Compte-rendu de fin de projet

Projet ANR-16 CE20-0008-01

PotyMove

Appel à projets générique 2016

Sécurité alimentaire et défi démographique

A IDENTIFICATION

Acronyme du projet	**PotyMove**
Titre du projet	Host factors involved in potyvirus cell-to-cell movement: new sources of resistance ?
Coordinateur du projet (société/organisme)	Sylvie GERMAN-RETANA (INRAE, UMR BFP 1332)
Période du projet (date de début – date de fin)	Début : 01/10/2016 Fin : 30/03/2023 (fin scientifique du projet reportée pour cause de crise Covid) Rapport final devant être soumis avant le 30/06/2023
Site web du projet, le cas échéant	

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Date de rédaction	.June 2023

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B RESUME CONSOLIDE PUBLIC

B.1 INSTRUCTIONS POUR LES RESUMES CONSOLIDES PUBLICS

B.2 RESUME CONSOLIDE PUBLIC EN FRANÇAIS

Comment les potyvirus se déplacent-ils dans les plantes ?

Le projet PotyMove vise à mieux connaître les facteurs de plante qui permettent aux potyvirus de se mouvoir d'une cellule à l'autre

Les potyvirus provoquent de graves dommages chez les plantes cultivées. L'infection repose sur une succession d'interactions entre protéines virales et protéines de l'hôte. L'absence ou la mutation d'un facteur essentiel au virus entraîne la résistance de la plante. On parle alors de résistance passive (empêcher une fonction du virus de s'accomplir) en opposition à une résistance active (la plante déclenche une panoplie de réactions pour lutter contre le virus). Lors du mouvement de cellule à cellule, les virus exploitent les plasmodesmes, des petits canaux reliant les cellules végétales entre elles. Les virus sont capables de modifier ces canaux pour se déplacer grâce à l'action de protéines virales de mouvement. Cette étape est un véritable goulot d'étranglement pour l'infection virale. Connaître les mécanismes sous-jacents permet d'identifier de nouvelles sources de résistance bloquant ou réduisant ce mouvement au niveau local, ce qui au niveau de la plante entière, se traduit par une réduction de l'accumulation virale, mais aussi une réduction du risque d'apparition et de propagation de souches virales contournant d'autres résistances déjà présentes. Le projet PotyMove vise donc à mieux connaître les facteurs de plante qui permettent aux potyvirus de se mouvoir d'une cellule à l'autre.

Utiliser les protéines de mouvement virales comme appâts pour piéger leurs partenaires de plante et valider leur rôle dans le mouvement des potyvirus

Lors du mouvement de cellule à cellule, les potyvirus exploitent les plasmodesmes en produisant des protéines permettant le passage du génome viral, nommées protéines de mouvement. En utilisant comme appâts ces protéines virales, le but est de piéger leurs partenaires chez la plante, grâce à une approche originale de protéomique des plasmodesmes et une approche d'analyses d'interactions entre protéines chez la levure. Le rôle des facteurs de plante ainsi identifiés (appelés candidats), dans le transport de cellule à cellule des potyvirus, doit ensuite être validé. Ceci est réalisé grâce à l'obtention de plantes mutées pour ces facteurs et en suivant scrupuleusement l'infection de virus étiquetés à l'aide d'une protéine fluorescente dans les plantes mutées. En parallèle, deux approches génétiques complémentaires sont menées chez la plante modèle Arabidopsis pour identifier d'autres candidats en partant de l'observation de plantes potentiellement affectées dans le mouvement du virus et en identifiant les gènes responsables. La validation fonctionnelle des candidats et/ou l'identification des gènes par approches génétiques sont les étapes du projet les plus longues.

Résultats majeurs du projet

Deux nouveaux facteurs de plante ont été identifiés et validés comme étant impliqués dans le mouvement des potyvirus : la protéine Remorine, que l'on retrouve associée à des domaines membranaires particuliers appelés radeaux lipidiques chez les plantes et aussi au niveau des plasmodesmes. Une deuxième protéine (en cours de publication), partiellement associée aux plasmodesmes, elle aussi impliquée dans le mouvement des potyvirus, semble faire partir d'un réseau plus large de protéines capables de modifier des structures particulières du réticulum endoplasmique de la cellule végétale. Le transfert de ces résultats à la tomate est encore en cours.

Le projet a permis la publication dans des revues internationales scientifiques de 9 publications. Une dixième publication sera soumise en septembre 2023.

Le projet PotyMove est un projet de recherche fondamentale coordonné par Sylvie German-Retana (équipe de Virologie, UMR BFP, INRAE-Nouvelle Aquitaine Bordeaux), en collaboration avec Sébastien Mongrand (Laboratoire de Biogenèse membranaire, LBM, CNRS-Bordeaux) et Jean-Luc Gallois (équipe GAFL, INRAE-Avignon). Le projet ANR PotyMove débuté en 2017 et en cours jusqu'en 2023 (car au départ prévu pour 5 ans, l'éligibilité des dépenses a été prolongée pour cause de pandémie) rassemble un consortium à forte complémentarité (généticiens, virologues, biochimistes, spécialistes de l'ultrastructure des plasmodesmes ou PD), pour caractériser de nouvelles cibles mais également valider leur potentiel de transférabilité à des espèces d'intérêt agronomique (la tomate). Il a bénéficié d'une aide ANR de 570 k€ pour un coût global de l'ordre de 3 864 k€.

B.3 RESUME CONSOLIDE PUBLIC EN ANGLAIS

How do potyviruses move through plants?

The PotyMove project aims to gain a better understanding of the plant factors that enable potyviruses to move from one cell to another.

Potyviruses cause severe damage to cultivated plants. Infection is based on a succession of interactions between viral proteins and host proteins. The absence or mutation of a factor essential to the virus leads to plant resistance. This is referred to as passive resistance (preventing a virus function from being carried out), as opposed to active resistance (the plant triggers a panoply of reactions to combat the virus). As they move from cell to cell, viruses exploit plasmodesmata, small channels linking plant cells. Viruses are able to modify these channels in order to move, thanks to the action of viral movement proteins. This step is a real bottleneck for viral infection. Knowing the underlying mechanisms makes it possible to identify new sources of resistance that block or reduce this movement at local level, which at whole-plant level translates into a reduction in viral accumulation, but also a reduction in the risk of the appearance and spread of viral strains bypassing other resistances already present. The PotyMove project therefore aims to gain a better understanding of the plant factors that enable potyviruses to move from one cell to another.

Using viral movement proteins as baits to trap their plant partners and validate their role in potyvirus movement

During cell-to-cell movement, potyviruses exploit plasmodesmata by producing proteins enabling the passage of the viral genome, known as movement proteins. Using these viral proteins as bait, the aim is to trap their partners in plants, thanks to an original approach of plasmodesmata proteomics and an approach of protein-protein interactions in yeast. The role of the plant factors thus identified (called candidates), in the cell-to-cell transport of potyviruses, must then be validated. This is achieved by obtaining plants mutated for these factors and carefully monitoring the infection of viruses tagged with a fluorescent protein in the mutated plants. In parallel, two complementary genetic approaches are being carried out in the model plant Arabidopsis to identify other candidates based on the observation of plants potentially affected in the movement of the virus, and by identifying the genes

responsible. Functional validation of the candidates and/or identification of the genes by genetic approaches are the longest stages of the project.

Key project results

Two new plant factors have been identified and validated as being involved in potyvirus movement: the Remorin protein, which is found associated with particular membrane domains called lipid rafts in plants, and also in plasmodesms. A second protein (currently being published), partially associated with plasmodesmata and also involved in potyvirus movement, appears to be part of a wider network of proteins capable of modifying specific structures in the plant cell's endoplasmic reticulum. The transfer of these results to tomato is still in progress.

The project has resulted in 9 publications in international scientific journals. One publication is in preparation for a submission planned in September 2023.

The PotyMove project is a fundamental research project coordinated by Sylvie German-Retana (Virology team, UMR BFP, INRAE-Nouvelle Aquitaine Bordeaux), in collaboration with Sébastien Mongrand (Membrane Biogenesis Laboratory, LBM, CNRS-Bordeaux) and Jean-Luc Gallois (GAFL team, INRAE-Avignon). The ANR PotyMove project, which began in 2017 and runs until 2023 (because initially planned for 5 years, the eligibility of expenses was extended due to the pandemic), brings together a highly complementary consortium (geneticists, virologists, biochemists, specialists in plasmodesmata ultrastructure or PD), to characterize new targets but also validate their transferability potential to species of agronomic interest (tomato). It received ANR funding of 570 k€ for a total cost of around 3 864 k€.

C MEMOIRE SCIENTIFIQUE

Mémoire scientifique confidentiel : **non**

C.1 RESUME DU MEMOIRE

Ce résumé peut être repris du résumé consolidé public.

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C.2 Enjeux et problematique, etat de l'art

Plant health is of prime importance to improve and secure the food supply for an increasing world population. Viruses rank as number one of among pathogens that cause emerging infectious diseases in plants (Anderson et al., 2004). When the project started in 2017, an overall increased incidence of diseases induced by plant viruses was observed, connected to the intensification of agricultural practices, the geographical expansion of insect vectors, strongly influenced by global warming and extensive exchanges of plant material in the global market (Bebber et al., 2013). This situation was confirmed during the last 6 years of the PotyMove project. Indeed, the emergence in Europe of tomato brown rugose fruit virus (ToBRFV, tobamovirus) represented a high risk for tomato and pepper producers in Europe, and this virus was added to the alert list of the European and Mediterranean Plant Protection Organization (EPPO) in January 2019. Since the ban of the use of the neonicotinoid-insecticide against aphid vectors, Barley/Cereal yellow dwarf viruses (YDVs) (Luteoviridae) have also been re-emerging during the last years and endanger cereal yields in Europe (Mc Namara et al., 2020).

Compared to other plant pathogens, viruses are particularly unpredictable and difficult to combat. As a matter of fact, cultural practices involving the use of crop rotation, sanitation and proper planting dates, generally do not prevent virus spread. Producers often attempt to limit virus-induced diseases by using pesticides to control their vector organisms but this measure, now banned in Europe, is generally inefficient, particularly for viruses that are transmitted in a non-persistent manner, such as potyvriuses. In view of this major economic problem, the development and use of cultivars that are genetically resistant to viruses still remains a critical factor of competitiveness for both breeders and producers and one of the key stakes for sustainable agriculture.

The genus Potyvirus is considered as one of the largest among plant RNA viruses, causing considerable economical damage in vegetable and fruit crops worldwide (Inoue-Nagata et al., 2022). To invade plants, those obligatory parasites have developed tactics to reroute host cellular functions for their own benefits. The completion of the viral cycle results from a complex interplay between virus- and host-encoded factors also called susceptibility factors. In this scheme, absence or non-adequacy of a single susceptibility factor leads to full or partial resistance to viruses. Partners P1-BFP and P3-GAFL were among the first to demonstrate this concept of loss-of-susceptibility resistance genes in crops, through the identification of eukaryotic initiation factors as key players in plant-potyvirus interactions. In addition, they demonstrated the "transferability" of a potential recessive resistance from one crop to another (German-Retana et al., 2008; Nicaise, 2003; Nicaise et al., 2007; Ruffel et al., 2002).

Initially, eukaryotic initiation factor eIFiso4E was identified as an interactor of a viral bait through a Yeast Two Hybrid screen by P1-BFP'collaborator (Jean-François Laliberté) illustrating the high potential of virus-target based approach to discover resistance genes. The host-factor eIFiso4E was subsequently identified by screening a mutagenized population for resistance to a potyvirus (Lellis et al., 2002). Considering the high adaptative potential of plant viruses and resulting resistance breakdown, a more extensive screen of host-virus protein-protein interactors can lead to the identification of new host factors conferring recessive resistance to potyviruses. The ability to combine in the same plant, mutations affecting eukaryotic initiation factors and newly identified genes involved in other steps of the viral cycle, should lead to higher durability of the resistance. Therefore, any other plant proteins with a role in the potyviral cycle and whose modification is associated with a loss-of-susceptibility is of particular interest to create resistant plants to potyviruses.

In this context, the aim of PotyMove was to identify new plant factors involved in the virus cell-to-cell movement. This is a key-step of the viral infection, considered as a major putative obstacle to viral exponential expansion in the plant by generating population bottlenecks (Gutiérrez et al., 2015; Miyashita and Kishino, 2010; Monsion et al., 2008) thus, an excellent target for resistance: indeed a defection of plant factors required by the virus for its cell-to-cell movement would cause the virus to be confined or restricted to its primary infection focus, thus delaying or preventing its systemic spreading. Also, by increasing the genetic drift, reduced cell-to-cell virus movement could increase resistance durability (Quenouille et al., 2013).

At the beginning of the PotyMove project this step was still very little documented for the potyvirus genus, as it did not fall into any of the known categories of plant virus movement strategies. However, during the last 6 years, a significant amount of data concerning potyvirus movement has been published and was recently reviewed

(Solovyev et al., 2022; Wang, 2021). However, no synthetic review compiling and integrating all information relevant to our current understanding of potyvirus transport was really available. Therefore, in the frame of PotyMove project, we decided to write a review where we highlight the complexity of potyvirus movement pathways and present three potential non-exclusive mechanisms based on i) the use of the host endomembrane system to produce membranous replication 6K2-vesicles that are targeted to PD and move cell-to-cell ii) the movement of extracellular viral vesicles in the apoplasm iii) the transport of virions particles or ribonucleoprotein complexes through PD. We also present and discuss experimental data supporting these different models as well as the aspects that still remain mostly speculative (**Xue et al., 2023, in revision, Annexe 1**).

In the PotyMove project, as potyviruses have developed strategies to hijack the host secretory pathway and plasmodesmata for their transport (Laliberté and Sanfaçon, 2010), consequently we aimed at identifying membrane and plasmodesmata proteins that interact with three key viral proteins baits involved in cell-to-cell movement: Cylindrical Inclusion helicase (CI), "Pretty Interesting Potyviridae ORF" (PIPO) and the "second 6K molecular-weight membrane anchoring protein" (6K2). In parallel high throughput plasmodesmata proteomics and genetics approaches would allow to identify new host factors involved in potyviral cell-to-cell movement.

C.3 Approche scientifique et technique

The molecularly well characterized potyvirus turnip mosaic virus (TuMV) was chosen to address the different questions of the project. Turnip mosaic virus is both an excellent model system because it infects Arabidopsis (for which numerous tools and experimental approaches are available), and is also a major pathogen that infects at least 418 host species (Gibbs et al., 2015). Resistance-breaking TuMV isolates can be easily scored for resistance durability analysis (Gallois et al., 2010). Molecular tools derived from TuMV were available in both P1-BFP and P3-GAFL laboratories.

Four complementary Tasks were performed in parallel. In Task1, two candidate genes, already available thanks to a collaboration between P1-BFP, collaborators, and P2-LBM, were analyzed right from the beginning of the project in an attempt to validate their involvement in potyvirus movement. The Presequence metalloprotease (PreP) protein, a P3N-PIPO interactor identified through a Yeast Two Hybrid screen, and the remorin (REM) a potential regulator of TuMV propagation. The methods developed and tested on these two candidates were also used for functional validation of the selected candidate genes further identified in Task 2.

Task2 intended to identify new membrane- and/or plasmodesmata-associated protein interactors of three key viral baits by means of two strategies: in vivo screening using Split-ubiquitin membrane Yeast Two Hybrid system (SUY2H) and pull-down assays from enriched fractions of plasmodesmal proteins, coupled with plasmodesmata comparative proteomics (Task2). Indeed, as we reasoned that PD may contain sets of proteins which control MP or viral movement complex contacts at PD during viral propagation, we hypothesized that the cell-to-cell movement of potyvirus could induce potential changes in the PD proteome in infected plants. Therefore, the third objective in Task2, was to perform a comparative analysis of PD proteome in healthy and infected cells, and to select promising candidates for further functional validation in Task1.

In Task 3, in order to identify candidate genes potentially involved (directly or indirectly) in potyvirus movement, high throughput genetic approach complementary to the proteomics approaches described in Task-2 were developed. Two independent forward genetic screening approaches were done: one based on Arabidopsis natural variability, the other by screening for resistance gene to a resistance-breaking viral strain, in a mutagenized Arabidopsis population. In both cases, we aimed at proceeding until cloning the underlying genes responsible for limiting the virus movement within the plant.

Task4, was the only task dependent on the three others. This ask aimed at benchmarking the candidates characterized in previous tasks for their potential in plant breeding. In particular, we aimed at providing experimental evidence that the candidate genes identified in Arabidopsis could be transferred to crops.

In summary, the PotyMove scientific and technical program was of multidisciplinary nature, involving both targeted approaches (focused on PreP and REM) and interactomics, proteomics, direct and forwards genetics approaches.

C.4 Resultats obtenus

*The references indicated **in bold** correspond to publications of PotyMove partners*

Task1. Functional validation of PreP, REM and other candidate genes in potyvirus movement

Objective

The aim of Task1 was to perform functional validation assays for two available candidate genes, PreP and REM. The methods developed and tested on these two candidates were planned to pave the way for the functional validation of other selected candidate genes further identified in Task2 and Task3 throughout the duration of the project.

Results

PreP candidate: Presequence metalloprotease (PreP) is a P3N-PIPO interactor identified by Dr. WA Miller (Univ. Iowa) using Y2H. TuMV infection assays in At-PreP1-PreP2-KO lines (Salk_048944-133220) showed that systemic accumulation of TuMV does not reproducibly differ between KO and WT lines. P3-GAFL performed in parallel quantitative phenotyping by following the infection of TuMV-GFP using a GFPCam system **(Bastet et al., 2018)** involving P1-BFP and P3-GAFL. No difference in the propagation of TuMV-GFP could be observed in At-PreP1-PreP2-KO lines compared to Col-0 WT. Therefore, this candidate was not functionally validated, and we decided to focus on REM candidate and on one of the four other candidates identified in Task2 (AtHVA22) see below.

REM candidate: REMORIN proteins belong to a plant-specific multigene family that localize in plasma membrane nanodomains and in plasmodesmata. We previously showed that in *Nicotiana benthamiana*, group 1 StREM1.3 limits the cell-to-cell spread of a potexvirus without affecting viral replication. This prompted us to check whether an effect on viral propagation could apply to potyvirus species *Turnip mosaic virus* (TuMV) and *Potato virus A* (PVA). Our results show that StREM1.3 transient or stable overexpression in transgenic lines increases potyvirus propagation, while it is slowed down in transgenic lines underexpressing endogenous NbREMs, without affecting viral replication. TuMV and PVA infection do not alter the membranous localization of StREM1.3. Furthermore, StREM1.3-membrane anchoring is necessary for its agonist effect on potyvirus propagation. StREM1.3 phosphocode seems to lead to distinct plant responses against potexvirus and potyvirus. We also showed that StREM1.3 interacts in yeast and *in planta* with the key potyviral movement protein CI (cylindrical inclusion) at the level of the plasma membrane but only partially at plasmodesmata pitfields. TuMV infection also counteracts StREM1.3-induced plasmodesmata callose accumulation at plasmodesmata. Altogether, these results showed that StREM1.3 plays an agonistic role in potyvirus cell-to-cell movement in *N. benthamiana*. Those results were published in the paper **(Rocher et al., 2022)**, involving P1-BFP and P2-LBM.

In the frame of PotyMove, P2-LBM in collaboration with P1-BFP, made also novel observations that REM mobility, phosphorylation status and nanodomain organization are changed by potexvirus infection. Those results were also published in two publications shared between P2-LBM and P1-BFP (**Gouguet et al., 2020; Perraki et al., 2018**).

Functional validation of the candidate AtHVA22a (identified in Task2) in TuMV propagation

Using Split-Ubiquitin membrane yeast two hybrid assays (Task2), we screened an Arabidopsis cDNA library for interactors of TuMV-6K2. We isolated AtHVA22a (*Hordeum vulgare abscisic acid responsive gene 22*) that belongs to a multigenic family of transmembrane proteins, homologous to Reep/DP1/Yop1 family proteins in animal and yeast. HVA22/DP1/Yop1 family genes are widely distributed in eukaryotes, but the role of HVA22 proteins in plants is still not well-known, although proteomics analysis of PD fractions purified from Arabidopsis suspension cells showed that AtHVA22a is highly enriched in a plasmodesmata proteome (Brault et al., 2019). We further confirmed the interaction between TuMV-6K2 and AtHVA22a in yeast, as well as in planta by using bimolecular fluorescence complementation. Furthermore, we showed that TuMV-6K2/AtHVA22a interaction occurs at the level of the viral replication compartment (VRC) during TuMV infection. Finally, we showed that the propagation of TuMV is increased when AtHVA22a is overexpressed in planta but slowed down upon mutagenesis of AtHVA22a by CRISPR-Cas9. Altogether, our results indicate that AtHVA22a plays an agonistic effect on TuMV propagation. The publication **Xue et al. in preparation**, shared by P1-BFP and P3-GAFL will be submitted to MPP before the end of 2023.

Outputs/Expected: 100%

We validated the role of Remorin in TuMV movement and invalidated the role of PreP in this process. We validated the role of one of the candidates identified in task 2 (AtHVA22a).

This Task involved two PhD students, one Master 2 student.

Task2: Identification of new host components involved in potyviral cell-to-cell movement via proteomic and biochemical approaches

Objective

We reasoned that PD may contain sets of proteins which control MP or viral movement complexes contacts at PD during viral propagation, and we hypothesized that the active process of potyvirus cell-to-cell movement could induce potential changes in the PD proteome in infected plants. We aimed at identifying PD and membrane-associated interactors of the three viral baits by pull-down experiments and SUY2H (Task2.1 and Task2.2) and performing a comparative analysis of PD proteome in healthy and infected cells (Task2.3).

Results

Task2.1 Identification of CI, 6K2 and P3N-PIPO plasmodesmal interactors through pull-down experiments from PD proteins extracts

The three key movement proteins, CI, 6K2 and P3N-PIPO, the negative control GFP and the positive control (2B movement protein of GFLV, known to recruit PDLP1, a PD marker) were successfully over-expressed in *Escherichia coli* as GST-fusions (P1-BFP) and used as baits for pull-down experiments from PD-enriched proteins (P1-BFP and P2-LBM). PD purification (the challenging step in this Task) was successfully achieved after mechanical disruption of Arabidopsis cells, isolating wall fragments containing intact PD. PD-derived membranes were then "released" from the extracellular matrix by using cell wall degrading enzymes (cellulase) and recovered by high-speed centrifugation. From the screening, two candidates (6K2 interactors) were selected for further functional validation in Task1: **MCTP6 (At1G22610), Multiple C2 Domains and transmembrane region protein**, involved in membrane-tethering at plasmodesmata (Brault et al., 2019), and **HIN1-like3 (At5G06320) (NHL3)**, a plasma membrane protein whose overexpression correlates with increased resistance to phytopathogenic bacteria.

We could not confirm by BiFC the interaction between MCTP6 and TuMV-6K2, suggesting that both proteins would not directly interact. Very recently, Christine Faulkner's team showed that NHL3 is involved in the regulation of PD permeability with other plasmodesmata located proteins (PDLPs) during pathogen infection (Tee et al., 2023). This team sent us the mutants lines described in Tee et al. (2023) to analyze TuMV-GFP propagation survey in those mutants, which remains to be done.

Task2.2. Identification of CI, 6K2 and P3N-PIPO membrane-associated interactors through an exploratory screen based on SUY2H

The screening of an Arabidopsis SUY2H cDNA library available in P2-LBM, allowed the identification of 68 candidates among which 12 were selected according to criteria described in Task 2.4 of the project. These candidates were cloned and the validation of the interactions observed in yeast was further done in planta by BIFC (Task 1). Two of these candidates were selected for further functional validation in Task1: a **CI-interactor, DIRIGENT (DIR) protein (AT1G55210)**, a defense-related protein, for which we validated the interaction with CI by BiFC, but functional validation of the role of this protein in TuMV movement could not be done by lack of time.

A 6K2-interactor, **HVA22a (AT1G74520)** was chosen for functional validation in Task 1. AtHVA22a protein belongs to the multigene HVA22 family (Chen et al., 2002; Shen et al., 1993, Shen et al., 2001) which gathers plant homologs of proteins responsible for the constriction of ER tubules in mammals and yeast, the Reep/DP1/Yop1 family (Chen et al., 2002). Yop1 regulates vesicle trafficking in stressed yeast cells (Brands, 2002). However, the role of HVA22 gene family proteins is very poorly known in plants, besides their potential involvement in autophagy and dormancy (Chen et al., 2009), response to abiotic stresses (Chen et al., 2002; Gomes Ferreira et al., 2019; Wai et al., 2022) and in plant immunity towards fungal infection in rice (Meng et al., 2022). In Arabidopsis, eleven HVA22 homologues, designated AtHVA22a to k, can be separated into four subfamilies, with AtHVA22a, b and c grouped in the group. Among the HVA22 family in Arabidopsis, only the two closely related members AtHVA22a and ATHVA22c are highly enriched in a PD proteome (Brault et al., 2019). This is why we also performed functional assays (see Task4) with this second AtHVA22c member and also showed that it is probably involved in TuMV propagation (Doctorate of MS Xue, defense planned on the 25th of September 2023).

Task.2.3. Comparison of PD proteome and structure in healthy and infected cells

This was probably one of the most challenging Tasks of the PotyMove project: production of transgenic arabidopsis cell suspensions expressing inducible-viral MP and TuMV-GFP replicon. The CI, 6K2, P3N-PIPO and GFP were cloned into the binary vector pTA7002 with a dexamethasone-inducible promoter (IE-CDD, P1-BFP). *Agrobacterium tumefaciens*–mediated transformation of Arabidopsis cell suspensions was then performed.

Unfortunately, no clonal arabidopsis cell cultures transgenic for each of the pTA7002-MP constructs could be obtained yet, despite multiple repeats. A potential remediating option was initiated at the mid-stage aiming at generating Arabidopsis transgenic plants lines expressing inducible-viral MP (using the same constructs as those used for cell suspension transformation) in a collaboration between P1-BFP and P3-GAFL. However, no transgenic lines could be obtained so far for reasons we do not explain.

Outputs/expected: 66%

Task2 allowed us (i) to identify membrane-associated and/or PD proteins interacting with the movement proteins CI and 6K2 (ii) to select four promising candidates for further functional validation. However, we could not establish the third expected output, the list of PD proteins that are differentially expressed in "healthy" vs "infected" PD- cell suspensions.

Two master 2 participated to Tasks 2.1 and 2.2.

Task3: Identification of new host components involved in potyviral cell-to-cell movement via genetic approaches

Objective

In order to identify other candidate genes potentially involved (directly or indirectly) in potyvirus movement, another high throughput genetic approach complementary to the proteomics approaches described in Task2 was performed by P1-BFP and P3-GAFL, using forward genetic approaches based on natural diversity or induced diversity screening.

Results

Task3.1 Cloning of resistance genes affecting potyvirus-movement in natural Arabidopsis accessions

P1-BFP focused on positional cloning of candidate genes on chromosome 1. The genomic region spans around 60 genes, 18 presenting a non-silent mutation between the 2 parents (susceptible vs resistant). In silico analysis of mutations effects was conducted, but did not reveal any effect on protein translation nor folding. F2 genotyping has been done. Recombinants were selected for phenotyping, combined with HIFs and RILs. This task has not been completed so far.

Task 3.2 and 3.4 Screening of an Arabidopsis mutagenized population and mapping

Using the large-scale inoculation method developed by P3-GAFL combined with the GFP-camera imaging, more than 4000 mutagenised Arabidopsis have been screened for resistance to a Resistance-Breaking (RB) TuMV-GFP. Ten independent families have been collected, showing at least partial resistance to the RB TuMV-GFP. F2 mapping populations have been developed for 5 mutations. In the frame of the Doctorate of Mamoudou Diop (University of Marseille), Segregating F2 populations have been analysed for segregation of the resistance to the TuMV-RB isolate. We have studied in depth the resistance associated with a dwarf arabidopsis mutant, named 11D. We could show by segregation analyses and backcross that the mutation associated with resistance to TuMV-RB is likely to be recessive and is not associated with the single mutation associated with the dwarf phenotype. Indeed, whereas mapping analyses clearly showed that the dwarf mutation is linked to chromosome 3, we could not find any marker associated with the resistance to TuMV-RB. We resorted to building bulks of resistant and susceptible F2 plants and went directly to whole genome sequencing of the bulks (see below). F2 populations were tested also for two other candidates (53A and 19D). Overall, 10 bulks of resistant/susceptible plants have been sequenced by illumina. Bioinformatic analyses have been delayed but will be carried out shortly to find potential SNPs possibly underlying the resistances.

Outputs/Expected: 33%

We expected to identify recessive resistance genes blocking the potyvirus movement in Arabidopsis later in infection other than the well-known eukaryotic initiation factors. However; the project did not allow us to clone candidate genes resulting from the screening of the mutant collection. However, this is in a very good underway for the Task 3.2.

One Master 2 and one PhD student participated to Task3.

Task4: Evaluation of candidate genes for breeding durable resistance to potyviruses

Objective

A major drawback of using genetic resistance is the emergence of resistance-breaking strains. Thus, for durable resistance, it is recommendable to select resistance sources affecting essential but distinct virus factors

and acting on various steps of the virus life cycle, to counter-act the resistance-breaking variants. In Task4, we aimed at evaluate the resistance spectrum conferred by the Arabidopsis KO candidate genes plants toward different potyvirus species, to pyramidize KO for identified candidate genes together with KO for *eIFiso4E*, and to check whether the level of resistance durability towards TuMV in Arabidopsis is increased. We also aimed at providing experimental evidence that the candidate genes identified in PotyMove could be transferred to a crop species, tomato.

Results

Task 4.1 Evaluation of the resistance spectrum.

P3-GAFL has obtained isolates of agro-inoculable GFP-tagged potyviruses: GFP-ClYVV and GFP-WMV from Dr Nakahara (Hokkaido University, Japan) and Dr Desbiez (INRA PV AVIGNON), respectively. The GFP camera imaging has been validated in Arabidopsis wild type Columbia for ClYVV-GFP. This helped to assay putative quantitative resistance of our first candidates (REM, HVA22a, see Task1). P3-GAFL further assessed how the targeted allele modification of the arabidopsis eIF4E1 factor can lead to a large and efficient resistance to the major group of potyviruses, including TuMV-RB variant. This study established a proof of concept for editing susceptibility genes in order to create new genetic resistances and expand resistance spectrum without affecting the plant development and agronomic traits, useful for the Task4 of PotyMove (See **Bastet et al., 2018,** P1-BFP and P3-GAFL). Furthermore, P3-GAFL established how base editing technology can be used to introduce point mutation in the plant genome (**Bastet et al., 2019**, P3-GAFL), a technique that will was also used for the validation of the role of Hva22a in Tomato (see Task 4.3).

For the candidate AtHVA22a, P3-GAFL obtained four independent homozygous CRISPR-Cas9 mutants affected in the C-terminal cytoplasmic domain of AtHVA22a that were used for the functional validation in Task1.

Task 4.2 Can we control RB events?

A high-throughput RB evaluation protocol, combining large-scale plant inoculation with a non-RB TuMV inoculum and GFP detection of RB events, has been set up. Given the absence of resistance associated with the double knock-out in both AtPrep1 and AtPrep2 (see Task 1), we have not carried out the introgression in eifiso4e. This task should have been resumed by knocking out eIFiso4E directly in AtHVA22a mutants but it could not be done by lack of time.

Task 4.3 Can we transfer the candidate resistances to crops.

As soon as the project began, homology search revealed a unique PreP homolog in tomato (Solyc01g108600). A construct targeting its first exon has been designed to generate CRISPR/Cas9-mediated gene inactivation. Despite the very high number of regenerated callus generated by P3-GAFL (more than a thousand), only chlorotic explant could be regenerated, in both genetic backgrounds tested, one fully susceptible to the potyvirus PVY and one with a frequently overcome eIF4E1 allele (see (Lebaron et al., 2016). This suggests that unlike in Arabidopsis, the Prep gene could encode an essential protein for tomato development in line with its function. Because of that, and because of the lack of resistance phenotype associated with Atprep1 Atprep2 double KO in Arabidopsis (See Task1), this experiment has been stopped.

Instead, we have started focusing on the candidates isolated by Partner 1-BFP in Task 2.2. Orthologs of the Arabidopsis genes AtHVA22a and AtHVA22c have been characterized in tomato as, respectively Solyc03g116350 and Solyc05g007300. Specific RNA guides for both genes have been cloned independently, targeting the second and third exons of HV22a, and only the third exon for HVA22c, respectively. CRISPR-Cas9 constructs have been generated and independently transformed into the two above-mentioned tomato genetic background (S.lyc cv WVA106 and S.lyc cv microtom). Edition events were genotyped for both constructs in both genetic backgrounds. 2 independent lines were isolated for HVA22A gene, and 4 independent lines for HVA22c gene in which the edition event result in gene knockout. Plants were selfed and the T-DNA was segregated away. We therefore obtained stabilized knock-out edited plants on T2 generation for hva22a and on T1 generation for hva22c. To stack mutations, we crossed the single KO mutants for hva22a and hva22c. 4 lines homozygous for both mutations were obtained in WVA106 background and 1 line in microtom.

Mutant analyses.

As a first step, we have assessed the potential effect of these edited lines, on resistance of PVY N605. No complete resistance was obtained, neither on simple mutant lines nor on double mutant lines. Future tests will be carried out by analyzing the cell-to-cell movement of a GFP-tagged PVY clone (under way at P3-GAFL) to mirror the experiments performed by P1-BFP on CRISPR-Cas9 AtHVA22a Arabidopsis mutants obtained by P3-GAFL (Xue et al., 2023, in preparation), and with other viruses (such as potexvirus). We have also started looking at potential side effects (for plant breeding) of inactivating HVA22a and HAV22c in tomato. Indeed, we reported several plant and fruit abnormal phenotypes associated with those genes' knock-out, such as a reduction in plant size on the

double mutant lines, or abnormal flower and fruit shape and content for mutant hva22a. This will be carried out with P1-BFP. Analysis of cell to cell permeability in those mutants will be performed.

Outputs/Expected: 50%
Arabidopsis and Tomato edited lines were obtained for the two main candidates HVA22a and HVA22c, genotyped and the PVY-GFP infection still remains to be analysed.
One PhD student participated to Task4

C.5 EXPLOITATION DES RESULTATS

The major advances and discoveries of the proposed research in PotyMove have been be published in scientific journals as well as presented at international and national meetings. All Partners have been successful in publishing in scientific journals, as illustrated by the list of articles published by the group members in international research journals (MPP, Plant Cell, Plant Physiol., Plant Biotechnology Journal, J. Virol., Plant Cell, Viruses…). Partners and collaborators of the project have regularly discussed their common valorization strategy for results of the PotyMove project. Results remaining and coming out from the shared project will also be divulgated in a concerted manner respecting the contribution of each Partner.

C.6 DISCUSSION

Globally, the degree of achievement of the initial objectives of PotyMove reaches about 60%.
We identified and validated two plant factors involved in potyvirus movement. Using the major crop Tomato and through genome editing approaches, we also started to assess how the knowledge on the role of HVA22 protein can be used to propose genetic resistances to breeders.

In Task2, the project initially planned to identify CI, 6K2 and P3NPIPO interactor, finally focused on the 6K2 protein, for which we identified the most interesting cellular partners (HVA22, NHL3, MCTP6). Although the functional validation of those three candidates in potyvirus movement could not be performed in the frame of PotyMove, they are still identified as potential players in Potymove movement and or replication.

The technical lock linked to the difficulty to extract PD proteins from Arabidopsis cells suspension for pull-down experiments in Taks2 was successfully lifted. On the other side, the technical challenge of the obtention of an Arabidopsis suspension cell culture system based on inducible release of viral TuMV RNA replicons from stably integrated DNA “pro-replicons” or even individual movement proteins, was not lifted. This explains that the deliverables D2.3 a, b and c were abandoned.

The two complementary genetics screens for resistance genes affecting potyvirus-movement, using Arabidopsis natural and induced population diversity (Task3), also took much more time than what was expected. However, 10 bulks of resistant/susceptible plants have been sequenced by illumina, and bioinformatic analyses will be carried out shortly to find potential SNPs possibly underlying the resistances in those mutants, independent of the eIF4E-medited resistance.

Although we showed that Remorin and HVA22 proteins are involved in potyvirus movement, it still remains to unravel the molecular mechanisms behind their positive effect on viral propagation.

The 6K2 protein is a small membranous viral protein crucial for potyvirus infection, being responsible for the formation of 6K2-induced vesicles from the ER, key players in coupling potyvirus replication and intercellular movement (**Xue et al., 2023, Annexe 1**). However, it is still unclear what host cell factors, lipids and other components may be involved in ER-derived vesicle maturation for TuMV, which is critical for both the replication and the cell-to-cell movement of the virus.

During the PotyMove project, we’ve explored another path, after having observed that TuMV infection strongly induces the biogenesis of lipid droplets, that locate in the proximity of TuMV replication vesicles 6K2, where we also observed partial relocalization of the candidate HVA22a (**Xue et al., in preparation**). Lipid droplets (LDs) are organelles composed of a single phospholipid layer enclosing neutral lipids, involved in energy production, membrane biogenesis and stress signaling. Recently, LDs have also been shown to be required by some animal (+) RNA viruses for their replication, and we obtained strong preliminary results revealing that it is probably the case for potyviruses and that 6K2 plays a central role in this process. We’ve submitted the LiDroVir project (AAPGeneric 2023 ANR call, final answer mid-July 2023) that aims at understanding of the involvement of lipid droplets (LDs) in plant virus infection.

Interestingly, in plants, LDs may also contribute to refurbishing the PD interior (Paul et al., 2014). In animals, the homologs of HVA22 (Reep proteins) have been shown to regulate lipid metabolism by interacting with

seipin (a structural protein of LDs). Although totally disconnected from the PotyMove project, the LiDroVir project (if funded) might allow to replace the candidates identified in Potymove in a network of plant proteins involved in LDs and crucial for potyvirus replication and movement.

C.7 Conclusions

In conclusion, the PotyMove project was an ambitious project, but the quality of the consortium and the complementarity of the approaches allowed to overcome some of those technical and scientific barriers, and lead to the identification of promising host factors involved in potyvirus movement.

C.8 References

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D LISTE DES LIVRABLES

N°	Intitulé	Nature*	Date de fourniture			Partenaires (souligner le responsable)
			Prévue initialement	Replanifiée	Livrée	
D0-a	Progress report	Report	12, 24, 36, 48	18, 30, 60	18, 30, 60	1, 2 & 3
D0-b	Final scientific report / expenses	Report	48	60	60	1, 2 & 3
D1.a	Validation of the role of REM and PreP in potyvirus movement in Arabidopsis and Tomato	Milestone	24, 36		30, 60	1, 2 & 3
D1.b	Validation of the role of new factors from Task2 in potyvirus movement in Arabidopsis	Milestone	24, 42	54	60	1 & 3
D1.c	*In planta* fine subcellular localization of the interactions between MP and PD and/or membrane associated factors	Milestone	48		60	1
D2.1-a	PD-proteins purified from Arabidopsis cell suspensions	Molecular tools	3, 6, 12		3, 6, 12	1 & 2
D2.1-b	GST-MP fusions purified from *E. coli*	Molecular tools	6, 14		14	1
D2.1-c	List of PD interactors of viral MPs	Report (Deliverable)	18, 24	30	30	1 & 2
D2.2	List of membrane-associated interactors of viral MPs (SY2H)	Milestone	18	30	30	1
D2.3-a	Transgenic Arabidopsis cell lines displaying inducible GFP-MP and TuMV-GFP expression	Milestone	24	48	Abandoned	1 & 2
D2.3-b	PD-proteins purified from induced and non-induced Arabidopsis transgenic cell suspensions	Milestone	30	Depends on D2.3-a	Abandoned	1 & 2
D2.3-c	List of differential PD-proteins changes in infected vs healthy conditions	Report (Deliverable)	36	Depends on D2.3-a	Abandoned	1 & 2
D2.4	List of candidate genes for Task1.1	Report (Deliverable)	18, 36		18, 30	1, 2 & 3
D3.1	Isolation of natural or EMS Arabidopsis lines with resistance to potyvirus movement	Milestone	24	40	Unachieved	1 & 3
D3.2	Underlying resistance genes cloned	Milestone	24, 42	42	Unachieved	1 & 3
D3.3	Functional validation of genes (from D3.2) performed	Milestone	30, 48	Depends on D3.2	Unachieved	1 & 3
D4.1	Resistance spectrum and durability of candidate genes in Arabidopsis	Milestone	12, 36	40	Partially-Achieved	1 & 3
D4.2	Role of PreP and REM candidates in tomato	Milestone	48		Partially Achieved 60	1 & 3

E IMPACT DU PROJET

E.1 INDICATEURS D'IMPACT

Nombre de publications et de communications (à détailler en E.2)

		Publications multipartenaires	Publications monopartenaires
International	Revues à comité de lecture	5	4
	Ouvrages ou chapitres d'ouvrage		
	Communications (conférence)	6 oral presentations (invited) 6 posters	
France	Revues à comité de lecture	1	
	Ouvrages ou chapitres d'ouvrage		
	Communications (conférence)	4 oral presentations (invited) 7 oral presentations 5 posters	
Actions de diffusion	Articles vulgarisation		
	Conférences vulgarisation		
	Autres (Rapports de stages de Master2, manuscrits de thèse)	4 PhD manuscripts	4 Master 2 reports

Autres valorisations scientifiques (à détailler en E.3)

Le projet PotyMove n'a donné lieu à aucune autre valorisation scientifique autre que les publications, ou manuscrits de thèse ou rapports de stage de Master 2.

E.2 LISTE DES PUBLICATIONS ET COMMUNICATIONS

I. International

1. Revues à comité de lecture

A. Bastet, B. Lederer, N. Giovinazzo, X. Arnoux, S. German-Retana, C. Reinbold, V. Brault, D. Garcia, S. Djennane, S. Gersch, O. Lemaire, C. Robaglia and J.L. Gallois. (**2018**). Trans-species synthetic gene design allows resistance pyramiding and broad spectrum engineering of virus resistance in plants. Plant Biotechnology Journal. pp. 1–13. doi: 10.1111/pbi.12896. (P3-GAFL in collaboration with P1-BFP).

A. Perraki, J. Gronnier, P. Gouguet, M. Boudsocq, A.F. Deroubaix, V. Simon, S. German-Retana, C. Zipfel, E. Bayer, S. Mongrand and V.Germain. (**2018**). The plant calcium-dependent protein kinase CPK3 phosphorylates REM1.3 to restrict viral infection. PlosPathogens, https://doi.org/10.1371/journal.ppat.1007378. (P2-LBM in collaboration with P1-BFP).

J.L. Gallois, B. Moury and S. German-Retana (**2018**). Role of the Genetic Background in Resistance to Plant Viruses. Int. J. Mol. Sci. 2018, 19, 2856; doi:10.3390/ijms19102856. (P1-BFP in collaboration with P3-GAFL).

Bastet, A., Zafirov, D., Giovinazzo, N., Guyon-Debast, A., Nogué, F., Robaglia, C. et al. (**2019**) Mimicking natural polymorphism in eIF4E by CRISPR-Cas9 base editing is associated with resistance to potyviruses. *Plant Biotechnology Journal*, 17, 1736– 1750. https://doi.org/10.1111/pbi.13096 (P3-GAFL)

Gouguet P., Gronnier J., Legrand A., Perraki A., Jolivet M.-D., Deroubaix A.-F., German-Retana S., Boudsocq M., Habenstein B., Mongrand S., Germain V. (**2021**). Connecting the dots: from nanodomains to physiological functions

of REMORINs. Plant Physiology, 185 (3), 632-649, https://dx.doi.org/10.1093/plphys/kiaa063, https://hal.inrae.fr/hal-03205360 (P2-LBM in collaboration with P1-BFP).

Zafirov, D., Giovinazzo, N., Bastet, A. and Gallois, J.L. (**2021**) When a knockout is an Achilles' heel: Resistance to one potyvirus species triggers hypersusceptibility to another one in Arabidopsis thaliana. Mol. Plant Pathol. 22, 334–347. https://doi.org/10.1111/mpp.13031 (P3-GAFL)

Rocher, M. Simon, Jolivet, MD, Sofer, L. Deroubaix, AF., Germain, V. Mongrand, S. German-Retana, S (**2022**). StREM1.3 REMORIN Protein Plays an Agonistic Role in Potyvirus Cell-to-Cell Movement in N. benthamiana. Viruses. 14, 574. https://doi.org/10.3390/v14030574. (P1-BFP in collaboration with P2-LBM).

Diop, M.; Gallois, J.-L. Exploring New Routes for Genetic Resistances to Potyviruses: The Case of the Arabidopsis thaliana Phosphoglycerates Kinases (PGK) Metabolic Enzymes. *Viruses* **2022**, *14*, 1245. https://doi.org/10.3390/v14061245 (P3-GAFL)

Xue, MS, Arvy, N. German-Retana, S. (**2023**). The mystery remains: how do potyviruses move between cells? Accepted for publication in Molecular Plant Pathology. Under revision (P1-BFP).

To be submitted
Xue, MS, Sofer, L., Arvy, N., Diop, M., Lion, R., Beucher, G., Bordat, A., Tilsner, J., Gallois, JL and German-Retana, S. AtHVA22a, a plant-specific homolog of Reep/DP1/Yop1 family proteins is involved in turnip mosaic virus movement. Will be submitted to MPP.

2. Ouvrages ou chapitres d'ouvrage
RAS

3. Oral Communications in International Conferences

German-Retana Sylvie. "Plant resistance to viruses (eIF4E)". International Advances in Plant Virology. Greenwich University, London, 7-9 September 2016. Key Note session

Mongrand Sébastien. «Group 1 REMORIN phospho-status defines its plasma membrane nanodomain organization, a link between phoshoinositides, nanodomain and actin cytoskeleton ». International worshop on plant membrane biology. Glasgow, UK, 7-13 juillet 2019. Invited speaker.

Mongrand Sébastien. « Discusion leader » de la session « Lipids at the interface with other players » + Présentation orale suite à sélection sur poster : « Group 1 REMORIN phospho-status defines its plasma membrane nanodomain organization, a link between phoshoinositides, nanodomain and actin cytoskeleton ». Gordon Research Conference on plant lipids. Galveston Texas, USA27 Jan-1 fev 2019.

Mongrand Sébastien. «Involvement of the plasma membrane in immunity against virus : the case of protein REMORINS». Webinar for DiSTAP-Disruptive and Sustainable Technologies for Agricultural Precision, Singapoure. Invitation N-H CHua., 29 oct 2021. Conférencier Invité

Mongrand Sébastien. "Role of the plasma membrane signalling during plant virus propagation". Hot Topics and Advances in Plant Cell Biology – Viktor Zarsky 65th birthday seminar. Prague, République tchèque, 3-4 mai 2023. Conférencier Invité

Mongrand Sébastien. « Plant plasma membrane nanodomain organisation : the example of protein REMORINS » Seeing is believing" Imaging workshop. Silke Robatzek and Kay Schneitz. 13 Octobre 2022 Conférencier Invité

4. Posters in international meetings:
V. Simon, A. Perraki J. Gronnier, A. F. Deroubaix, V. Germain, S. Mongrand & S. German-Retana. Remorin: a membrane raft plasmodesmal protein involved in potyvirus propagation? EMBO Workshop. Intercellular communication in development and disease. 10-15 July 2017, Berlin, Germany.

L. Sofer, K. Darling, G. Beucher, K. Elapata, E. Bayer, S. German-Retana & J. Tilsner. Candidate proteins for membrane constriction of the plasmodesmal endoplasmic reticulum. 18th International workshop on plant membrane biology. 7th-12th July 2019, Glasgow, Scotland.

A. Perraki, J. Gronnier, A. F. Deroubaix, V. Simon, P. Gouguet, M. Bouscocq, E. Bayer, S. German-Retana, S. Mongrand and V. Germain. Remorin: a membrane raft plasmodesmal protein limiting or promoting virus cell-to-cell movement of two distinct virus genera. IS-MPMI Meeting XVII, 14th-18th July 2019, Glasgow, Scotland.

MS Xue, L. Sofer, V. Simon, R. Lion, J. Tilsner and S. German-Retana. Plasmodesmal Components Involved in Cell-to-cell Transport of Potyviruses---Focus on HVA22a candidat. EMBO workshop Intercellular communication in development and disease (planned at St Andrews, Scotland, postponed as a visio-conference, July 2021).

MS Xue, L. Sofer, V. Simon, R. Lion, N. Arvy, M. Diop, JL. Gallois, J. Tilsner and S. German-Retana. A plant-specific homolog of DP1/Yop1 family proteins plays a proviral role in potyvirus infection. American Society for Virology Annual Meeting (ASV), Madison, USA, 14-21 July, 2022.

MS Xue, L. Sofer, V. Simon, R. Lion, N. Arvy, M. Diop, JL. Gallois, J. Tilsner and S. German-Retana. A plant-specific homolog of Reep/DP1/Yop1 family proteins plays a proviral role in potyvirus infection. ICPP2023 Meeting, Lyon, France, 20-25 July 2023.

II. France

1. Revues à comité de lecture

J.L Gallois and S. German-Retana. (2023). Rôle des facteurs d'initiation de la traduction 4E dans la résistance des plantes aux potyvirus : de la découverte des résistances naturelles à l'édition des gènes. Virologie (in press)

2. Ouvrages ou chapitres d'ouvrage

RAS

3. Communications (conférence)

Oral communications

Gronnier, A. Perraki, P. Gouguet, M. Boudsocq, A.F. Deroubaix, V. Simon, S. German-Retana, E.Bayer, S. Mongrand & V. Germain. Making a functional link between plasma membrane nanodomain features, regulation of cell-to-cell connectivity and Potato Virus X propagation. 16èmes Rencontres de Virologie Végétale, Aussois 15-19 janvier 2017.

A. F. Deroubaix, P. Gouguet, J. Gronnier, S. German-Retana, Bouscocq M., Ott T, S. Mongrand and V. Germain. PlAMV/Arabidopsis pathosystem for studying Remorins and membrane nanodomains in potexvirus cell-to-cell movement. 17 èmes Rencontres de Virologie Végétale, Aussois 27-31 janvier 2019.

Gallois Jean-Luc. Résistance aux virus: Quand la variabilité naturelle guide l'édition de gènes. AFBV 9ème Colloque des Biotechnologies Végétales Paris, France Invited speaker. (2019)

S. Mongrand « Plant-specific membrane-bound REMORIN proteins involved in immunity against phytovirus » Microbiology Day in Bordeaux. Invitation H Wodrich, Bordeaux, 20 Février 2020.

Xue MS., Sofer L., Vincent S., Lion R., Tilsner J., German-Retana S. (2021). Plasmodesmal Components Involved in Cell-to-cell Transport of Potyviruses---Focus on HVA22a candidate. 18èmes Rencontres de Virologie Végétale, Aussois, Septembre 2021.

Gallois Jean-Luc. Resistance to viruses by loss-of-susceptibility: from natural variation to edited genes. XXIII Journées Francophones de Virologie conférence en ligne Invited speaker (2021).

Gallois Jean-Luc. (2021) Resistance to viruses by loss-of-susceptibility in tomato: from natural variation to edited genes. 10ème journée du réseau EFOR. Invited speaker

Xue MS., Sofer L., Vincent S., Lion R., Tilsner J., German-Retana S. (2021). HVA22a and HVA22c, two candidate proteins involved in potyvirus replication and movement. Presented at : Journée de l'École Doctorale des Sciences de la Vie et de la Santé de Bordeaux-, Bordeaux, France (2021-05-03 - 2021-05-04), Prix de la meilleure présentation.

Xue MS. HVA22a: a candidate protein involved in Potyvirus replication and movement. Journée de l'École Doctorale des Sciences de la Vie et de la Santé de Bordeaux-, Bordeaux, France (2022).

L. Jambou, L. Sofer, N. Arvy, V. Simon, JL. Gallois, D. Coulon, C. Brehelin and S. German-Retana. Lipid droplets: new actors of the plant virus infection. 19èmes Rencontres de Virologie Végétale, Aussois 15-19 janvier 2023.

MS Xue, L. Sofer, V.Simon, N. Arvy, A.Bordat, R. Lion, M. Diop, JL. Gallois, J. Tilsner, and Sylvie German-Retana. A plant-specific homolog of DP1/Yop1 family proteins plays a proviral role in potyvirus infection. 19èmes Rencontres de Virologie Végétale, Aussois 15-19 janvier 2023

Posters presentation

L. Sofer, V. Simon, A. Barra, *L. Giordano**², T. Michon, J. Walter & S. German-Retana. Towards the identification of plamodesmal and membranous host components involved in potyvirus movement. 17èmes rencontres de virologie végétale, 15-19 janvier 2017, Aussois, France.

V. Simon, *A. Perraki** J. Gronnier, *A. F. Deroubaix**, V. Germain, S. Mongrand & S. German-Retana. Remorin: a membrane raft plasmodesmal protein involved in potyvirus propagation? 17èmes rencontres de virologie végétale, 15-19 janvier 2017, Aussois, France.

L. Sofer, V. Simon, X. Arnoux, A. Barra, T. Michon, J. Walter and S. German-Retana. Characterization of plamodesmal and membranous plant candidates potentially involved in cell to cell movement of Turnip mosaic virus. 18èmes rencontres de virologie végétale, 27-31 janvier 2019, Aussois, France.

V. Simon, A. F. Deroubaix, V. Germain, S. Mongrand and S. German-Retana. Remorin: a membrane raft plasmodesmal protein involved in potyvirus propagation? 18èmes rencontres de virologie végétale, 27-31 janvier 2019, Aussois, France.

C. Lebaron and JL Gallois. Developing gene editing strategies in Tomato as a method to translate new genetic resistance to viruses. 19èmes rencontres de virologie végétale, 15-19 janvier 2023, Aussois, France.

III. Actions de diffusion

Rapports de Master2

(*Ne sont listés ici que les rapports des Stagiaires ayant bénéficié d'une gratification de stage financée par le projet PotyMove)*

Jules Dupuis 2017, Master BioVGPA, co-habilité Université d'Angers, de Bretagne Occidentale de Nantes, de Poitiers, de Rennes 1, de Tours et Agrocampus Ouest. Identification de facteurs de plantes impliqués dans le mouvement de cellule à cellule des Potyvirus (Task 2.)

Mathilde Aime 2018, Master 2 Biologie Agrosciences, Université de Bordeaux. Plasmodesmal components involved in cell-to-cell transport of potyviruses (Task 2.)

Guillaume Beucher, 2019, Master 2 Microbiologie et Immunologie, Université de Bordeaux. Validation fonctionnelle du rôle du facteur cellulaire AtHVA22a dans le mouvement du virus de la mosaïque du navet. (Task 1.)

Charles Bastide, 2020, Master 2 Biologie Agrosciences, Metabolic variability and genetic architecture of *Arabidopsis thaliana* in response to *Turnip Mosaic Virus* in common garden. (Task 3)

Manuscrits de thèse

Anna Bastet. 2018. Élargissement du spectre de résistance aux potyvirus : Utilisation de la plante modèle Arabidopsis thaliana. Docotral thesis, Aix-Marseille Université, 168p.

Anne-Flore Deroubaix. 2019. Rôle de la rémorine et des nanodomaines membranaires dans la signalisation de la réponse aux phytovirus. Doctoral Thesis, University of Bordeaux, 260p.

Mamoudou Diop. 2022. Vers la mise en place de nouvelles résistances génétiques aux potyvirus Par l'exploitation de facteurs de sensibilité Chez *Arabidopsis thaliana*. Doctoral thesis, Aix-Marseille Université, 181p.

Mingshuo Xue. Planned 25/09/2023. HVA22a, a plant-specific homolog oe endoplsmic reticulum shaping proteins, is involved in potyvirus propagation. Doctoral thesis, University of Bordeaux.

E.3 LISTE DES ELEMENTS DE VALORISATION

RAS

E.4 BILAN ET SUIVI DES PERSONNELS RECRUTES EN CDD (HORS STAGIAIRES)

Ce tableau dresse le bilan du projet en termes de recrutement de personnels non permanents sur CDD ou assimilé. Renseigner une ligne par personne embauchée sur le projet quand l'embauche a été financée partiellement ou en totalité par l'aide de l'ANR et quand la contribution au projet a été d'une durée au moins égale à 3 mois, tous contrats confondus, l'aide de l'ANR pouvant ne représenter qu'une partie de la rémunération de la personne sur la durée de sa participation au projet.
Les stagiaires bénéficiant d'une convention de stage avec un établissement d'enseignement ne doivent pas être mentionnés.
Les données recueillies pourront faire l'objet d'une demande de mise à jour par l'ANR jusqu'à 5 ans après la fin du projet.

Identification				Avant le recrutement sur le projet			Recrutement sur le projet				Après le projet				
Nom et prénom	Sexe H/F	Adresse email (1)	Date des dernières nouvelles	Dernier diplôme obtenu au moment du recrutement	Lieu d'études (France, UE, hors UE)	Expérience prof. Antérieure, y compris post-docs (ans)	Partenaire ayant embauché la personne	Poste dans le projet (2)	Durée missions (mois) (3)	Date de fin de mission sur le projet	Devenir professionnel (4)	Type d'employeur (5)	Type d'emploi (6)	Lien au projet ANR (7)	Valorisation expérience (8)
Xavier Arnoux	H	Unknown	June 2019	Master 2	France	CDD-IE Cobra-ANR project (2 years)	P1-BFP	Engineer (IE)	24 month	29/02/2019	No news	ND	ND	ND	ND
Roxane Lion	F	Roxane.lion@inrae.fr	2023	Licence Pro	France	CDD-TR (Sanofi, Mas Seeds,	P1-BFP	Technician (TR)	30 month	31/10/2021	CDD	INRAE	AI	No	Yes
Marion Rocher	F	marion.r94@laposte.net	2023	Master 2	France	None	P2-LBM	Engineer (IE)	30 month	30/04/21	Ingénieure Maïs Adour	Privé	Ingénieure	Non	Yes

Aide pour le remplissage

(1) Adresse email : indiquer une adresse email la plus pérenne possible
(2) Poste dans le projet : post-doc, doctorant, ingénieur ou niveau ingénieur, technicien, vacataire, autre (préciser)
(3) Durée missions : indiquer en mois la durée totale des missions (y compris celles non financées par l'ANR) effectuées sur le projet
(4) Devenir professionnel : CDI, CDD, chef d'entreprise, encore sur le projet, post-doc France, post-doc étranger, étudiant, recherche d'emploi, sans nouvelles
(5) Type d'employeur : enseignement et recherche publique, EPIC de recherche, grande entreprise, PME/TPE, création d'entreprise, autre public, autre privé, libéral, autre (préciser)
(6) Type d'emploi : ingénieur, chercheur, enseignant-chercheur, cadre, technicien, autre (préciser)
(7) Lien au projet ANR : préciser si l'employeur est ou non un partenaire du projet
(8) Valorisation expérience : préciser si le poste occupé valorise l'expérience acquise pendant le projet.

Annexe 1

Review accepted for publication in MPP (under revision in July 2023)

The mystery remains: how do potyviruses move between cells?

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Running head: How do potyvirus move between cells?
Word count of article: 7240 words (Summary: 172, Introduction: 983, Main text: 5508, Acknowledgments: 63, Table and Figure legends: 512)

SUMMARY

The genus *Potyvirus* is considered as one of the largest among plant single-stranded (+) RNA viruses, causing considerable economical damage in vegetable and fruit crops worldwide. Through the coordinated action of four viral proteins and a few identified host factors, potyviruses exploit the endomembrane system of infected cells for their replication, intra- and intercellular movement to and through plasmodesmata (PD). Although a significant amount of data concerning potyvirus movement has been published, no synthetic review compiling and integrating all information relevant to our current understanding of potyvirus transport is available. In this review, we highlight the complexity of potyvirus movement pathways and present three potential non-exclusive mechanisms based on i) the use of the host endomembrane system to produce membranous replication vesicles that are targeted to PD and move cell-to-cell ii) the movement of extracellular viral vesicles in the apoplasm iii) the transport of virions particles or ribonucleoprotein complexes through PD. We also present and discuss experimental data supporting these different models as well as the aspects that still remain mostly speculative.

INTRODUCTION

To invade the whole plant after their replication, plant viruses must move intracellularly to reach plasmodesmata (PD), the symplasmic tunnels between cells that are the gateways for this movement, then cross them to enter in neighboring cells, and finally enter sieve elements. Viruses are then passively transported with the source-to-sink flow of photoassimilates and are unloaded distantly from sieve elements into sink tissues (Schoelz et al., 2011). PD are structures unique to plants, crucial for the communication between plant cells, that allow the regulated passage of signals, from small molecules to macromolecules involved in plant growth, development and defense (Reagan and Burch-Smith, 2020). PD can be defined by three major structural components: the membrane-bound tube that leads to plasma membrane (PM) and cytoplasmic continuity between adjacent cells, the central desmotubule, which is an appressed form of the endoplasmic reticulum (ER) and the cell wall surrounding the PM (Reagan and Burch-Smith, 2020). Cell wall glucan deposition, insoluble glucans or callose, densifies at the neck regions of PDs, modulating PD permeability and affecting their size exclusion limit (SEL) (Benitez-Alfonso et al., 2010; German et al., 2023). Despite the symplasm continuity between cells, the SEL of PD constitutes a physical barrier that must be overcome by a virus to successfully move from cell-to-cell. Indeed, viral nucleic acids or ribonucleoprotein complexes are too large to move through the PD on their own and virions sizes are too large for them to passively cross PD. According to Schoelz et al. (2011) "if PD are the doorway out of the cell, then plant viruses must possess the tools to find the door as well as the keys to unlock the door". Indeed, plant viruses use active mechanisms to move from the site of replication within the cell to the PD for cell-to-cell movement. Plant virus genomes encode so-called Movement Proteins (MP), that interact with host proteins to modify PD and permit cell-to-cell transportation of the viral genome (Heinlein, 2015). Viral movement proteins have been identified in nearly all plant viruses (Tilsner et al., 2014) and display a range of common functions such as the ability to: i) non-specifically bind nucleic acids (Citovsky et al., 1992) ii) target themselves to PD and mediate their own cell-to-cell movement (Oparka et al., 1997; Wei et al., 2010b) and iii) increase PDs SEL, also known as gating (Howard et al., 2004; Oparka et al., 1997). Although a variety of models for how different plant viruses transport their genomes within and between cells have been proposed (Kumar and Dasgupta, 2021), two main categories have been described. The first one, exemplified by tobacco mosaic virus (TMV) (tobamovirus), may be common to all viruses that do not move as intact virions, including the triple gene block proteins (TGBp) containing potex- and hordeiviruses. Those viruses are considered as "non-tubule forming viruses" (because they do not morphologically drastically alter PD). Other viruses, illustrated for example by *Secoviridae*, *Bromoviridae* or *Caulimoviridae* members, move as intact virions. The MPs of these viruses form large tubules that insert into PD to allow the transport of intact virions between cells. Those viruses are considered as "tubule-forming viruses" and induce drastic PD alterations, with elimination of the desmotubule.

However, the cell-to-cell movement mechanism of the largest family of plant RNA viruses, the *Potyviridae*, does not fall into any of the two previous categories. The *Potyvirus* genus comprises plant viruses impacting crops of economic interest, with important pathogens such as plum pox virus (PPV), potato virus Y (PVY), soybean mosaic virus (SMV) or turnip mosaic virus (TuMV) (Inoue-Nagata et al., 2022). Potyviruses are non-enveloped, flexuous rod-shaped particles of 680- 900 nm in length and 11-15 nm in diameter. The viral genome is a positive sense, single-stranded RNA (+ssRNA) of around 10 kb, linked to a viral protein (VPg, Viral Protein Genome-linked) at its 5' end, and polyadenylated at its 3' end. When the virus enters host plant cells, it is uncoated and the viral RNA is translated into a large viral polyprotein sequentially cleaved into 10 proteins (Figure 1). This cleavage is performed by three viral proteins P1, HC-Pro and NIa-Pro. The 11th protein called PIPO for "Pretty Interesting Potyviridae ORF" is embedded in the P3 cistron. During the viral replication, RNA polymerase slippage leads to insertion of an additional adenine in some genomic RNA progeny molecules, bringing the PIPO-encoding sequence in frame with the P3 protein gene. As a result, a fusion protein named P3N-PIPO, which contains the N-terminal of P3 fused to the PIPO sequence is produced (Chung et al., 2008; Olspert et al., 2015; Rodamilans et al., 2015) (Figure 1). As many other viral proteins, potyviral proteins are multitasks and are involved in multiple functions such as replication, intracellular movement, cell-to-cell and long-distance systemic movement (Table 1).

Although several recent models have been proposed (Martínez-Turiño and García, 2020; Solovyev et al., 2022; Wang, 2021), the cell-to-cell movement of potyviruses still remains poorly understood. Two non-exclusive cell-to-cell transport of potyviruses pathways were recently reviewed: one mediated by the "second 6 kDa membrane-anchoring protein" (6K2), in which potyviruses use the host endomembrane system to produce membranous vesicles able to move between cells, in a fashion reminiscent of the way some animal viruses utilize membrane-derived vesicles for exit from infected cells and entry into healthy ones (Solovyev et al., 2022) and another mechanism where virions or ribonucleoprotein complexes are transported through PD (Wang, 2021). A third, very original mechanism was proposed based on the observation in the extracellular space and cell wall (apoplasm) of TuMV-induced vesicles, illustrating a potential change of paradigm for virus cell-to-cell transport (Movahed et al., 2019; Wan and Laliberté, 2015). The aim of the present review is to compile and integrate all available information relevant to the current understanding of potyvirus transport, in one complete and synthetic review. We present and discuss experimental data supporting these different models as well as the aspects that still remain mostly speculative.

I. Four viral multitasks proteins are involved in potyvirus movement

For potyviruses, there is no dedicated movement protein *per se*. Reverse genetics and cell biology studies performed on a large number of potyvirus species have shown that at least four potyviral proteins are involved in potyvirus transport (Revers and García, 2015; Wang, 2021). These are i) the Cylindrical Inclusion helicase or CI (a protein that composes the pinwheel-shaped inclusion bodies in the cytoplasm of infected cells, a unique feature of *Potyviridae*) ii) P3N-PIPO iii) the 6K2 and iv) the capsid protein (CP). A potential role for the Helper-Component proteinase (HC-Pro) in movement has also been suggested as it was shown to increase PD SEL (Rojas et al., 1997; Valli et al., 2018), but this could as well be associated to

an ability to move autonomously from cell-to-cell and long distance, needed to fulfill its silencing suppression function rather than to being involved in virus movement (Kasschau and Carrington, 2001; Kumar and Dasgupta, 2021).

In addition to replication, the CI protein is involved in cell-to-cell and long-distance movement. Previous alanine-scanning mutagenesis experiments reviewed by Sorel et al. (2014) and Deng et al. (2015) revealed that mutations in the N-terminal part of the CI impair cell-to-cell spread of tobacco etch virus (TEV) and PPV (Carrington et al., 1998; Gomez de Cedron et al., 2006). The ability of TuMV CI to target PD and interact with CP seems to be associated with its role in viral cell-to-cell movement (Deng et al., 2015). It has since been reported that P3N-PIPO recruits the CI protein to the cell wall near PD, and depends on the secretory pathway but not on actin or myosin motors for that function (Wei et al., 2010b).

Before the discovery of P3N-PIPO by Chung et al. (2008), it had already been noted that a silent mutation in the P3 cistron of wheat streak mosaic virus altered viral movement. This mutation is now known to prevent the polymerase slippage event and thus P3N-PIPO production (Choi et al., 2005). A few studies show that P3N-PIPO can be considered as a MP for potyviruses: GFP-tagged P3N-PIPO targets PD and is able to spread between cells, being thus seemingly capable of increasing the PD SEL and promoting viral movement (Cui et al., 2017; Vijayapalani et al., 2012). The N-terminal domain of P3N-PIPO conditions its localization to PD in the case of sugarcane mosaic virus (ScMV) (Cheng et al., 2017) and stop codons introduced in the PIPO sequence decrease the intercellular spread of soybean mosaic virus while they do not affect virus accumulation (Wen and Hajimorad, 2010).

The 6K2 of potyvirus is a transmembrane protein involved in rearrangement of the ER, leading to the generation of membranous viral vesicles, important for replication (Beauchemin et al., 2007) as well as for intracellular and intercellular movement (Cotton et al., 2009; Grangeon et al., 2013). The 6K2 protein is predicted to contain a central hydrophobic transmembrane domain necessary for vesicle formation (Schaad et al., 1997), a N-terminal tail exposed in the cytoplasm involved in vesicles' export from ER and a C-terminal tail exposed in the ER lumen or in vesicles (Jiang et al., 2015).

Besides the three non-structural proteins listed above, the fourth protein important for potyviral transport is the capsid protein (CP) (Martínez-Turiño and García, 2020). The potyviral CP presents three structural domains, with both N- and C-terminal regions exposed on the virion surface, and a conserved core subunit structure (Dolja et al., 1994). A large number of reverse genetics experiments have shown that mutations in all domains of the CP may impact viral movement (Martínez-Turiño and García, 2020).

II. 6K2-induced vesicles: key players in coupling potyvirus replication and intercellular movement

II.1 The 6K2 protein induces the formation of ER-derived vesicles

The first description of 6K2-induced vesicles dates back 25 years ago, when Schaad et al. (1997) showed that the TEV 6K2 protein induces, alone or during TEV infection, some discrete 2-10 µm diameter vesicles derived from the ER. Since then, the dynamics and ultrastructure of TuMV 6K2-induced vesicles have been extensively studied by confocal and transmission electron microscopy (TEM) (Grangeon et al., 2012; Li et al., 2020; Wan et al., 2015a). At an early stage of TuMV infection, the accumulation of convoluted membranes (CMs) connected to the rough ER is observed. Then, at TuMV infection midstage, CMs turn into single-membrane vesicle-like (SMVL) structures, which are viral RNA replication sites according to immunogold labeling studies using anti-RNA-dependent RNA polymerase (RdRP) and anti-double-stranded RNA (dsRNA) polyclonal antibodies (Wan et al., 2015a). Late in infection, in addition to the SMVLs, double-membrane vesicles like structures (DMVLs) are produced as well as virion bundles associated with vacuoles where encapsidation could occur (Li et al., 2020; Wan et al., 2015a). Wan et al. (2015a) suggested that DMVLs are likely underlying the perinuclear globular-like structures previously observed by confocal microscopy and composed by an amalgamation of ER, Golgi bodies, coat protein complex II (COPII) coatomers and chloroplasts (Grangeon et al., 2012; Wei et al., 2010a). This perinuclear globular structure contains viral RNA, numerous 6K2-induced vesicles as well as viral proteins involved in replication [P3, 6K1, VPg, NIaPro, NIb (RdRp) and CI helicase] (Cronin et al., 1995; Cui and Wang, 2016; Cui et al., 2010; Klein et al., 1994; Lõhmus et al., 2016; Wei et al., 2010a), together with host translation factors [eIF(iso)4E, poly(A)-binding protein (PABP), and eukaryotic translation elongation factor 1A (eEF1A)] (Beauchemin and Laliberte, 2007; Dufresne et al., 2008; Lõhmus et al., 2016; Thivierge et al., 2008). Even though the ER and Golgi apparatus have lost their characteristic organization in this perinuclear globular structure, they remain connected to the host secretory pathway, that is likely important for the generation of peripheral 6K2-induced vesicles, shown to exit from the globular structure (Grangeon et al., 2012). Potyvirus replication and probably viral translation occur at the level of those 6K2-vesicles, each being derived from a single viral genome (Cotton et al., 2009). This is confirmed by experiments with TuMV-infected protoplasts, in which VPg-Pro, RdRp, and CI, colocalize in vesicular punctate structures (likely 6K2-vesicles) with immunofluorescence-stained dsRNA replicative forms or neosynthesized 5-bromouridine-labeled RNA (Cotton et al., 2009).

In summary, the 6K2-dependent reorganization of the ER in potyvirus infected cells leads to the formation of two types of structures that are both involved in replication: the large perinuclear globular structure and the mobile 6K2-vesicles located at the cell periphery. This led to some confusion in the terminology used in the literature, where different terms are employed to design the perinuclear structure and 6K2-vesicles, such as "viral replication complexes (VRCs)", "vesicular structures", "punctate structures", "replication complex vesicles", "membrane-derived replication complexes", "viral replication vesicles", "VRO (viral replication organelles)", "vesicular VRCs", "VRC-associated membranous structures". Therefore, in this review, for clarity, the term "VRC" (viral replication compartment) will be used to design the perinuclear structure (in reference to similar perinuclear replication compartments observed for different plant virus genera), whereas we will refer to the 6K2-induced mobile vesicles as "6K2-vesicles".

The formation of potyvirus VRC and 6K2-vesicles induces drastic endomembrane rearrangements and relies on the early secretory pathway. Indeed, the detachment of 6K2-vesicles from the ER membrane involves the cellular mechanism of COPII-mediated formation of transport vesicles, which bud from the ER membrane and deliver cargo to the Golgi cisternae (Brandizzi and Barlowe, 2013; He et al., 2023; Wei and Wang, 2008). In particular, TuMV-6K2 interacts with the COPII coatomer Sec24a, the subunit interacting with cargo proteins for their packaging into COPII-vesicles (Jiang et al., 2015). The N-terminal cytoplasmic domain of 6K2 is crucial for this interaction and mutation of a conserved tryptophan residue at position 15 leads to partial retention of the 6K2 protein in the ER, alters TuMV replication and inhibits TuMV cell-to-cell movement (Jiang et al., 2015). Furthermore, the Atlastin-like GTPase ROOT HAIR DEFECTIVE3 (RHD3), which plays an important role in the generation of the interconnected tubular ER network and in membrane shaping, interacts with 6K2, and is required for the maturation of TuMV 6K2-vesicles in replication competent vesicles (Movahed et al., 2019b). Another striking point highlighted by studies on TuMV is that the 6K2-vesicles are mobile, and their different fates, after they detached from the ER, are detailed below.

II.2 6K2-vesicles are mobile and target multiple cellular compartments during viral replication and intercellular movement

Mobile 6K2-vesicles located at the cell periphery are observed, deriving from the VRC (Grangeon et al., 2012). The intracellular mobility of potyvirus 6K2-vesicles relies both on components of the early secretory pathway and on the actin network (but not on microtubules), while the vesicles trafficking along actin microfilaments depends on myosins XI-2 and XI-K (Agbeci et al., 2013; Cotton et al., 2009; Grangeon et al., 2012; Wei and Wang, 2008). LatrunculinB, a drug that disrupts actin microfilaments, impedes not only the intracellular movement of 6K2-vesicles (Cotton et al., 2009) but also TuMV cell-to-cell transport (Agbeci et al., 2013) suggesting that 6K2-vesicles could be involved in both intra- and intercellular movement of TuMV.

II.2.1 6K2-induced vesicles are targeted to chloroplasts

Some 6K2-vesicles traffic from the ER to chloroplasts where they amalgamate and induce chloroplast membrane invaginations. Both the vesicular transport pathway and actomyosin motility systems are involved in this process (Wei et al., 2010a). Viral RNA, dsRNA, and viral replicase components are concentrated at the level of 6K2-vesicles associated with chloroplasts in infected cells, suggesting that these chloroplast-bound 6K2-vesicles can also be the site for potyvirus replication (Wei et al., 2010a). This association of 6K2-vesicles at the periphery of chloroplasts is observed at 48 hours post infection (hpi). Later in infection (96hpi), 6K2-vesicles form an elongated tubular structure at the junctions between two adjacent chloroplasts (Wei et al., 2013). Some regulators of the secretory pathway, the soluble N-ethylmaleimide sensitive factor attachment protein receptors (SNAREs), are crucial for the targeting of 6K2-vesicles to the chloroplast. In the case of TuMV, the ER SNARE protein Syp71 colocalizes with the chloroplast-bound 6K2-vesicles that make elongated tubular structures (Wei et al., 2013). Downregulation of Syp71 inhibits the formation of such structures and reduces TuMV accumulation and virus systemic infection (Wei et al., 2013). However, Syp71 does not interact directly with the 6K2 protein suggesting that the recruitment of Syp71 to the 6K2-vesicles associated with chloroplasts is not direct. Plant dynamin-related proteins 1 (DRP1) and 2 (DRP2) are large multidomain GTPases that play key roles in endocytosis and post-Golgi trafficking in plants and are also co-opted by TuMV for infection (Wu et al., 2020). Arabidopsis AtDRP1 and 2 interact with TuMV-6K2. AtDRP1-labelled endosomes colocalize with the chloroplast-associated TuMV 6K2-vesicles and VRCs, suggesting that AtDRP1 or the AtDRP1-labelled endosomes are co-opted by TuMV to support virus replication (Wu et al., 2020). AtDRP2 is also recruited to the VRCs in TuMV-infected cells (Wu et al., 2018). As AtDRP2 plays an essential role in membrane remodeling and fusion, it is possible that TuMV coopts AtDRP2 for VRC assembly. However, the exact mechanical roles of AtDRP1 an AtDRP2 in the VRC assembly remains to be further elucidated (Wu et al., 2018; Wu et al., 2020).

II.2.2 Some 6K2-induced vesicles follow an unconventional trafficking route based on the endosomal /post-Golgi Multivesicular Bodies (MVB) pathway

Although a proportion of 6K2 vesicles does traffic to the Golgi apparatus, a large number of 6K2 vesicles bypass the Golgi through an unconventional trafficking route involving the late endosome (LE)/multivesicular bodies (MVB) pathway (Cabanillas et al., 2018). The central transmembrane domain of TuMV-6K2 that contains a GxxxG motif (x being any amino acid) is very important for vesicles production and this unconventional trafficking. Indeed, a mutation of Glycine (G) residues in this motif, leads to relocalization of 6K2 vesicles to Golgi bodies and the plasma membrane, and severely affects virus replication (Cabanillas et al., 2018). Furthermore, TuMV-6K2 interacts with Sec22, a SNARE protein that, when overexpressed, has a negative effect on the ER-Golgi equilibrium by impairing the fusion of transport vesicles between the ER and the Golgi apparatus. The overexpression of Sec22 impairs the fusion of 6K2-vesicles with the Golgi apparatus membrane, and enhances TuMV intercellular movement, supporting the hypothesis that the Golgi compartment could be a dead-end for TuMV 6K2-vesicles when it comes to viral movement (Cabanillas et al., 2018). Furthermore, instead of being addressed to the Golgi, the 6K2-vesicles can be targeted to MVB. Indeed, the 6K2 protein is colocalized and copurifies with Vti11 (Vps10-interacting protein11), another SNARE protein localized to the trans-Golgi network and LE/MVB, where dsRNAs are also detected by immunogold electron microscopy (Cabanillas et al., 2018). The biological role of the targeting of 6K2-vesicles to MVB is strongly supported by the resistance to TuMV infection of an Arabidopsis *vti11* knock-out line (Cabanillas et al., 2018). Interestingly, Vti11 associates with VSR1, a member of the Vacuolar Sorting Receptor family of proteins, which localizes to prevacuolar compartments (PVC)/LE/MVB (Song et al., 2006). Recently, it was shown that another member of the VSRs family, VSR4, interacts with TuMV-6K2, and plays a key role in targeting 6K2-vesicles to MVB (Wu et al., 2022). In parallel, TuMV infection is impaired in Arabidopsis *vsr4* knock-out lines (Wu et al., 2022). Altogether those results show that the intracellular transport

of potyvirus 6K2-vesicles, which are detached from the ER, can bypass the Golgi and for some of them, use post-Golgi membrane compartments to reach MVB.

II.2.3 6K2-vesicles are addressed to PD and can move cell-to-cell

In TuMV-infected cells, Grangeon et al (2013) showed that the 6K2-vesicles traffic towards the plasma membrane (PM) where they can be observed in close vicinity to PD, and have been even reported to move cell-to-cell (Grangeon et al., 2013). This very intriguing conclusion is based on confocal microscopy observations performed with 6K2 fused to a photoactivable GFP (6K2:PAGFP) expressed in mock- or in TuMV-infected cells. After PAGFP activation, 6K2:PAGFP-tagged vesicles can be observed moving to the cell periphery and, across the cell wall, to neighboring cells. This was however only observed in the infected condition (Grangeon et al., 2013). These results support the hypothesis that intercellular movement of TuMV may occur in the form of a membrane-associated viral RNA vesicular complex induced by 6K2 (Grangeon et al., 2013).

Importantly, when expressed alone (in the absence of virus infection), the 6K2 protein can induce vesicles that are morphologically identical to those observed in infected cells (Figure 2), but these vesicles do not move cell-to-cell (González et al., 2019; Grangeon et al., 2013; Lõhmus et al., 2015). However, when co-expressed with 6K2, P3N-PIPO and CI are necessary and sufficient for the targeting of 6K2-vesicles to PD and for their intercellular movement. The CI and P3N-PIPO proteins would therefore compose the minimal complex required for the intercellular movement of the 6K2-vesicles (Movahed et al., 2017). The current hypothesis is that P3N-PIPO interacts with and recruits the CI to PD via the PIPO domain (Wei et al., 2010b). P3N-PIPO does not interact directly with 6K2 but interacts with the P3 protein via their shared P3N domain, and this interaction has been suggested to allow to establish a connection between CI and 6K2 vesicles at the PD level (Chai et al., 2020). These results support the notion that the replication and cell-to-cell movement of potyviruses are coupled through the anchoring of 6K2-vesicles at the entrance of PD.

II.2.4 6K2-vesicles could move as extracellular vesicles

Extracellular vesicles have in the past few years emerged as a potential additional route for macromolecule transport in plants, and in particular, for delivering RNA molecules to distant tissues (Kehr and Kragler, 2018). Extracellular vesicles exist in plants and their proteome significantly overlaps with the PD proteome, suggesting that PD might be a hotspot for extracellular vesicles/exosome transfer between cells (Rutter and Innes, 2017; Rutter and Innes, 2018). Extracellular vesicles can be derived from MVB intraluminal vesicles released upon fusion of the MVB boundary membrane with the plasma membrane (Rutter and Innes, 2018). Interestingly, the Vti11 SNARE that plays a critical role in TuMV infection (Cabanillas et al., 2018) is also found in the proteome of extracellular vesicles isolated from Arabidopsis (Rutter and Innes, 2017). The functions of Vti11 in both TuMV infection and MVB-plasma membrane fusion, suggest the possible existence of extracellular viral vesicles produced through the fusion of TuMV-MVB with the plasma membrane. This hypothesis is strongly supported by the confocal microscopy observations of 6K2-induced aggregates present in the extracellular space of infected leaves, probably an amalgamation of 6K2-vesicles containing vRNA and the viral RdRp as shown in xylem-conducting tubes (Wan et al., 2015b). This suggests that 6K2-vesicles can enter the extracellular space by exocytosis and possibly thus move between cells (Grangeon et al., 2013; Movahed et al., 2019a). Indeed, by using TEM, both MVB-plasma membrane fusion events and vesicles present in the extracellular space can be observed in infected tissues and contain dsRNA (Movahed et al., 2019a). Furthermore, the presence of 6K2-vesicles in xylem vessels suggests that viral components could also be found in other apoplastic regions of the plant (Wan et al., 2015b; Wan and Laliberté, 2015). Altogether, these results highlight a potential novel pathway for intercellular movement of potyviruses that would represent an important change of paradigm for plant virus movement (Richardson, 2019).

In the previous part, we've highlighted the progress made in understanding how potyviruses recruit the host plant endomembrane system and cytoskeleton to move intracellularly to the cell wall and PD, with a special focus on the role played by 6K2-vesicles in this process. A major question remains to be answered: what is the nature of the viral "entity" that crosses the PD? As discussed above, 6K2-vesicles are able to transit to non-infected cells, however, the exact composition of their viral load still remains to be determined, which is very technically challenging. At this point, it cannot be excluded that potyviral RNA moves cell-to-cell through PD, associated with still unknown plant factors and in a 6K2-independent way. In this hypothesis, the 6K2-vesicles could have the function of allowing the synthesizing and bringing the viral material (genomic RNA, viral proteins) close to PD. It is also not known whether the encapsidation process precedes the transport of virions or whether the genomic RNA is transported in the form of a ribonucleoprotein complex. The demonstration that only replication-competent RNA can be encapsidated into mature potyviral particles suggests that viral RNA replication, virion assembly and viral movement are closely interconnected for potyviruses (Gallo et al., 2018).

III. A still opened question: do potyviruses move between cells through plasmodesmata as ribonucleoprotein complexes or as virions?

III.1 Potyviruses counteract viral-induced callose deposition at PD

Plant infection by viruses has been associated with callose regulation at PD level. Callose is a 1,3-beta-glucan that accumulates in cell wall microdomains surrounding PD, regulating the cell-to-cell transport of macromolecules by changing the SEL (German et al., 2023). A negative role of callose accumulation in PD permeability has been confirmed experimentally, yet the roles of cytoskeletal elements and many PD-associated proteins in this phenomenon still remain unclear. Many viruses have been reported to induce PD callose accumulation, including potyviruses (Zavaliev et al., 2013). Nevertheless, for efficient movement, potyviruses must somehow overcome the effect of callose deposition at PD. Potyvirid infection is associated with the up-regulation of genes involved in callose degradation, such as cell wall beta-1,3-glucanases, as observed during cassava

brown streak ipomovirus infection (Anjanappa et al., 2018) and zucchini yellow mosaic virus infection (Amoroso et al., 2022). In response to SMV infection, soybean endo-1,3-beta-glucanase (GmGLU) expression is also increased and GmGLU regulates callose deposition at the PD (Shi et al., 2020).

The roles of potyviral CI and P3N-PIPO in regulating callose deposition at PD have been highlighted in different studies. The ectopic expression in *N. benthamiana* of the potato (*Solanum tuberosum*) REMORIN (StREM1.3), a plant protein that localizes at lipid rafts and PD, increases PD-callose deposition. However, ectopic over-expression of REMORIN in *N. benthamiana* affects differently virus spread: the progagation of tomato mosaic virus (tobamovirus) and the movement of the potexvirus PVX are restricted, while the propagation of two potyviruses, TuMV and potato virus A (PVA) is enhanced (Perraki et al., 2018; Sasaki et al., 2018). The observation that TuMV-CI interacts with StREM1.3 at the plasma membrane and at PD levels, suggests that the CI could counteract the role of StREM1.3 in promoting callose deposition at PD to favor TuMV propagation (Rocher et al., 2022). Furthermore, the presence of the CI of pea seedborne mosaic virus (PSbMV) at PD level, is linked with an apparent transient reduction in callose deposit in the vicinity of PD (Roberts et al., 1998), suggesting that the CI might transiently increase the SEL to allow viral movement. This notion is however not supported by microinjection experiments performed by Rojas et al. (1997), who showed that the CP can increase the SEL of PD but not the CI. On the other hand, the PD SEL has been shown to be increased by P3N-PIPO, which interacts with both CI and a divalent cation-binding plasma membrane protein (PCaP1) via its C-terminal PIPO domain (Chai et al., 2020; Vijayapalani et al., 2012). It has thus been proposed that P3N-PIPO could control Ca^{2+} concentration around PD, which could in turn affect the SEL by regulating callose deposition and severing the actin system (Cheng et al., 2017; Cheng et al., 2020). Indeed, both actin and myosin localize in the PD pore, and regulate not only the gating to but also the trafficking through it (Diao and Huang, 2021).

A cell wall loosening protein, NbEXPA1, a PD-located *N. benthamiana* alpha-expansin, plays a role in leaf development and growth, potentially by remodeling the cell wall for PD maturation. When ectopically expressed, NbEXPA1 promotes TuMV replication and intercellular movement, suggesting that this PD-specific cell wall loosening protein could be involved in PD modification and facilitate potyviral cell-to-cell movement (Park et al., 2017).

Altogether those results show that potyviruses probably modulate callose deposition and possibly other cell wall parameters at the PD level in order to favor their cell-to-cell movement, although the exact underlying mechanism(s) still remain(s) to be determined.

III.2 Docking of potyviral "movement proteins" at PD level

Different ultrastructural analyses reviewed by Sorel et al. (2014) reveal that cylindrical inclusions are localized in close vicinity to the plasmalemma during early infection. In particular, the "step-by-step" ultrastructural analysis of PSbMV infection of pea cotyledons, has shown that CI inclusions are detected at both sides of PD connecting two cells at the front edge of the viral infection (Roberts et al., 1998). Other studies with different potyviruses confirm that the CI is associated with pathognomonic cone-shaped structures close to PD, and in the vicinity of the CI cytoplasmic inclusions, where CP and viral RNA are also detected (Otulak and Garbaczewska, 2012; Roberts et al., 1998; Rodriguez-Cerezo et al., 1997; Rojas et al., 1997). Furthermore, a TuMV CI mutated on N-terminal charged residues failed to form conical inclusions and to interact with the virus CP at PD, and the corresponding mutants are altered in their cell-to-cell movement, suggesting a functional link between CI localization at PD and potyvirus movement (Deng et al., 2015).

The recruitment of the CI to PD is mediated by its interaction with P3N-PIPO and the CI could then serve as a docking point for the intercellular movement of viral material or intact virions (Deng et al., 2015; Movahed et al., 2017; Wei et al., 2010b). The anchoring of P3N-PIPO to PD depends on its interaction with plasma membrane-associated PCaP1 (Vijayapalani et al., 2012). Indeed, the knock-out of *PCaP1* in *A. thaliana* induces a strong reduction of TuMV movement (Vijayapalani et al. 2012). Furthermore, the regulated plasma membrane protein NbDREPP in *N. benthamiana*, considered as the homolog of AtPCaP1, interacts with both P3N-PIPO and CI of tobacco vein banding mosaic virus (TVBMV) at PD level. Its knockdown impedes the cell-to-cell movement of TVBMV, confirming the key role played by PCaP1 at the PD level for potyvirus transport (Geng et al., 2015). It is hypothesized that PCaP1 recruits TuMV-P3N-PIPO to PD and the actin filament-severing activity of PCaP1 is required for TuMV intercellular movement (Cheng et al., 2020). However, Arabidopsis AtREM1.2 negatively regulates the cell-to-cell movement of TuMV via competition with PCaP1 for binding actin filaments (Cheng et al., 2020). This negative effect of AtREM1.2 on potyvirus movement contradicts the positive effect observed for StREM1.3 (Rocher et al., 2022), adding another level of complexity concerning the functions of REMORINs in viral infections.

Another protein involved in membrane trafficking, the synaptotagmin SYTA, has been shown to regulate P3N-PIPO intercellular trafficking (Uchiyama et al., 2014). SYTA localizes to the sites where the ER makes contact sites with the PM, such as at the PD sites. TuMV propagation and the cell-to-cell movement of P3N-PIPO-GFP fusions are delayed in *Arabidopsis syta-1* knockdown mutant. The authors suggest that SYTA could play a role in the way P3N-PIPO, with its cargo of CI and viral material, could be directed to PD. However, such an hypothesis is not supported by the fact that no interaction between P3N-PIPO and SYTA has been described so far (Uchiyama et al., 2014).

III.3 Do potyvirus virions cross the PD?

The structure capsid protein (CP) is also part of the potyviral multicomponent transport system (Martínez-Turiño and García, 2020). However, as detailed below, it is still not clear whether the form of potyvirus transport through PD occurs as virions, or as non-virions ribonucleoprotein complexes.

Studies on filamentous plant virus particles using atomic force microscopy (AFM) revealed the presence of protruding tip structures on PVY and PVA virions (Gabrenaite-Verkhovskaya et al., 2008; Torrance et al., 2006). At the level of these

protruding tips at the 5' end of the PVA particles, the CI is detected, together with the VPg and HC-Pro proteins (Gabrenaite-Verkhovskaya et al., 2008). The role of those structural supplements at the virion end is still unknown and may potentially play roles in different steps of the viral infection, such as virus assembly/disassembly, movement and vector transmission (Torrance et al., 2006). A possible hypothesis, based on the CI ATPase and RNA helicase activities, is that the CI could behave as a molecular motor both to disassemble virions and translocate the viral genome through PD (Gabrenaite-Verkhovskaya et al., 2008).

Pioneering studies such as that of Weintraub et al. (1974) suggest that PVY moves cell-to-cell as particles, but due to limitations of imaging technologies, as well as to the absence of specific labeling of the CP in these experiments, this conclusion remains highly speculative. Even after immunolabeling in other studies, it is difficult to conclude whether CP labeling within PD corresponds to virions or to CP involved in non-virion ribonucleoprotein complexes (Riedel et al., 1998; Roberts et al., 1998; Varrelmann and Maiss, 2000). Indeed, based on immuno-electron microscopy observations, the CP has been detected in the central core of the CI pinwheel inclusions, leading to the hypothesis that CI could position and even guide, like a funnel, viral particles across the cell wall (Roberts et al., 1998). Linear shaped CP "complexes", inside or attached to cylindrical inclusions near PD connections in tobacco leaves infected with tobacco vein mottling virus (TVMV) or potato leaves infected by PVY were also observed, but it was not possible to conclude whether these complexes are virions targeted for transport, or non-virions ribonucleoprotein complexes (Otulak and Garbaczewska, 2012; Rodriguez-Cerezo et al., 1997). Even though viral particles are observed near PD, late in infection (Roberts et al. 1998), neither a "queue" of "waiting" virions, nor "engaged" virions in PD have been observed or reported in any of published electron microscopy studies.

As reviewed above, reverse genetics experiments showed that in addition of being the potyvirus structural protein, mutations or deletions of CP affect the intercellular movement of TEV and PPV, without impairing viral replication but hampering assembly of the particles (Roberts et al., 1998; Varrelmann and Maiss, 2000). This essential role of multiple conserved aromatic residues in the core CP, in CP accumulation and virus movement, was confirmed for TVBMV, watermelon mosaic virus (WMV) and PVY (Yan et al., 2021). Mutations of three charged residues Arginine R245, Histidine H246 and Aspartic acid D250 in the C-terminal domain also impair intercellular movement of SMV (Seo et al., 2013), while deletions in the CP core negatively impact TuMV particle assembly (Yuste-Calvo et al., 2020) and impair the TEV cell-to-cell movement and production of virus particles (Dolja et al., 1994; Dolja et al., 1995). All these results suggest that some potyviruses likely move as virions. On the other hand, for TuMV, intact virions are not needed for the intercellular spread, since deletions in the N-terminal part of CP induce the formation of aberrant long forms of virions without impairing cell-to-cell nor systemic spread (Dai et al., 2020). In parallel, encapsidation is not sufficient for cell-to-cell or long-distance movement of TEV and PVY as those potyviruses can form virions despite the deletion of the C-terminal part of their CP that abolish movement (Dolja et al., 1995; Kežar et al., 2019). Other mutations in the CP, leading to movement-defective viruses, can be partially rescued by trans-complementation in CP transgenic plants, but without visible virions formation (Dolja et al., 1994; Dolja et al., 1995), supporting the assumption that cell-to-cell and long-distance transports of potyviruses are facilitated by their CP but without an absolute requirement for virion formation. Therefore some conclusions about potyviruses moving as virions, based on reverse genetics experiments but without any experimental proof of virions crossing the PD (Seo et al., 2013), should likely be considered with caution. In summary, there is currently conflicting evidence about the need for encapsidation for potyvirus movement. In some cases, mutations have been identified that simultaneously prevent viral particle formation and potyviral movement, supporting the view that mature virions are responsible for virus movement. As discussed above, other experimental results show that cell-to-cell movement can occur without accumulation of viral particles, which greatly challenges the need for virions assumption. Indeed, in addition to particle encapsidation, mutations in the CP could also modify its interactions with the viral RNA or with other viral or cellular proteins, impacting vesicular transport and cell-to-cell spread. As a result, potyvirus transport through PDs could occur as virions, as non-virions ribonucleoprotein complexes involving the CP or even possibly as a combination of these two non-exclusive modes.

Conclusion

Taken together, data from this review including genetic, cellular, and ultrastructural studies, highlight how the potyvirus transport involves a large network of interactions between viral and cellular proteins. Despite now extensive investigations, whether potyviruses move between cells as virions or other movement complexes such as ribonucleoproteins or 6K2-vesicles, still remains an unsolved conundrum, setting asides potyviruses from the majority of plant viruses. However, evidence obtained so far shows that viral replication and viral movement are very closely interconnected in the case of potyviruses. Observations of 6K2-vesicles trafficking between cells and of viral extra-cellular vesicles containing dsRNA (potential replicative forms of viral RNA) being penetrating and possibly even crossing the cell wall, are highly innovative results that suggest the existence of a totally novel mechanism for plant virus cell-to-cell spread. Whether those results can be generalized to all potyvirus species besides TuMV and may apply to other plant virus genera remains to be determined.

Based on data gathered in this review, we propose an updated working model integrating all the identified pathways potentially involved in potyvirus movement (Figure 3). It must be noted that in this comprehensive model of the intracellular and intercellular movement of potyviruses, the chronological order shown in Figure 3 is provided for presentation clarity but probably does not reflect the biological reality, as some described events can occur simultaneously. Figure 3 shows that upon entry into the cell of the viral particle via aphid transmission or mechanical injury, the virion is uncoated (**1**). The (+) RNA viral genome is translated and the synthesized viral proteins, including the 6K2 and co-opted host factors, induce a drastic remodeling of the ER and Golgi endomembrane systems associated with the formation of a large perinuclear globular structure (VRC) (**3**) and the release of mobile 6K2-vesicles that move intracellularly following transvacuolar actin strands (**2**). These

6K2-vesicles contain the viral RNA together with host and viral proteins involved in translation and replication. Some 6K2-vesicles target chloroplasts, where replication can occur (**4**). Others bypass the Golgi apparatus and become associated with the PVC/LE/MVB (**5**). MVB can then fuse with the plasma membrane and release their vesicular content in the extracellular space where the vesicles can move through pores in the cell wall to the neighboring cell (**5a**). The 6K2-vesicles can also reach the plasma membrane and become be associated with PD, a process relying on the P3N-PIPO and CI proteins, in association with host factors such as PCaP1 for PD gating and the docking of 6K2-vesicles (**5b**). The delivering of the 6K2-vesicles to the PD probably increases the concentration of viral replication products nearby. After downloading of the content of the vesicles, newly synthesized genomic RNAs can be packaged by CP in virions in the vacuoles, or be transported through PD either as viral-ribonucleoprotein complexes involving the CP (**5c**) or as nascent virions (**5d**). Investigations are still in dire need in order to sort out or to reconcile all these potential routes of intracellular and intercellular propagation of potyviruses, and if possible, to establish a chronology linking them.

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Figures legends

Figure 1. **Genomic organization of potyviruses**. The long open reading frame encodes the polyprotein cleaved by three viral proteases (P1, HC-Pro and NIa-Pro) into 10 mature viral proteins. The PIPO ORF embedded in the P3 cistron is indicated as a striped area. The viral genome linked protein (VPg) is represented as a black ellipse at the 5' end of the genome. Arrows starting from the three proteases indicate their cleavage sites in the polyprotein. While P1 and HC-Pro cleave themselves autoproteolytically, NIa-pro, in addition to itself, releases the remaining viral proteins from the polyprotein (Adapted from Revers and Garcia, 2015).

Figure 2. 6K2-induced vesicles in *N. benthamiana* epidermal cells imaged using confocal microscopy. A) 6K2-vesicles observed when 6K2-GFP is expressed alone at 2 days after agroinfiltration. Chloroplast autofluorescence is shown in red. **B**) 6K2-vesicles observed during infection by a recombinant TuMV clone expressing a 6K2-mcherry fusion (TuMV-6K2mCherry) 3 days after inoculation. The 6K2-induced vesicles are indicated in magenta while chloroplast autofluorescence is shown in blue. In both cases 6K2-induced vesicles are observed in close association with chloroplasts.

Figure 3. Schematic illustration of the different models for potyviral intercellular transport. After entry of the virus via aphid stylets or through mechanical injury, virions are uncoated (**1**) and genomic RNA released in the cytoplasm where translation occurs. The viral 6K2 protein induces the formation of ER-derived 6K2-vesicles involved in replication (**2**) and a large perinuclear globular structure (VRC) associated with a drastic remodeling of the ER and Golgi endomembrane system (**3**). A subpopulation of 6K2-vesicles targets the Golgi apparatus and chloroplasts where replication occurs (**4**). The 6K2-vesicles can also bypass the Golgi apparatus and are associated with the prevacuolar compartments/ late endosomes/multivesicular bodies (PVC/LE/MVB) (**5**). MVB can fuse with the plasma membrane and release their vesicular content in the extracellular space where the vesicles can move through pores in the cell wall to neighboring cell (**5a**). 6K2-vesicles can also reach the plasma membrane and associate with PDs, a process relying on the P3N-PIPO and CI proteins, in association with host factors such as PCaP1 for PD gating. The CI protein associated with 6K2-vesicles can serve as a docking point at the PDs for their intercellular movement (**5b**). The delivering of the 6K2-vesicles to PDs probably increases the concentration of viral replication products nearby. After downloading of the content of the vesicles, newly synthesized genomic RNAs can be packaged by CP in virions in the vacuole, or be transported through PD either as viral-ribonucleoprotein complexes associated with the CP (**5c**) or as nascent virions (**5d**).

Table 1: Relevant features of multitask potyviral proteins. The column "References" corresponds to pioneering studies and the column "Reviews" to recent reviews focused on a particular potyviral protein. The asterisk indicates a function that is mainly described for TuMV species. $ Sweet potato feathery mottle virus (SPFMV) P1 protein is the largest among all potyviral P1 proteins, ranging from 74.1 to 80 kDa (Mingot et al., 2016); Sweet potato virus G (SPVG) CP is 39.4 kDa, the largest of all known species of potyviruses (Li et al., 2012).

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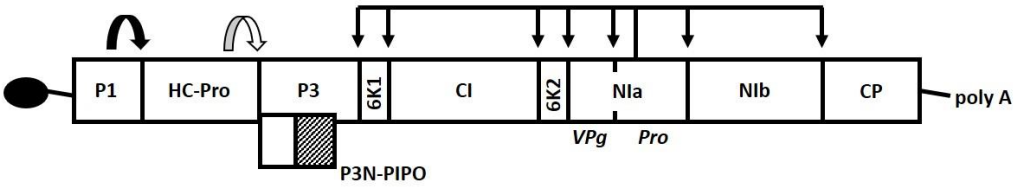

Figure 1. Genomic organization of potyviruses. The long open reading frame encodes the polyprotein cleaved by three viral proteases (P1, HC-Pro and NIaPro) into 10 final viral proteins. The PIPO ORF embedded in the P3 cistron is indicated as a striped area. The viral genome linked protein (VPg) is represented as a black ellipse at the 5' end of the genome. Arrows starting from the three proteases (P1, HC-Pro, and NIa-Pro) indicate their cleavage sites in the polyprotein. While P1 and HC-Pro cleave themselves autoproteolytically, NIa-pro, in addition to itself, releases the remaining viral proteins from the polyprotein (Adapted from Revers and Garcia, 2015).

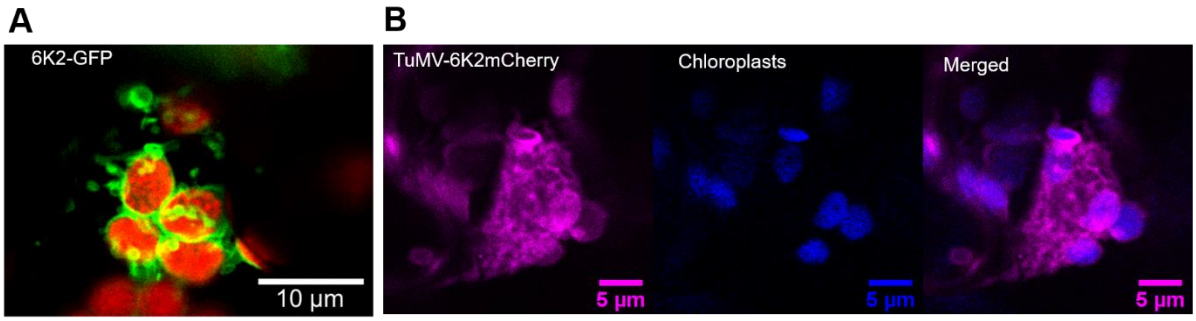

Figure 2. 6K2-induced vesicles in *N. benthamiana* epidermal cells imaged using confocal microscopy. A) 6K2-induced vesicles observed when 6K2-GFP is expressed alone at 4 days after agroinfiltration. The chloroplast autofluorescence is shown in red. **B)** 6K2-induced vesicles observed during infection by a recombinant TuMV clone expressing a 6K2-mcherry fusion (TuMV-6K2mCherry) 4 days after inoculation. The 6K2-induced vesicles are indicated in magenta and the chloroplast autofluorescence is shown in blue. In both cases 6K2-induced vesicles are observed in close association with chloroplasts.

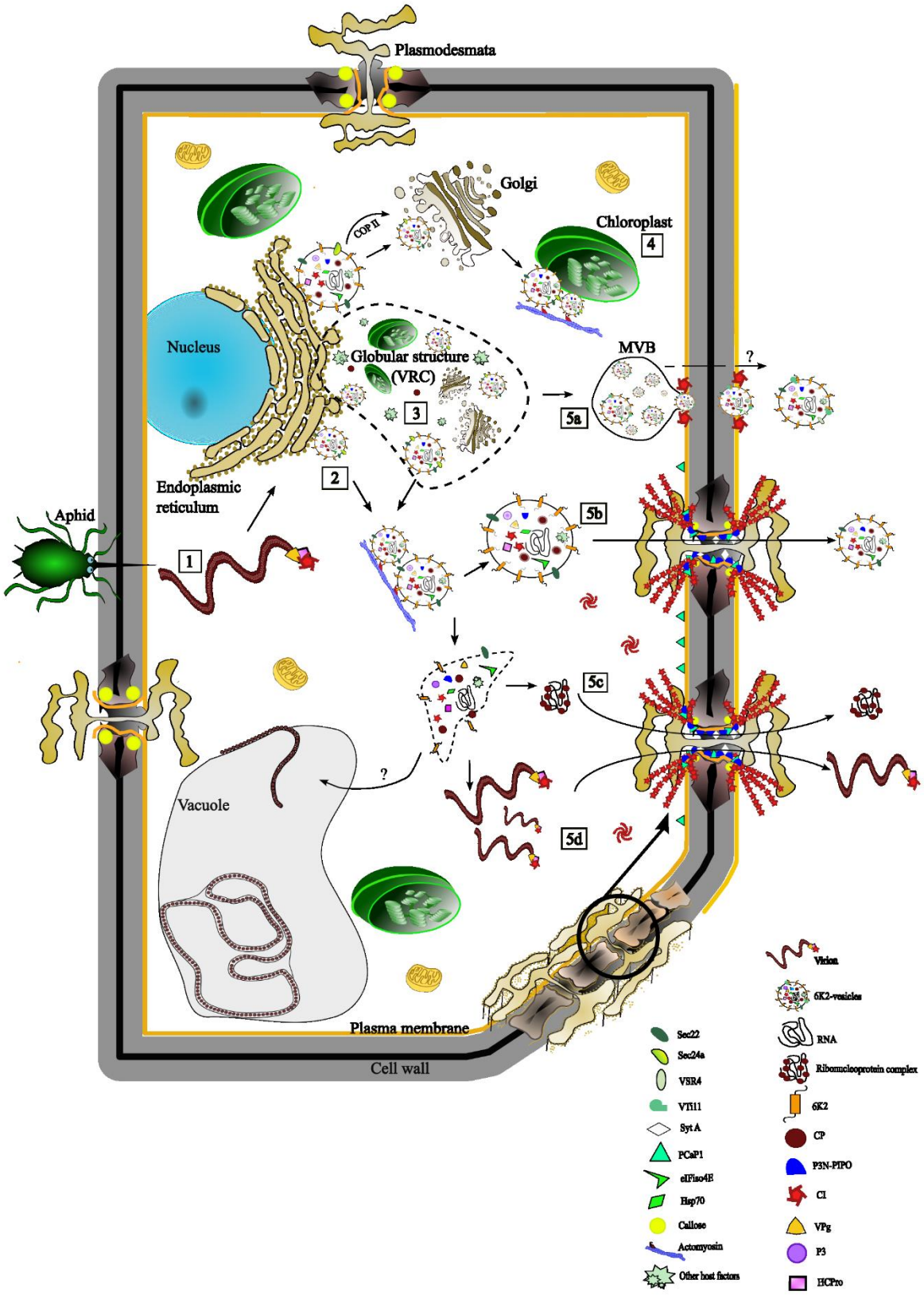
Plasmodesmata
Golgi
COP II
Chloroplast
4
Nucleus
Globular structure
(VRC)
3
MVB
?
5a
2
Endoplasmic
reticulum
Aphid
1
5b
5c
?
Vacuole
5d
Plasma membrane
Cell wall
Sec22
Sec24a
VSR4
VTI11
Syt A
PCaP1
eIFiso4E
Hsp70
Callose
Actomyosin
Other host factors
Virion
6K2-vesicles
RNA
Ribonucleoprotein complex
6K2
CP
P3N-PIPO
CI
VPg
P3
HCPro

Figure 3

Table 1. Relevant features of multitask potyviral proteins. The column "References" corresponds to pioneering studies and the column "Reviews" to recent reviews focused on a particular potyviral protein. The asterisk indicates a function that is mainly described for TuMV species. $ Sweet potato feathery mottle virus (SPFMV) P1 protein is the largest among all potyviral P1 proteins, ranging from 74.1 to 80 kDa (Mingot *et al.*, 2016); Sweet potato virus G (SPVG) CP is 39.4 kDa, the largest of all known species of potyviruses (Li *et al.*, 2012).

Protein	Molecular Weight (kDa)	Protein Functions	References	Reviews
P1	30-80$	Serine protease; RNA-binding; Accessory factor for virus replication; virus adaptation to host	(Verchot *et al.*, 1991; Brantley and Hunt, 1993; Verchot and Carrington, 1995; Valli *et al.*, 2007)	(Rohožková and Navrátil, 2011; Revers and García, 2015)
HC-Pro	~56	Cysteine protease ; Aphid transmission ; RNA silencing suppression.	(Govier and Kassanis, 1974; Carrington *et al.*, 1989; Anandalakshmi *et al.*, 1998; Kasschau and Carrington, 1998; Carrington *et al.*, 1996)	(Valli *et al.*, 2018; Gadhave *et al.*, 2020)
P3	~37	Virus adaptation to host; Virus replication and movement	(Salvador *et al.*, 2008; Klein *et al.*, 1994; Cui *et al.*, 2010)	(Revers and García, 2015)
P3N-PIPO	~14	Virus cell-to-cell movement	(Wei *et al.*, 2010; Vijayapalani *et al.*, 2012; Cui *et al.*, 2017; Chai *et al.*, 2020)	(Wang, 2021)
6K1	6	Virus replication; Reduction of plant proteases activity to increase virus accumulation	(Cui and Wang, 2016; Geng *et al.*, 2017; Bera *et al.*, 2022; Hu *et al.*, 2023)	
CI	~79	Pinwheel formation; RNA helicase; Virus replication; Virus cell-to-cell and long-distance movement;	(Edwardson, 1966; Lain *et al.*, 1990; Fernández *et al.*, 1995; Calder and Ingerfeld, 1990; Wei *et al.*, 2010; Otulak and Garbaczewska, 2012; Deng *et al.*, 2015)	(Sorel *et al.*, 2014)
6K2	6	Formation of viral replication complex vesicles; Virus replication; Virus intra/inter-cellular and long-distance movement*;	(Mary C. Schaad *et al.*, 1997; Cotton *et al.*, 2009; Grangeon *et al.*, 2012; Grangeon *et al.*, 2013; Wan *et al.*, 2015)	(Grangeon *et al.*, 2010; Solovyev *et al.*, 2022; He *et al.*, 2023)
VPg	~21	RNA replication and translation; Virus cell-to-cell and long-distance movement; RNA silencing suppression; Virus phloem loading;	(Siaw *et al.*, 1985; Keller *et al.*, 1998; Nicolas *et al.*, 1997; M C Schaad *et al.*, 1997; Rajamäki and Valkonen, 2002; Rajamäki and Valkonen, 2009; Tavert-Roudet *et al.*, 2017; Cheng and Wang, 2017)	(Jiang and Laliberté, 2011; Rantalainen *et al.*, 2011; Eruera *et al.*, 2021; Jaramillo-Mesa and Rakotondrafara, 2023)
NIa-Pro	~27	Cysteine protease DNAse	(Schaad *et al.*, 1996; Flint and Ryan, 1997; Anindya and Savithri, 2004)	(Adams *et al.*, 2005)
NIb	~58	RNA replicase (RNA dependent RNA Polymerase, RdRP); Assembly and activation of viral replication complexes (VRCs); *Suppressor of host defense;	(Hong and Hunt, 1996; Sanfaçon, 2005; Cheng *et al.*, 2017; Li *et al.*, 2018)	(Mäkinen and Hafrén, 2014; Shen *et al.*, 2020)
CP	30-40$	Virion assembly; RNA binding Virus translation; Virus cell-to-cell and long-distance movement; Aphid transmission;	(Shukla and Ward, 1989; Atreya *et al.*, 1991; Dolja *et al.*, 1995; Blanc *et al.*, 1997; Kendall *et al.*, 2008; Hafrén *et al.*, 2010; Seo, 2013)	(Revers and García, 2015; Gadhave *et al.*, 2020; Martínez-Turiño and García, 2020)