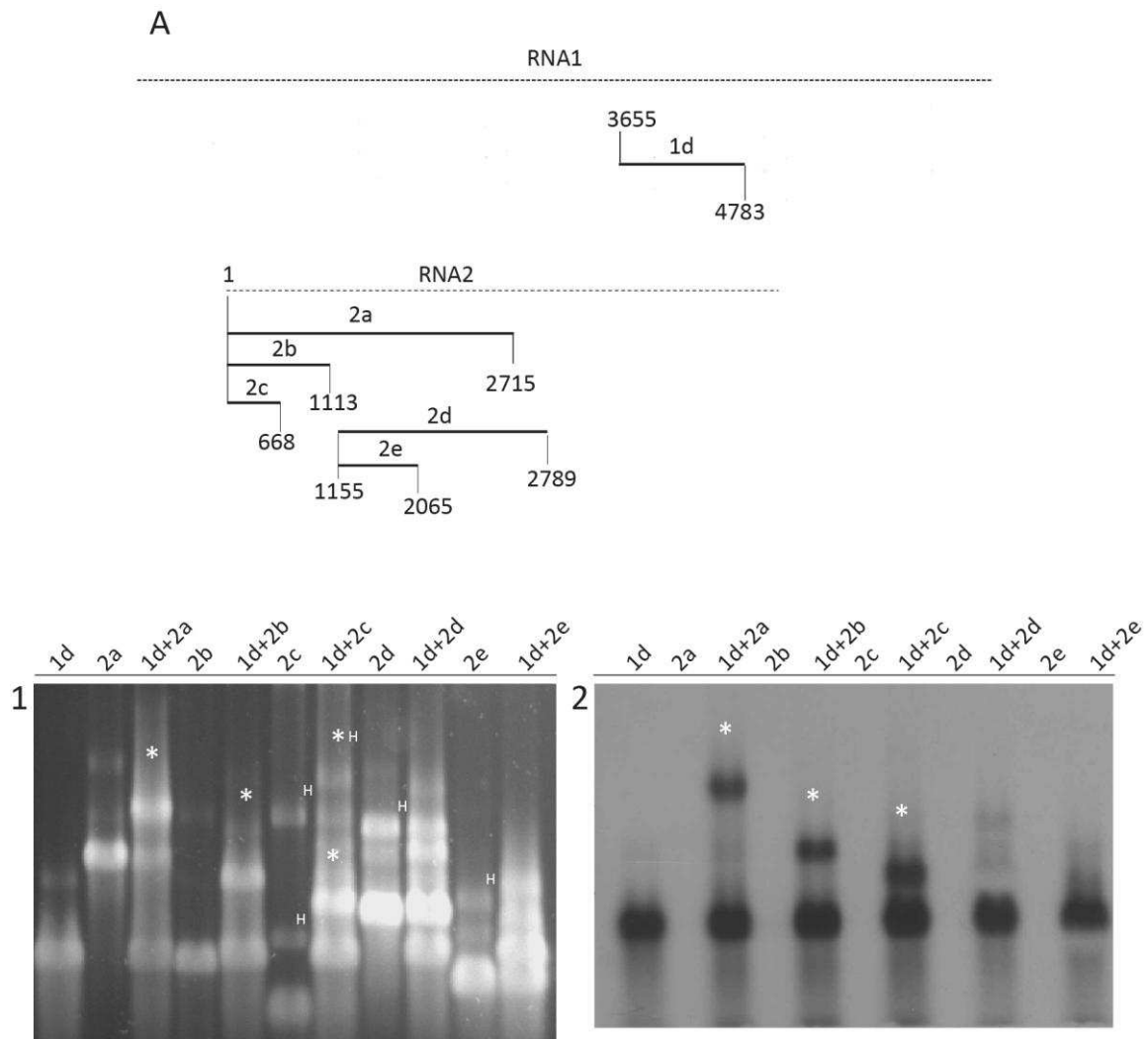


Figure 4 EMSA II: (A) representation of the sequences covered by RNA1d and RNA2a, b, c, d, e partial clones used for RNA/RNA interaction analyses. TBE agarose gel reveals several band-shifts (1) caused by hetero-dimer formation marked with asterisks and homo-multimers marked with H letter; (2) RNAs were transferred onto membranes and RNA1 revealed using radio-labeled ODNs 3739 R (Table 3).

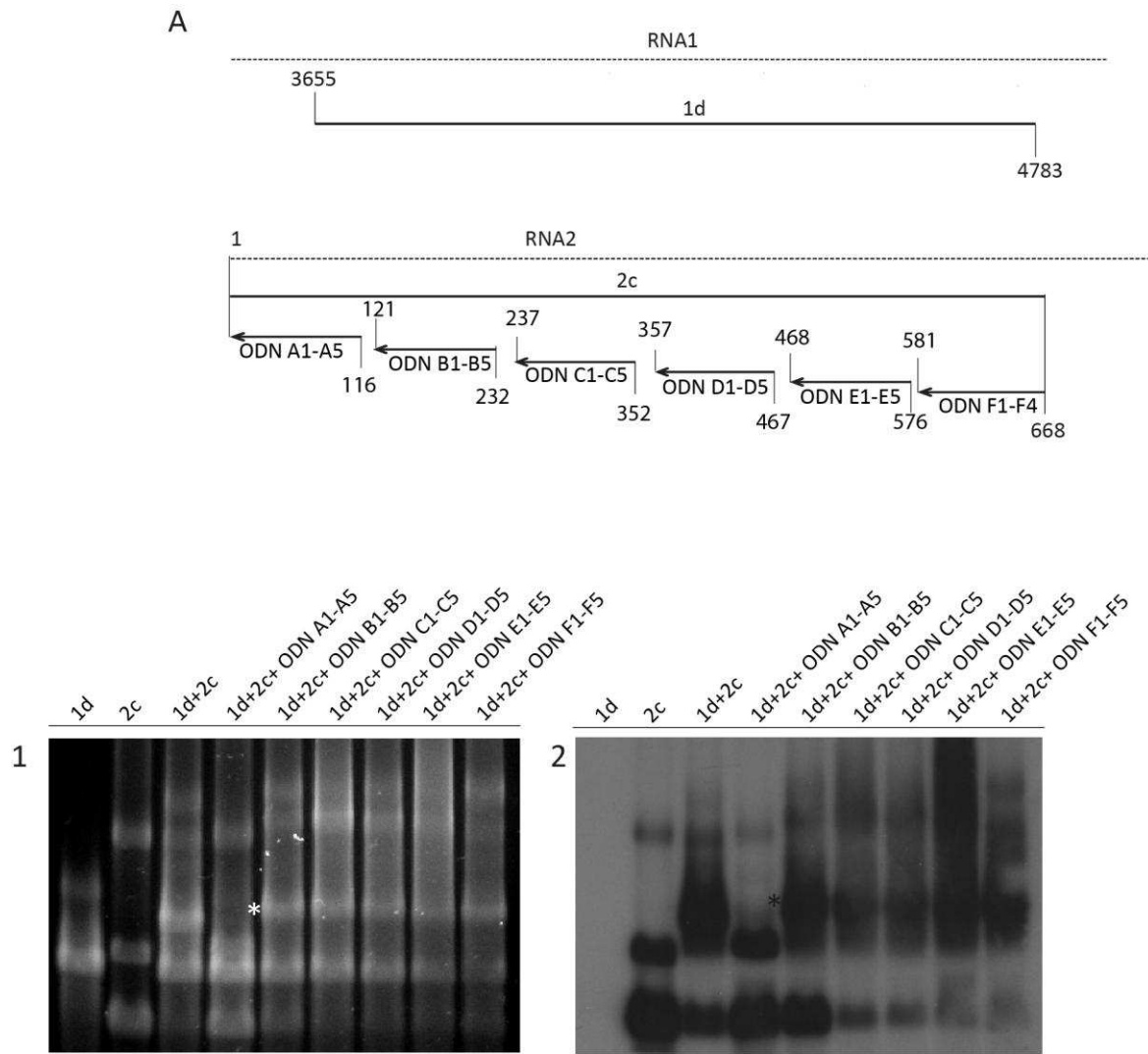


Figure 5 EMSA III: (A) representation of the sequences covered by RNA1d and RNA2c partial clones and six sets of five ODNs (ODN A1-A5, B1-B5, C1-C5, D1-D5, E1-E5, F1-F4 detailed in Table 6). These ODN are complementary to the first 668 nts of RNA2. The interference of ODN on RNA1d/RNA2c interaction has been analysed in TBE gel (1). ODN set able to disrupt the interaction are highlighted by Asterisks. Northern blot (2) analysis has been performed using radio-labeled ODNs (B3 and F3) complementary to RNA2 (Table3).

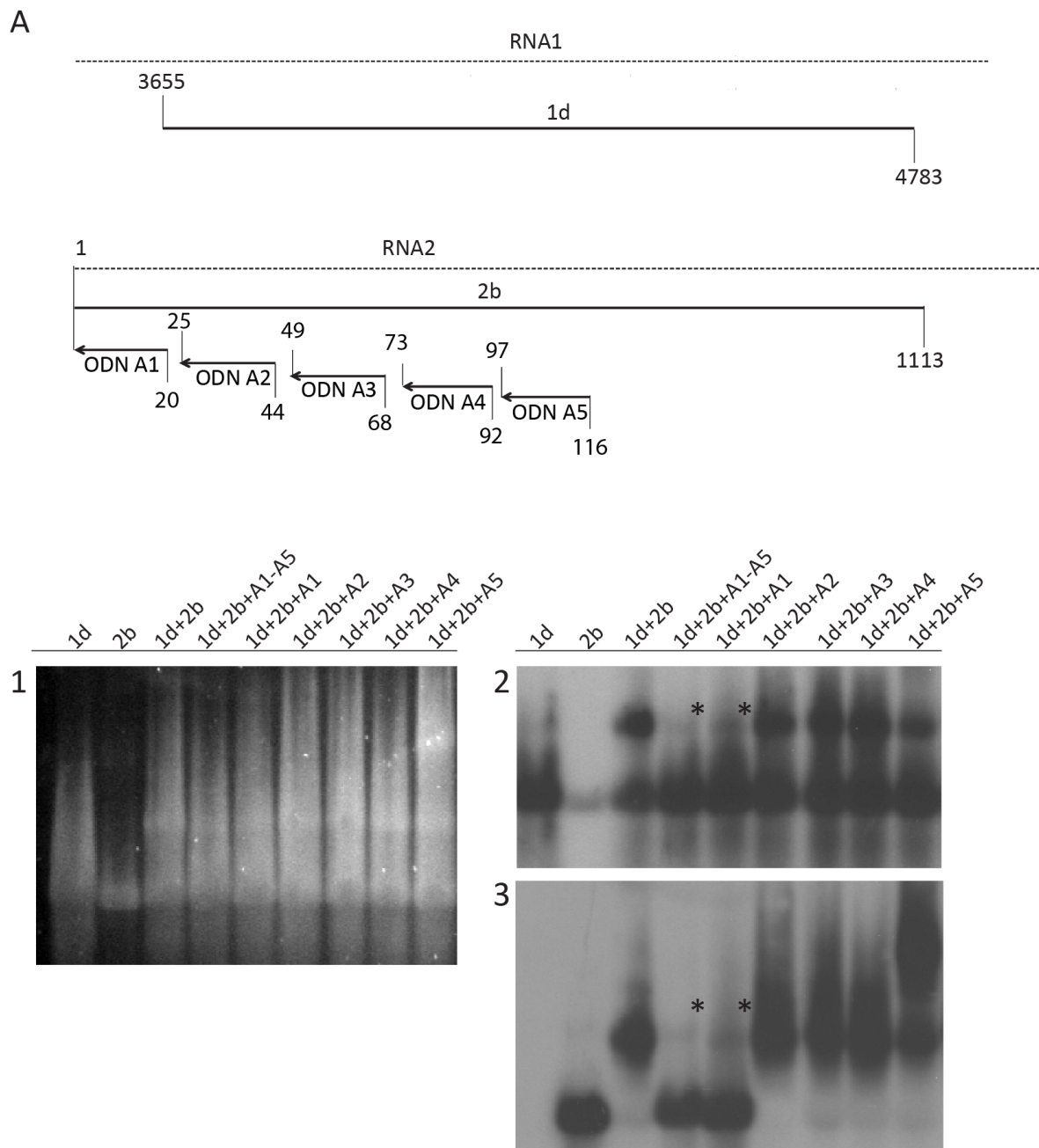


Figure 6 EMSA IV: (A) representation of the sequences covered by RNA1d , RNA2b and ODNs complementary to the first 116 nts of RNA2. RNAs from TBE gel (1) were northern blotted and RNA1d revealed using radio-labeled ODN 3739R (2) while RNA2 positions revealed using radio-labeled ODN 334R (3). Asterisks indicate the disruption of RNA1d/RNA2b hetero-duplex.

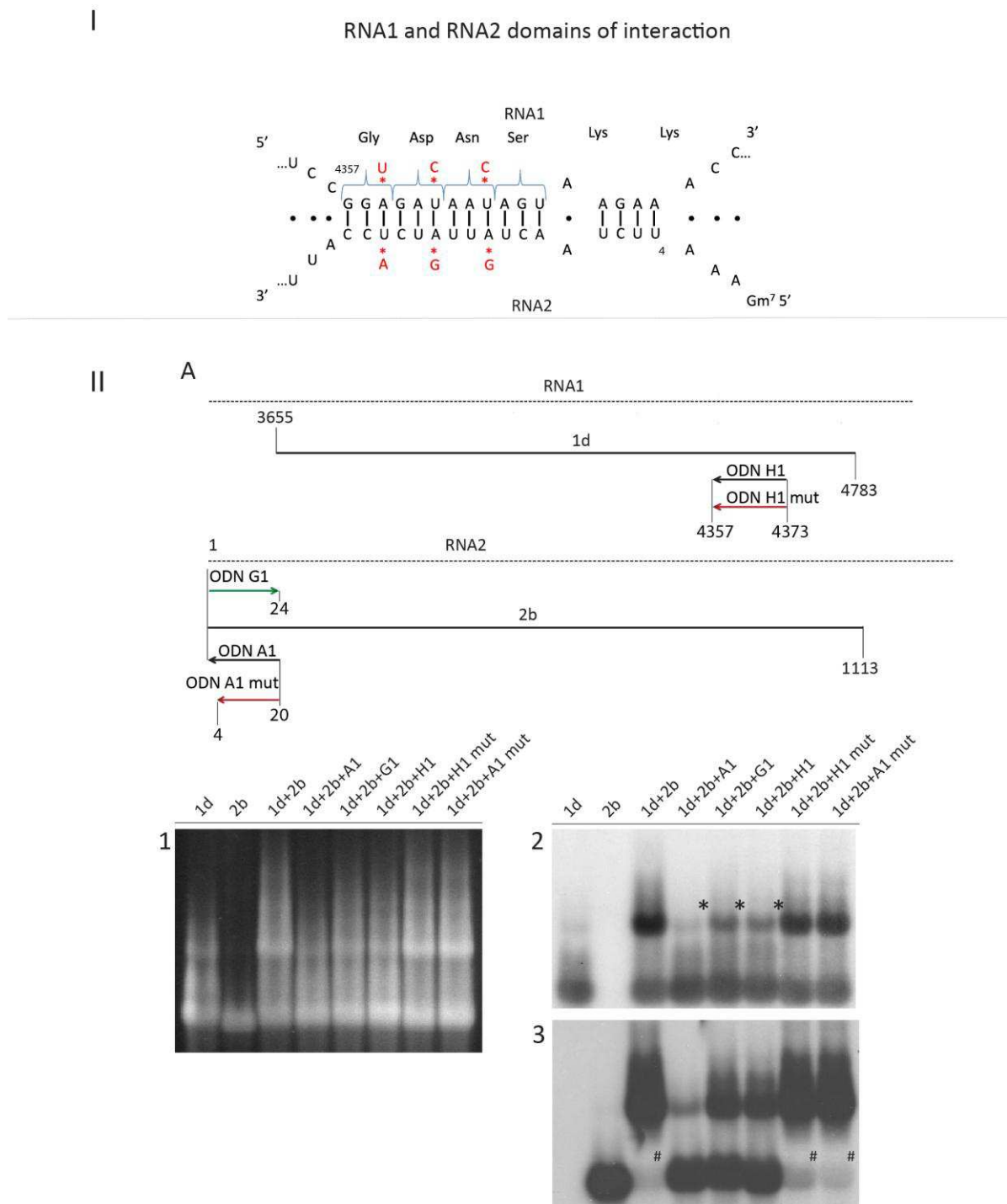


Figure 7 (I) RNA1 and RNA2 interaction domains is characterized by 17 nucleotides base pairing with a bulge due to two unpaired bases (A·A). Bases in red correspond to RNA1 synonymous mutation introduced and compensatory mutations on RNA2 to maintain the interaction. (II) EMSA V: panel A represents the sequences covered by RNA1d , RNA2b and ODNs complementary to both putative interaction domains (A1,H1) and carrying the designed mutations (A1 mut and H1 mut). The capacity to disrupt RNA1d/RNA2b interaction has been also evaluated with ODN G1 (corresponding to the first 24 nts of RNA2, table 6). TBE gel (1) has been blotted in to membrane and RNA1d revealed with radiolabeled ODN 3739R (2) while RNA2b position revealed with ODN 334R (3). Asterisks indicate disruption of RNA1d/RNA2b hetero-duplex and hashtags indicate depletion of RNA2b monomer form.

RNA2 A1	20	GGAGATAATAGTTAGAATTT ₁	RNA2 D1	376	GTGTCTGTGGAAACGGGTC ₃₅₇
RNA2 A2	44	ACAGACGGTGAAATTCTATT ₂₅	RNA2 D2	400	ACACAAGTGCACCATACATA ₃₈₁
RNA2 A3	68	CCCCCAGAACAAAATAAGA ₄₉	RNA2 D3	424	CTGGGTCAGACAGATTAACA ₄₀₅
RNA2 A4	92	TAAAGTAGGGCCCTGAATAA ₇₃	RNA2 D4	448	CCTTAGTCATTATCAACGCA ₄₂₉
RNA2 A5	116	CTACTTATTACTCGCACCTA ₉₇	RNA2 D5	467	GAATCAGTTAAAGTATTT A ₄₄₉
RNA2 B1	140	GTACTATCTTCTTCTGGACG ₁₂₁	RNA2 E1	484	CATTATCTGCTAACCCCT ₄₆₈
RNA2 B2	164	TATCTACCTTCACTCGACAT ₁₄₅	RNA2 E2	504	TCTACGTACATTAGCAGATG ₄₈₅
RNA2 B3	188	TGTGACATATCCTTCCATGT ₁₆₉	RNA2 E3	528	AGCTTTATTTCCAGACACCA ₅₀₉
RNA2 B4	212	CATCGATCGGTCATAAACTT ₁₉₃	RNA2 E4	552	ACCAGCAGTTTTACCGGATG ₅₃₃
RNA2 B5	232	CGACGTCCGAAACACG ₂₁₇	RNA2 E5	576	CGTATAAGCAGAGTTCTCAT ₅₅₇
RNA2 C1	256	CATGCGATTGTTTAATAACA ₂₃₇	RNA2 F1	600	TAAACCAGCAAGACTGACAG ₅₈₁
RNA2 C2	280	TCGAGCCTTGACAAGTCC ₂₆₁	RNA2 F2	622	CTTCAAGCCTAAGAGCTT ₆₀₅
RNA2 C3	304	AAGCAGTTTAAATTATAGAT ₂₈₅	RNA2 F3	646	ATTTATCCCGGTCCACATT ₆₂₇
RNA2 C4	328	ACCAACCCGAGCCTAATCCT ₃₀₉	RNA2 F4	668	GGTAATTTGAACCGGTCC ₆₅₁
RNA2 C5	352	GAGACACAAAAGGATTACTG ₃₃₃	RNA2 G1_(sense)	1	AAATTCTAACTATTATCTCCATTG ₂₄
RNA2 A1_{mut}	20	GGTGAACAAGTTAGAA ₄	RNA1 H1	4373	TTCTTACTATTATCTCC ₄₃₅₇
RNA2 I2_(sense)	113	GTAGCCGCCGTCCAGAAGAAG ₁₃₃	RNA1 H1_{mut}	4373	TTCTTACTGTTGTCACC ₄₃₅₇
			RNA1 I1	1421	ACCTCGAACAAATGCAACC ₁₄₀₃

Table 6 ODNs used as competitors in EMSAs and in *C. quinoa* protoplast experiments

3.2 *In vivo* experiment of RNA1/RNA2 interaction disruption by interfering of competitive RNA5 derived replicons.

RNA5 derived replicons (Rep5 [22]) carrying interactions domains have been used to evaluate a putative interference with RNA1/RNA2 interaction assembly (Figure 8, A). These replicons contain a 400 nts sequence of RNA1 or RNA2 encompassing the wild type or mutated interaction domain. Protoplasts were infected with RNA1 and RNA2 (Helper strain) supplemented or not with Rep5 constructions. Viral RNAs were analyzed 40 hpi. The presence of the Rep5 or Rep5 containing the wt or mutated RNA1 interaction domain (Rep5 RNA1 I.D., Rep5 RNA1 I.D. mut) did not interfere with the viral replication machinery, guaranteeing an intracellular accumulation of viral RNAs. However, when Rep5 contained the interaction domain of RNA2 (Rep5 RNA2 ID), no viral accumulation was observed, indicating a defective interfering effect (DI) [26] of the domain used. Such DI effect is partially alleviated with a mutated interaction domain provided by Rep5 (Figure 8, B). Experiments on *C. quinoa* leaves confirmed these results as no local lesion appeared on leaves inoculated with BN12 supplemented with Rep5 RNA2 I.D. However, the capacity of the virus to form loci of infection was partially restored when Rep5 carried RNA2 mutated domain (Figure 8, B). Finally, Rep5 containing the RNA1 domain (wt or mutated) did not interfere on the infection as indicated by the high number of local lesions formed in the

inoculated leaves (Figure 8, B). Molecular analysis of viral RNA accumulations confirmed the absence of RNA1 and RNA2 accumulation in the presence of Rep5RNA2 I.D. as observed in *C. quinoa* protoplasts. Interestingly, when the mutated version of the Rep5RNA2 I.D. was added to the inoculum, few lesions appeared (Figure 8, C) and their viral RNA content was similar to that observed on BN12 or BN12+ Rep5 inoculum of control (Figure 8, D).

3.3 *In vivo* experiment of RNA1/RNA2 interaction disruption by interfering of competitive ODNs

To discriminate between a DI effect of the Rep5 RNA2 I.D. and an effect of the RNA/RNA interaction, the replication RNA1 and RNA2 was evaluated on *C. quinoa* protoplasts inoculated in the presence or absence of ODN G1 (Table 6), complementary to the interaction domain of RNA1. Since RNA/DNA duplex can trigger cellular RNase H activity, ODNs complementary to other regions of RNA1 (I1) and to minus strand of RNA2 (I2) have been chosen as controls (Figure 9, A). When the concentration of ODN targeting RNA1 was 15 times higher to the concentration of genomic RNAs, a significant reduction of viral RNA accumulation was detected by Northern blot (Figure 9, B). However, the same behavior has been observed in the negative control when the ratio between ODNs and genomic RNAs was 50:1 probably due to a cytoplasmic RNase H activity.

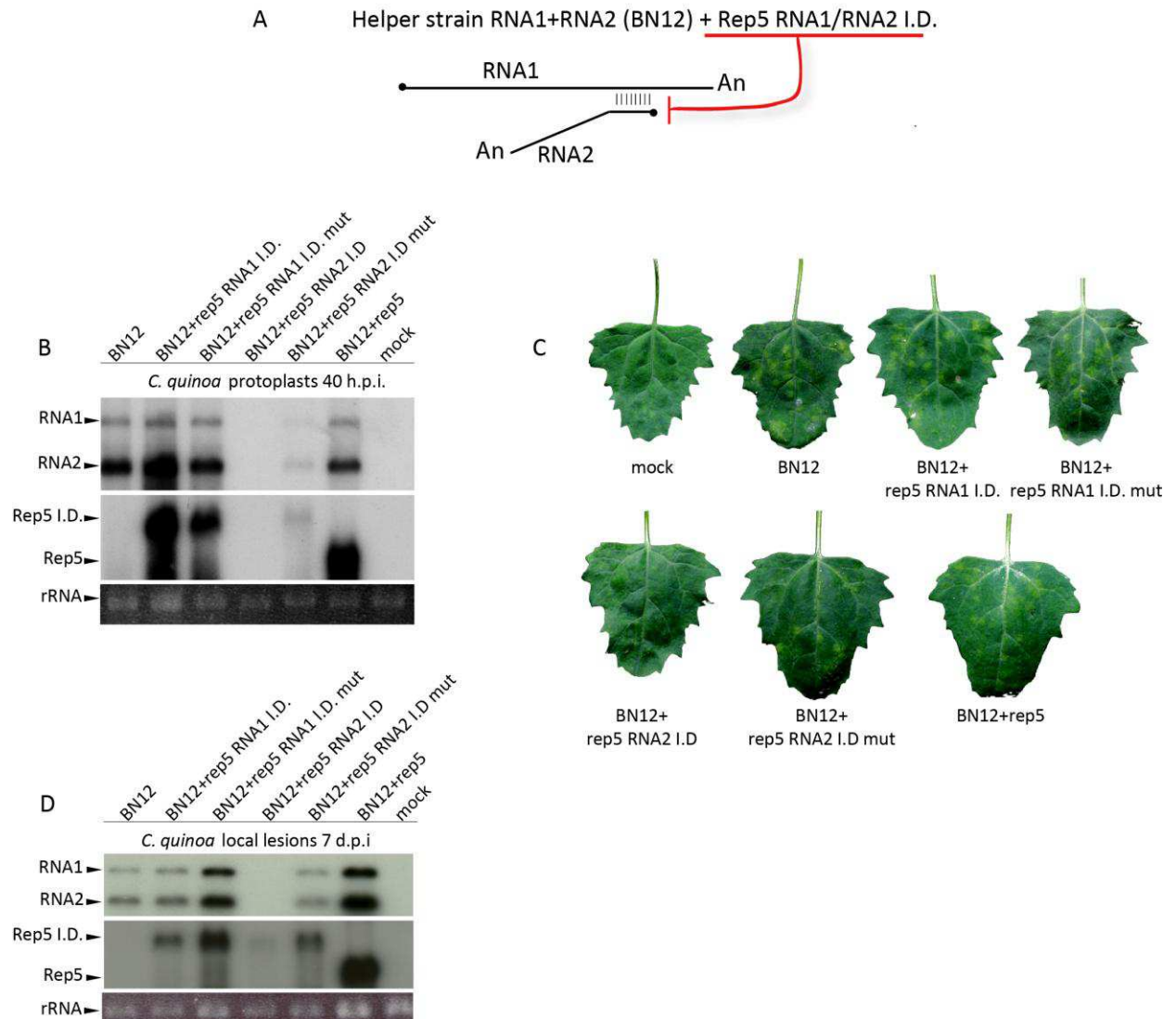


Figure 8 Disruption of RNA1/RNA2 interaction by competitive RNA5 derived replicons. Schematic representation of experimental strategy (A): Rep5 carrying or not an RNA1 or RNA2 interaction domain have been supplemented to RNA1 and RNA2 (BN12) for *C. quinoa* protoplast transfection or mechanical leaf inoculations. Northern blots (B) of vRNAs extracted from protoplast 40 hpi or (D) from leaf local lesions 7 dpi. RNA loads are visualized by Ribosomal RNAs (rRNA) staining. Number of leaf local lesions varies with the inoculum (C): comparable number of local lesions were obtained with BN12 alone or supplemented with Rep5 replicon containing or not wild type or mutated Rep5 RNA1 I.D. No local lesion was observed in leaves when rep5 RNA2 I.D. was added to the BN12 helper strain and few foci of infections were found on leaves inoculated with BN12+rep5 RNA2 I.D.mut.

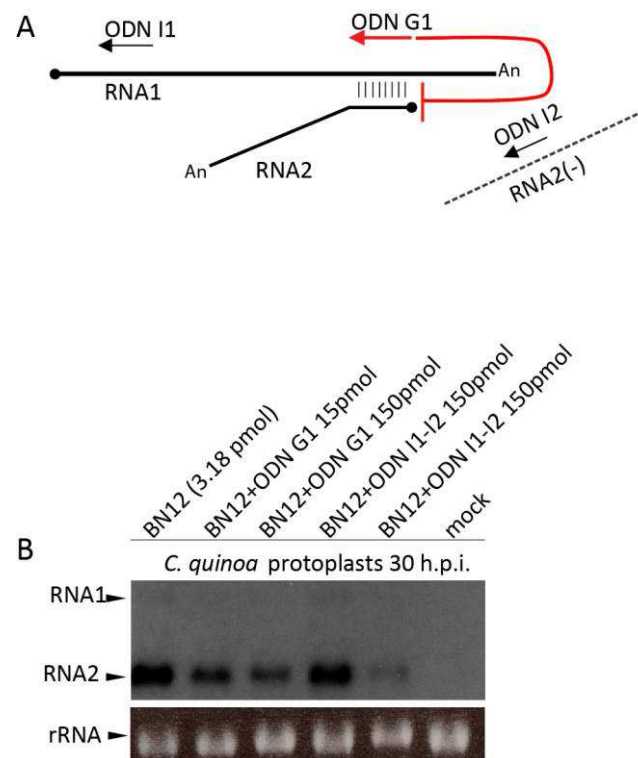


Figure 9 Disruption RNA1/RNA2 interaction with competitive ODNs. Schematic representation of experimental strategy (A): ODN G1 corresponding to the first 24 nts of RNA2 and complementary to RNA1 domain of interaction was used to disrupt RNA1/RNA2 interaction in *C. quinoa* protoplasts. ODNs complementary to other regions of RNA1 (I1) and to minus strand of RNA2 (I2) were chosen as negative controls. Northern blot of vRNAs 30 h.p.i. (B) evidence a significant reduction of viral RNA accumulation when ODN G1 is present even at low concentration (15 pmol). A similar behavior has been observed for the negative control when ratio between ODNs and genomic RNAs were 50:1. rRNAs staining served for RNA loading.

3.4 *In vivo* experiment of disrupted and restored RNA1/RNA2 interaction by synonymous and compensatory mutations

Synonymous and compensatory mutations were introduced within the 17 nts interacting sequences of RNA1 and RNA2 infectious T7 cDNA clones with the aim to prevent and/or restore RNA base pairing *in planta* and validate the biological significance of the RNA/RNA interaction identified. Vitality tests for the mutated genomic RNAs, RNA1 mut and RNA2 mut, were performed in *C. quinoa* plants to evaluate the essential functions of replication, cell to cell movement, and RNA silencing suppression. Wild type RNA1 (WT) + RNA2 MUT (BN1 WT+BN2 mut) and RNA1 MUT + RNA2 MUT (BN1 mut+BN2 mut) combinations did not allow the formation of local lesion on *C. quinoa* leaves probably due to the 5' promoter disruption on RNA2 MUT. Contrarily, RNA1 MUT + RNA2 WT (BN1 mut+BN2 WT) combination allowed the replication and cell to cell movement of the virus, however we

observed a dramatic decrease of local lesion number when compared to wild type combination (BN12) (Figure 8). BNYVV and *Beet soil borne mosaic virus* (BSBMV) are distinct but closely related species belonging to the *Benyvirus* genus within the *Benyviridae* family and possess the same genomic organization [27]. Under laboratory conditions genomic RNA exchanges between RNA1 and RNA2 of BNYVV and BSBMV result in functional chimeric viruses [23]. Using bioinformatics comparisons, we evidenced that RNA1 domain of interaction identified in BNYVV is not present on BSBMV RNA1, suggesting that an alternative network of RNA1/RNA2 interaction is established for BSBMV and for Benyviridae reassortants [11]. For this reason we used such opportunity to compare the behavior of BNYVV RNA1 WT + BSBMV RNA2 (BN1BS2) and BNYVV RNA1 MUT + BSBMV RNA2 (BN1 mut+ BS2) chimeras. In *C. quinoa*, numbers of local lesions were similar, demonstrating that the mutations introduced in BNYVV RNA1 were not affecting the replication and viral progression of the chimeras. In BNYVV context, the mutation affected the viral behavior on leaves, suggesting that indeed this RNA1/RNA2 interaction is orchestrating a regulatory network.

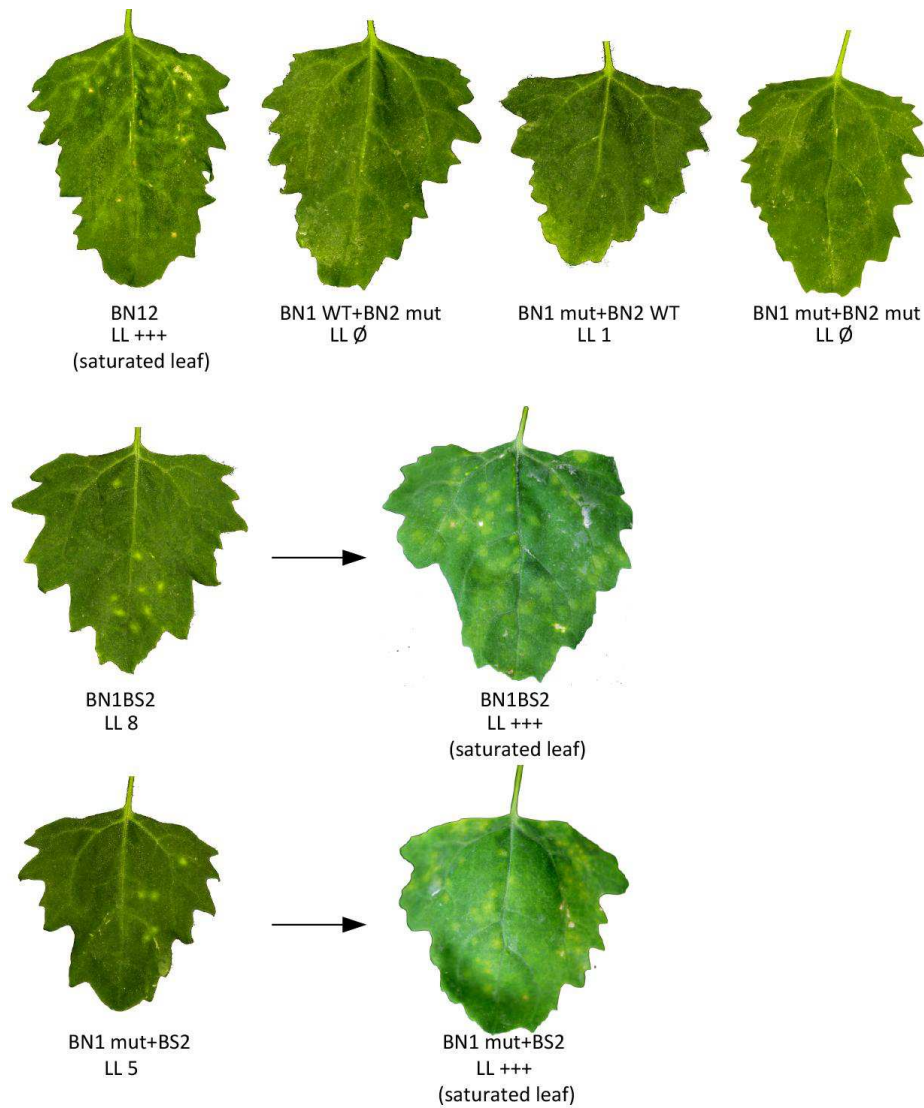


Figure 10 Disruption and restoration of RNA1/RNA2 interaction by synonymous and compensatory mutations. Number of local lesions (LL) in *C. quinoa* leaves 7 dpi of the following RNA combination: BN12, BN1 WT+BN2 mut, BN1 mut +BN2 WT, BN1 mut +BN2 mut, BN1BS2 and BN1 mut +BS2. Black arrows indicate three-fold increase of BSBMV RNA2 quantity in the inoculum

4. Discussion

The existence of interaction between BNYVV RNA1 and RNA2 has been confirmed *in silico* and *in vitro*. Such interaction involves two domains of 17 nts located in highly conserved regions of RNA1 (sequence codifying the polymerase) and RNA2 (5' end UTR). No additional interaction domains have been identified yet. However, we expect them to exist but

they might be unable to form interactions due to alternative structures that may arise in our experimental conditions. The exposition of these domains could depend on the presence of viral or cellular factor or be part of riboswitches. It has been proposed, for example, that *human immunodeficiency virus 1* (HIV-1) leader sequence can adopt two alternative conformations that differ in the presentation of the dimerization initiation site (DIS hairpin) and consequently in its ability to dimerize *in vitro* [28]. Furthermore the presence of viral factor as the nucleocapsid protein enhance, *in trans*, HIV-1 dimerization efficiency [29].

The biological significance of BNYVV RNA1/RNA2 interaction has been tested following two different experimental strategies: One was intended to interfere with the formation of RNA1 and RNA2 hetero-complexes using competitor replicon or ODNs. The second consisted on the disruption and restoration of RNA1/RNA2 pairing by the introduction of compensatory mutations in the interacting sequences. The first strategy demonstrated that Rep5 RNA2 D.I. as well as G1 ODN significantly reduced viral replication. However, the mechanism of this interference is still to be clarified. The use of oligoribonucleotide instead of ODN could be the alternative to understand the inhibition of RNA1/RNA2 interaction on the replication efficiency without triggering RNase H activity. The second strategy evidenced that the disruption of RNA1 interaction domain by synonymous mutation, lead to a strong decrease of the viral infectivity in *C. quinoa* leaves. However, in BN1BS2 chimeric context, the mutation effect disappeared indicating that the loss of function due to synonymous mutation was restored. Therefore, we conclude that RNA1 mut provides an efficient replicase machinery and therefore the infectivity reduction in *C. quinoa* leaves is related to the disruption of RNA1mut/RNA2 WT interaction. We suspect this interaction is involved in the coordination of the viral replication or cell-to-cell movement. Experiments on *C. quinoa* protoplast are required to validate one or the other hypotheses. The analysis of the viral progeny from *C. quinoa* lesions will help us to determine if a selection and the establishment of a *de novo* alternative network interaction between RNA1mut and RNA2WT can overcome the mutation on the RNA1 interaction domain. Such alternative interaction should already exist in BN1BS2 chimera. Its identification could provide new insights in the regulation of genomic reassortment between Benyviridae. BNYVV and BSBMV coexist within large areas of infection in the USA. These closely related viruses are frequently present in the same field infecting the same plant. Under laboratory conditions cognate RNA exchanges results in a functional chimeric virus. However, no natural chimeras have been described so far in field

conditions [23]. The best fitness of parental RNA combination could therefore be explained by a selection of the optimal RNA/RNA network interaction required for the formation of mobile infective units.

Another way to demonstrate the occurrence of RNA/RNA interaction during the viral spread in the host is to produce an additional RNA able to move long distance using an artificial RNA/RNA interaction with one of BNYVV genomic RNAs. For this purpose, BNYVV RNA3 derived replicon (Rep3) [30], unable to move long distance [31], and RNA2 eGFP fusion construct [32] could be used in an experiment of gain of function: Rep3 carrying 17 nucleotides long sequences and complementary to eGFP sequence should be tested together with the combination RNA1 + RNA2 eGFP in systemic infection of *Spinacia oleracea*. Occurring of Rep3 systemic spread, together with eGFP tagged helper strain would represent the demonstration of the crucial role of RNA/RNA interaction played in BNYVV movement.

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General Conclusion

The aim of my study was to investigate different aspects of the multipartite life style of the *beet necrotic yellow vein virus* (BNYVV) evaluating a RNA/RNA interaction model involved in the preservation of the viral genome integrity and in the regulation of the viral cycle. The proposed model minimizes the cost for a RNA multipartite virus to accomplish the “one genome one infected cell paradigm”. In this BNYVV model a network of RNA/RNA interactions that recalls *influenza A virus* lesson [1], determines the specific recognition and the mobilization of at least one of each genomic RNA in a modular ribonucleoprotein complex (RNP). This RNP complex should be considered as the mobile infectious unit required for the viral spread in the plant [2].

To date the unique study performed to evaluate the relative accumulation of each genomic RNA during the infection consisted of a particle count from *Tetragonia expansa* leaf dip preparations. Transmission electron microscopy evidenced the over representation of RNA3, RNA4 and RNA5 *versus* RNA1 and RNA2 containing viral particles [3]. In chapter I, I extended this investigation to different organ (leaf and root) of different host species (*Beta macrocarpa*, *Spinacia oleracea* and *Chenopodium quinoa*) using a more reliable approach. I determined the stoichiometry of the viral RNA species using a dual step reverse transcriptase droplet digital PCR (ddRT-PCR), and characterized on the one side total genomic RNAs and on the other side encapsidated RNAs. I evidenced a regulated organ and host adapted viral RNA relative abundances. These data indicate that genome partition gives the opportunity to regulate BNYVV gene expression in different contexts. In particular, I confirmed a tendency for ancillary RNAs to accumulate more than one order of magnitude than housekeeping gene-coding RNAs. This differential replication trends may correspond to a viral counter defense against plant defense systems. As it was shown for RNA3 and suspected for RNA4 [4,5], ancillary RNAs require a yet unidentified viral or disease induced cellular effector to promote their translation. Furthermore, RNA3 to RNA5 increased amplification could also saturate the RNA silencing machinery preserving RNA1 and RNA2 from degradation.

This differential accumulation of genomic RNAs provides as well a high multiplicity of infection (MOI). One should keep in mind that the number of the different genome entities required for the virus to propagate in new target cell corresponds to at least one copy of each genomic segment. Elevated MOI and differential viral RNA species accumulation have a direct consequence on the progression of the infection and the dissemination of the viral

segments. Over representation of ancillary RNAs induces biological costs probably unsustainable for the virus (and for the host) [2,6,7]. For this reason, in chapter II, the “one infected cell one viral genome” paradigm has been tested. I evaluated the presence of at least one of each segment in single cell. Qualitative duplex RT-qPCRs have been conducted on protoplasts collected from *S. oleracea* infected tissue. I determined the genomic integrity preservation in each singular event of infection with the sole exception of RNA4 founded alone for 20% of protoplasts. Taking this exception apart, this result indicates the requirement for a specific process that guarantees a coordinated transport of each segment within an infective collective unit. Such a process reduces the MOI to sustainable levels recalling for this reason the proposed model of RNA/RNA interaction.

In chapter III the existence of direct and specific interactions between genomic RNA1 and RNA2 have been evaluated using *in vitro* experiments combined with *in silico* predictions. RNA1 and RNA2 interaction depends on two conserved domains located within the RNA1 coding region and the 5' end of RNA2. Disruption of the RNA1/RNA2 pairing provided evidences on an existing regulatory function for such binding in the cell-to-cell movement and viral RNA accumulation. These results give new insights of the BNYVV lifestyle where a fine-tuning of the viral genome expression may occurs at different infection stages, from the first steps after transmission to the viral systemic movement. Once transmitted into the cytoplasm of a root cell, osmotic changes, ionic strength or the presence of cellular factor(s) induce the partial uncoating of the viral particles releasing 5' and 3' ends RNA extremities [5]. Taking in to the account the distance between the 5' end and the initiation codons, only RNA1 and RNA2 present reasonable 5' UTR for ribosomal scanning and translation initiation. RNA3 and RNA4 ORF remains inaccessible [5]. As described for *tobacco mosaic virus* [8], translation of genomic RNA1 allows its complete uncoating leading to the replicase production and initiation of minus strand synthesis. RNA2 may remain partially uncoated. This could be explained as its translation ends at the CP stop codon. Indeed, a CP read-through requires structural elements present downstream of this stop signal [9], yet covered by CP subunits. This RNA2 species as well as ancillary RNAs will be uncoated by the replicase during minus strand synthesis.

The next steps remain speculative but could consist on a reduced translation of RNA2 while interacting with RNA1 to prevent the premature RNA encapsidation. This allows the

complementary strand production required for subgenomic RNAs transcription and further translation of other housekeeping genes (movement proteins and VSR). The mobile infectious unit should be assembled at this moment to allow viral spread. The translation of RNA3 and RNA4 could then start excepted for species dedicated to the mobile unit. In late infection stages, RNA3 and RNA4 amplifications reach the relative frequencies described in chapter I. RNA2 expression occurs as well to provide enough structural proteins and allow encapsidation. This packaging can be considered as a way to preserve and transmit the virus but also to negatively regulate the translation of viral RNAs and modulate the assembly of RNP units, thus limiting the viral expression.

These dotted line drawing BNYVV life just require time, hands and money to be either erased or “written in stone”; that’s science.

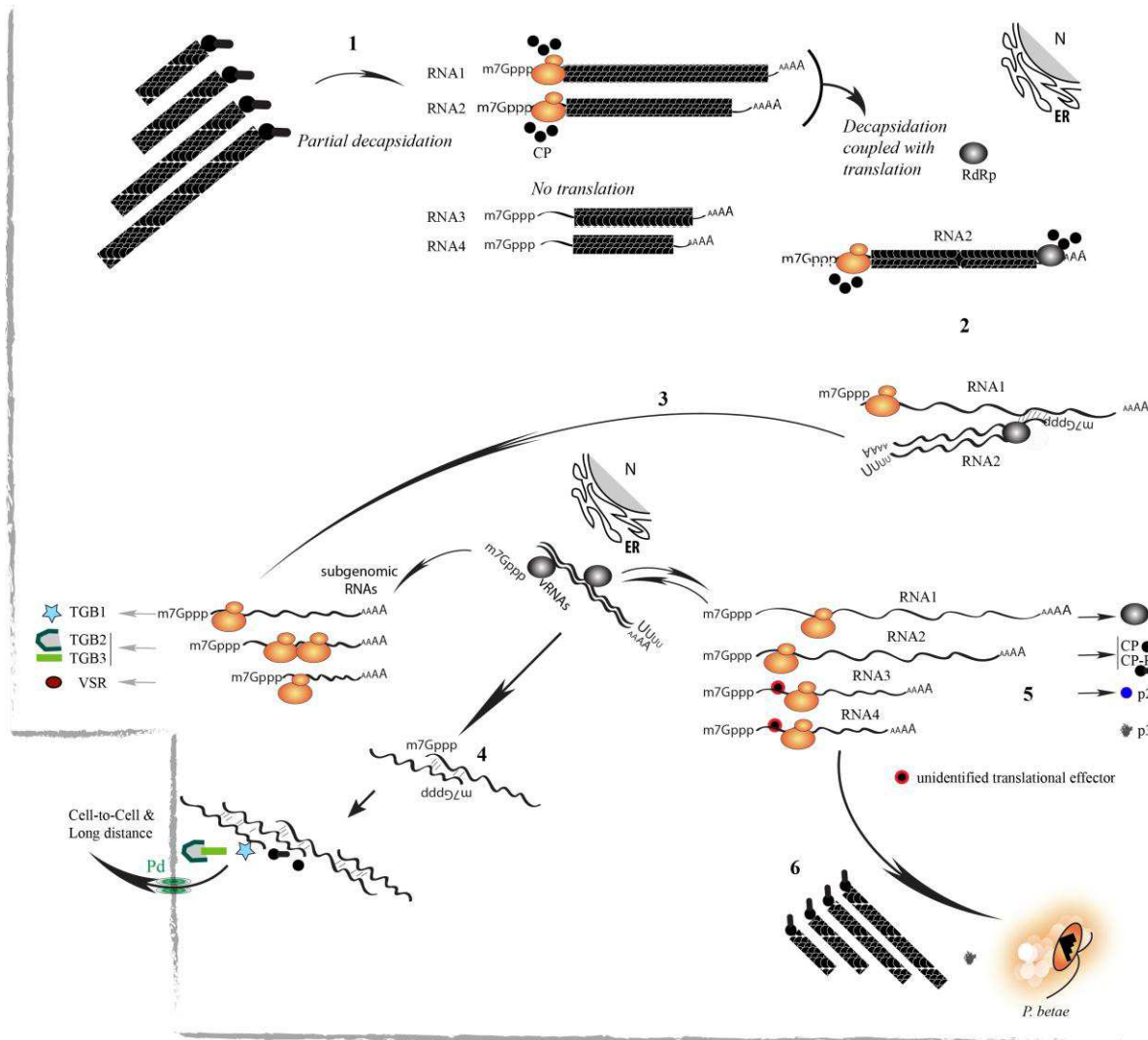


Figure 1 Drawing of a putative BNYVV infection cycle with insights derived from RNA/RNA interactions. Particles (black rods) are delivered in the cytoplasm of root cells by *Polymyxa betae* transmission. (1) Partial uncoating is coupled with ribosomal translation of RNA1 and RNA2. The complete translations of RNA2 (CP-RT) and RNA3 take place at later stages. In particular, RNA2 is uncoated by replicase (gray dots) progression and interacts with RNA1 through their interaction domains (2) covering 5' end sequences involved in the translation initiation complex formation. CP (black dots) expression stalling prevents the premature RNA encapsidation and allow the complementary strand production required for subgenomic RNAs transcription and translation of TGB proteins and VSR. Once produced, these viral proteins stabilize the RNA/RNA network in a RNP complex and move cell to cell or long distance (4). After accumulation of the yet unidentified translational effector, RNA3 and RNA4 can be expressed to produce p25 and p31 protein (5). RNA2 expression occurs as well to provide enough structural proteins required for the genomic RNA encapsidation necessary for *P. betae* transmission (6) and spread the infection in new hosts. Cap: m7Gppp, polyadenylated sequence: AAAA, N: nucleus, ER: endoplasmic reticulum, Pd: plasmodesmata (modified from Gilmer [5]).

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