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Par

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Molecular mechanisms involved in the pathogenesis of beet soil-borne viruses

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Alice Delbianco a été membre de la promotion Jane Goodall du Collège Doctoral Européen de l'Université de Strasbourg pendant la préparation de sa thèse de novembre 2010 au mars 2013. Elle a bénéficié des aides spécifiques du CDE et à suivi un enseignement hebdomadaire, au cours de ses séjours à Strasbourg, sur les affaires européennes dispensé par des spécialistes internationaux. Ses travaux de recherche ont été effectués dans le cadre d'une convention de cotutelle avec l'Université de Bologne (Italie) et l'Université de Strasbourg.

Alice Delbianco was a member of the European Doctoral College of the University of Strasbourg during the preparation of her PhD from November 2010 to March 2013, class name: Jane Goodall. She has benefited from specific financial supports offered by the College and, along with her mainstream research, has followed a special course on topics of general European interests presented by international experts. This PhD research project has been led with the collaboration of two universities: the University of Bologna (Italy) and the University of Strasbourg (France).

Alice Delbianco è stata membro della promozione Jane Goodall del Collège Doctoral Européen dell'Università di Strasburgo durante la preparazione della tesi da novembre 2010 a marzo 2013. Ha beneficiato dei sostegni del CDE e ha seguito una formazione settimanale, durante il suo soggiorno a Strasburgo, sugli affari europei impartita da specialisti internazionali. I suoi lavori di ricerca sono stati svolti nel quadro di una convenzione di cotutela tra l'Università di Bologna e l'Università di Strasburgo (Francia).

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"Mécanismes moléculaires à l'origine de la pathogénicité de phytovirus de betterave sucrière transmis par un vecteur tellurique"

Introduction

Les quatre virus de betterave les plus répandus et transmis par le protozoaire *Polymyxa betae* appartiennent aux genres *Benyvirus* et *Pomovirus. Le Beet soil-borne virus* et le *Beet virus Q* sont deux *Pomovirus* souvent retrouvés associés à l'un ou à l'autre des Benyvirus responsables de maladies virales de la betterave. Le virus des nervures jaunes et nécrotiques de la betterave (*Beet necrotic yellow vein virus*, BNYVV), est l'agent infectieux responsable de la rhizomanie de la betterave sucrière, une maladie caractérisée par une prolifération anarchique du chevelu racinaire. La rhizomanie de la betterave est retrouvée dans toutes les régions de culture de la betterave (Peltier et al., 2008). Deux types majeurs de BNYVV existent, le type A, retrouvé en Europe, Iran, Amérique du Nord, Chine et Japon et le type B, retrouvé en France, Allemagne, Suède, Chine, UK et Lituanie. Un troisième type, le type P, se caractérise par la présence d'un ARN surnuméraire : l'ARN-5 et est retrouvé dans certaines régions du Kazakhstan, d'UK, de Chine et du Japon.

Le *Beet soil-borne mosaic virus* (BSBMV) appartient également au genre Benyvirus mais n'est retrouvé qu'en Amérique du Nord (Lee et al., 2001 ; Gilmer & Ratti, 2012). Ce virus, identifié pour la première fois au Texas, est morphologiquement et génétiquement semblable au BNYVV mais sérologiquement éloigné. Compte tenu des différences moléculaires existant, le BSBMV et BNYVV correspondent à deux espèces virales distinctes (Lee et al., 2001).

Au champ, les isolats viraux renferment quatre ARN génomiques. L'ARN1 code pour une seule protéine possédant les caractéristiques des ARN-polymérases-

ARN dépendantes et helicases virales. L'ARN2 renferme six ORF permettant l'expression des protéines majeures et mineures de capside (CP et CP-RT), de trois protéines impliquées dans le mouvement de cellules à cellules (Triple Gene Block, TGB) et d'une protéine riche en cystéines qui est un suppresseur de l'interférence par l'ARN. L'ARN3 est impliqué dans l'expression des symptômes et la pathogénicité virale et l'ARN4 est essentiel pour la transmission du virus par le vecteur P. betae. L'ARN5 est retrouvé uniquement chez certains isolats caractérisés comme très agressifs.

Les ARN3 et 4 de BSBMB sont capables d'être pris en charge par le complexe de réplication du BNYVV et d'être encapsidés par la protéine CP du BNYVV (Ratti *et al*., 2009; D'Alonzo *et al*., 2012) .

Mon projet de thèse a consisté à étudier les interactions moléculaires entre le BNYVV et le BSBMV et rechercher les mécanismes impliqués dans la pathogénicité de ces deux virus.

Des clones complets cDNA infectieux du BNYVV étaient disponibles (Quillet et al., 1989) tout comme ceux des ARN1 et 2 de BSBMV (non publié), de l'ARN3 (Ratti et al., 2009) et 4 (D'Alonzo et al, 2012). Compte tenu de l'aspect versatile de l'obtention de transcrits infectieux de ces différents clones, j'ai entrepris de produire des clones cDNA de chacun des ARN viraux sous contrôle d'un promoteur constitutive végétal pour initier l'infection par agroinfiltration. J'ai également produit des constructions permettant la production des vecteurs d'expression viraux (réplicons) afin d'exprimer diverses protéines.

Les plantes hôtes Chenopodium quinoa et Nicotiana benthamiana ont été inoculées par des transcrits et agroinfiltrées pour initier l'infection virale et étudier l'interaction entre les ARN génomiques 1 et 2 des deux virus et étudier les propriétés de constructions chimères. En parallèle à ce travail, j'ai réalisé la caractérisation du suppresseur de RNA silencing du BSBMV en le comparant à celui du BNYVV.

Résultats et Discussion

La technique d'agroinfection (Grimsley, 1986) apparaît moins onéreuse et plus reproductible qu'une infection à partir de transcrits produits *in vitro*.

L'agroinfection consiste en l'infiltration des tissus végétaux par une suspension d'*Agrobacterium tumefaciens* renfermant un plasmide binaire contenant la copie complète cDNA d'un composant viral sous contrôle d'un promoteur végétal. Le transfert de l'ADN-T dans le génome de la plante permet la transcription *in vivo* de l'ARN viral biologiquement actif. Les clones complets cDNA des ARN de BNYVV, BSBMV et des réplicons exprimant la protéine GFP ont été introduits dans le vecteur pJL89 sous contrôle du promoteur *35S* du virus de la mosaïque du choux fleur et introduits dans *A. tumefaciens*.

L'agroinfection de feuilles de *N. benthamiana* et *Beta macrocarpa* a permis d'initier l'infection locale puis systémique des plantes. Les ARN viraux et les protéines virales ont été détectées par northern blot et western blot, respectivement, tout comme la production de la GFP par l'intermédiaire d'un réplicon. Les particules virales on pu être visualisées par microscopie électronique dans l'ensemble des tissus, dont les racines. La transmission du virus par le vecteur a également été observée, démontrant ainsi la fonctionnalité biologique complète de ce nouvel outil. Deux articles dont je suis premier et second auteur portent sur ces travaux (D'Alonzo et al., 2012 ; Delbianco et al. 2013).

L'inoculation de virus chimériques à partir de transcrits infectieux ou d'infiltration des suspension d'*A. tumefaciens* ont permis de contribuer à l'étude des interactions entre le BNYVV et le BSBMV. Les plantes ont été inoculées par les constructions virales sauvages (Stras12 pour BNYVV et Bo12 pour BSBMV) et par les constructions chimériques BoStras12 et StrasBo12 qui renferme l'ARN1 du premier virus et l'ARN2 du second. Les combinaisons Stras12, Bo12 et StrasBo12 ont produit des lésions locales chlorotiques mais la chimère BoStras12 induit des lésions nécrotiques sévères qui résultent probablement d'une réaction hypersensible de la plante. La nécrose n'apparaît pas quand le suppresseur de RNA silencing du BSBMV est exprimé par l'intermédiaire d'un réplicon. De tels résultats nous laissent penser qu'un isolat chimérique résultant d'une coïnfection d'une plante par les deux virus n'est pas viable et qu'il existe une relation entre l'ARN1 viral et le suppresseur de RNA silencing.

La protéine riche en cystéine codée par le BNYVV (protéine p14) est le suppresseur de RNAi du BNYVV et a été caractérisée dans le groupe du Prof. David Gilmer (IBMP, UdS). Cependant, la fonction de la protéine p14 du BSBMV restait à caractériser. J'ai mis en œuvre des tests d'agroinfiltration de plantes transgéniques exprimant constitutivement la GFP par des constructions déclenchant le PTGS, mis en présence de candidats suppresseur de RNAi. J'ai ainsi pu montrer que la protéine p14 du BSBMV est un suppresseur de RNAi qui induit une forte réduction du pool de siRNA secondaires sans affecter la production de siRNA primaires. J'ai poursuivi la caractérisation de la protéine p14 du BSBMV et montré que cette protéine se localisait à la fois dans le nucléole et le cytoplasme, qu'elle est capable de former des dimères (système double hydride et Fret-FLIM) et d'interagir avec une séquence d'ARN dénommée « coremin » à l'instar de la protéine p14 du BNYVV. La séquence coremin est essentielle au mouvement à longue distance et à la stabilisation d'un ARN non codant d'origine virale (Lauber et al., 1998 ; Peltier et al., 2012). Une partie des résultats exposés ci-dessus a été publiée dans « Molecular plant-microbes interaction » où je suis co-premier auteur (Chiba et al., 2013).

Conclusion and perspectives

Durant ma thèse, j'ai développé de nouveaux outils permettant l'étude du BNYVV et du BSBMV. Ces outils représentent une alternative économique permettant l'étude des interactions entre les deux benyvirus et permettra d'avancer rapidement dans l'étude des synergies / antagonismes existant entre les deux virus. Ces outils permettent également la mise en place de moyens de tests rapides pour caractériser les résistances des betteraves à grande échelle.

L'étude des virus chimères produits par mélange de transcrits infectieux des ARN1 et 2 de chaque virus ou produit après agroinfiltration a mis en évidence l'apparition de lésions nécrotiques sévères qui disparaissent spécifiquement en présence du suppresseur de RNA silencing du BSBMV, suggérant un lien entre l'ARN1 et la protéine p14 dont les caractéristiques ont été étudiées durant cette thèse. Les résultats obtenus ainsi que les outils développés permettront sans nul doute de progresser dans l'étude de la biologie des benyvirus. L'étude des

propriétés des suppresseurs de RNAi du BNYVV et BSBMV se poursuivent et leur lien avec le mouvement longue distance du virus semble lier à la production de l'ARN non codant viral, stabilisé par la séquence coremin reconnue par les protéines p14. Cet ARN non codant pourrait se comporter comme les VA-RNA produits par l'Adenovirus pour saturer la machinerie cellulaire impliquée dans le RNAi (Anderson et al., 2005 ; Peltier et al., 2012).

References

Andersson M. G., Haasnoot P. C., Xu N., Berenjian S., Berkhout B., Akusjarvi G. (2005). Suppression of RNA interference by adenovirus virus-associated RNA. *J. Virol.* 79, 9556–9565.

Chiba, S., Hleibieh, K., Delbianco, A., Klein, E., Ratti, C., Ziegler-Graff, V., Bouzoubaa, S.E., and Gilmer, D. 2012. The benyvirus RNA silencing suppressor is essential for long-distance movement, requires both Zn-finger and NoLS basic residues but not a nucleolar localization for its silencing suppression activity. Mol Plant Microbe Interact. (Sous presse).

D'Alonzo M., Delbianco A., Lanzoni C., Rubies Autonell C., Gilmer D., Ratti C. (2012). *Beet soil-borne mosaic virus* RNA-4 encodes a 32 kDa protein involved in symptom expression and in virus transmission through *Polymyxa betae*. *Virology*, 423, pg.187-194.

Delbianco, Alice; Lanzoni, Chiara; Klein, Elodie; Rubies Autonell, Concepcion; Gilmer, David; Ratti, Claudio (2013) Agroinoculation of Beet necrotic yellow vein virus cDNA clones results in plant systemic infection and efficient Polymyxa betae transmission. *Molecular Plant Pathology.* Sous presse.

Grimsley N., Hohn B., Hohn T., Walden R. (1986). Agroinfection, an alternative route for viral infection of plants by using the Ti plasmid. *Proc. Natl. Acad. Sci*. USA 83, 3282-3286.

Lauber E., Guilley H., Tamada T., Richards K.E., Jonard G. (1998). Vascular movement of *Beet necrotic yellow vein virus* in *Beta macrocarpa* is probably dependent on an RNA3 sequence domain rather than a gene product. *Journal of General Virology* 79, 385-393.

Lee L., Telford E.B., Batten J.S., Scholthof K.B., Rush C.M. (2001). Complete nucleotide sequence and genome organization of *Beet soil-borne mosaic virus*, a proposed member of the genus *Benyvirus*. *Arch. Virol.* 146, 2443-2453.

Peltier C., Hleibieh K., Thiel H., Klein E., Bragard C., Gilmer D. (2008). Molecular Biology of the *Beet necrotic yellow vein virus*. *Plant Viruses* 2, 14-24.

Peltier C., Klein E., Hleibieh K, D'Alonzo M., Hammann P., Bouzoubaa S., Ratti C., Gilmer D. (2012). *Beet necrotic yellow vein virus* subgenomic RNA3 is a cleavage product leading to stable non-coding RNA required for long-distance movement. *J. Gen. Virol*. 93 (Pt 5), 1093-102.

Quillet L., Guilley H., Jonard G., Richards K. (1989). In vitro synthesis of biologically active *Beet necrotic yellow vein virus* RNA. *Virology* 172, 293-301.

Ratti C., Hleibieh K., Bianchi L., Schirmer A., Rubies Autonell C., Gilmer D. (2009). *Beet soil-borne mosaic virus* RNA-3 is replicated and encapsidated in the presence of BNYVV RNA-1 and -2 and allows long distance movement in *Beta macrocarpa*. *Virology* 385, 392-399.

Gilmer, D., and Ratti, C. 2012. Benyvirus. In A.M.Q. King, M.J. Adams, E.B. Carstens, and E.J. Lefkowitz (ed.), Virus taxonomy: classification and nomenclature of viruses: Ninth Report of the International Committee on Taxonomy of Viruses. Elsevier, San Diego, p. 1133-1138.

Thesis Summary

"Molecular mechanisms involved in the pathogenesis of beet soil-borne viruses"

Introduction

The genus *Benyvirus* includes the most important and widespread sugar beet viruses transmitted through the soil by the plasmodiophorid *Polymyxa betae*. In particular *Beet necrotic yellow vein virus* (BNYVV), the leading infectious agent that affects sugar beet, causes an abnormal rootlet proliferation known as rhizomania. BNYVV is widespread in the sugar beet growing areas in Europe, Asia and America (for review see Peltier *et al*., 2008). According to nucleotide sequences analysis the existence of two types of BNYVV was revealed: A type (found in most European countries, Iran, North America, China and Japan) and B type (present in France, Germany, Sweden, China, United Kingdom and Lithuania). Additional types, characterized by the presence of 5 RNAs were identified in France (P type), Kazakhstan, United Kingdom, China and Japan.

Beet soil-borne mosaic virus (BSBMV) is also a member of the *Benyvirus* genus (Lee *et al*., 2001; Gilmer & Ratti, 2012), is widely distributed in the United States and, up to date has not been reported in others countries. It was first identified in Texas as a sugar beet virus morphologically similar but serologically distinct to BNYVV. Subsequent sequence analysis of BSBMV RNAs evidenced similar genomic organization to BNYVV but sufficient molecular differences to distinguish BSBMV and BNYVV in two different species (Lee *et al*., 2001).

Benyviruses field isolates usually consist of four RNA species. RNAs-1 contains a single long ORF encoding for RNA-dependent RNA polymerases (RdRp) and helicases. RNAs-2 contains six ORFs encoding for the capsid protein (CP), the read-through protein (RT), the triple gene block proteins (TGB) required for viral cell-to-cell movement and for a small protein of 14KDa that is a suppressor of post-transcriptional gene silencing. RNAs-3 are involved in disease symptoms and

in viral long distance movement, whereas RNAs-4 are essential for viral transmission through the vector *P. betae*. RNA-5 is only retrieved in some BNYVV field isolates that appear to be more aggressive.

BSBMV RNA-3 and -4 can be trans-replicated and trans-encapsidated by the BNYVV helper strain (RNA-1 and -2) allowing long distance movement and viral transmission through the vector (Ratti *et al*., 2009; D'Alonzo *et al*., 2012).

My PhD project aims to investigate molecular interactions between BNYVV and BSBMV and the mechanisms involved in the pathogenesis of these viruses.

BNYVV full-length infectious cDNA clones were available (Quillet *et al*., 1989) as well as full-length cDNA clones of BSBMV RNA-1, -2 (unpublished), -3 (Ratti *et al*., 2009) and -4 (D'Alonzo *et al*., 2012). Handling of these cDNA clones in order to produce *in vitro* infectious transcripts need sensitive and expensive steps, so I developed agroclones of BNYVV and BSBMV RNAs, as well as viral replicons allowing the expression of different proteins.

Chenopodium quinoa and *Nicotiana benthamiana* plants have been infected with *in vitro* transcripts and agroclones to investigate the interaction between BNYVV and BSBMV RNA-1 and -2 and the behavior of artificial viral chimeras. Simultaneously I characterized BSBMV p14 and demonstrated that it is a suppressor of post-transcriptional gene silencing sharing common features with BNYVV p14.

Results and Discussion

Agroinfection (Grimsley, 1986) is a useful technique that appears to be less expensive and more reproducible for plant infection compared to the use of *in vitro* transcripts. This method consists in tissue infiltration with a suspension of *Agrobacterium tumefaciens* cells carrying binary plasmids harboring full-length cDNA copy of a viral genome component. A plant-functional promoter and the cDNA of a viral RNA are transferred by the way of a T-DNA from *A. tumefaciens* into plant cells. The T-DNA transcription leads to the production of biologically active RNAs able to initiate the viral infection.

Full-length cDNA of BNYVV and BSBMV RNAs as well as viral replicons expressing different proteins and the green fluorescent protein (GFP) have been introduced

in the pJL89 binary vector downstream of the *Cauliflower mosaic virus* 35S promoter. These plasmids were transferred by electroporation into *A. tumefaciens* cells (strain C58C1).

Agroclones were used to agroinfect *N. benthamiana* and *Beta macrocarpa* plants, showing local and systemic symptoms. Viral RNAs and proteins were detected by northern and western blot. Furthermore, replicon-mediated GFP expression has been observed in *N. benthamiana* leaves and characteristic rod-shape particles have been observed through a Transmission Electron Microscope (TEM) in all tissues including roots. The capability of our clones to generate viral RNAs able to complete the viral cycle in *B. macrocarpa* plants, from replication to the transmission through the vector, has been demonstrated. Such results evidenced that our agroclones are perfectly functional and agroinfection represents indeed a useful strategy to carry on further experiments.

Agroclones, as well as infectious transcripts, have been used to investigate interaction between BNYVV and BSBMV in viral chimeras. Plants of *C. quinoa* have been infected with BNYVV and BSBMV RNAs infectious transcripts in different combinations named Stras12 (BNYVV RNA-1 and -2, control), Bo12 (BSBMV RNA-1 and -2, control), BoStras12 (BSBMV RNA-1 and BNYVV RNA-2) and StrasBo12 (BNYVV RNA-1 and BSBMV RNA-2). The combinations Stras12, Bo12 and StrasBo12 showed chlorotic lesions, while BoStras12 induced severe necrotic lesions, probably due to hypersensitive response of the plant. The necrosis disappeared when the plant was co-inoculated with BoStras12 together with a viral replicon expressing the BSBMV p14. Necrotic lesions arose even in *N. benthamiana* plants agroinfected with BoStras12, both in the infiltrated and not infiltrated leaves. These results evidenced a possible interaction between BSBMV p14 protein and the RNA-1 of the virus that requires further investigations.

BNYVV p14 is known to be a suppressor of post-transcriptional gene silencing and it has been characterized in the research group of Prof. David Gilmer (IBMP, University of Strasbourg) but the function of BSBMV p14 has never been investigated. I therefore started the study of BSBMV p14 testing its ability to suppress the PTGS through agroinfiltration of *N. benthamiana* (line 16C) plants constitutively expressing the Green Fluorescent Protein (GFP) transgene.

Challenged *N. benthamiana* 16C plants challenged with the GFP silencing trigger and BSBMV p14 retained the fluorescence in the infiltrated leaves whereas fluorescence disappeared in the controls. Tissue content analyses evidenced the presence of GFP mRNA and strong reduction of siRNAs, the hallmark of the RNA silencing pathway. Taken together, these results demonstrate that the BSBMV p14 is an efficient silencing suppressor protein (SSP).

I then wanted to investigate at which level the Benyviruses p14s interfere in the post-transcriptional gene silencing. Agroinfiltration of *N. benthamiana* plants with different constructs encoding the GFP target, an hairpin GFFG trigger and SSPs showed a normal amount of primary siRNA and a reduced amount of secondary siRNA suggesting that the p14s act downstream of the Dicer proteins without interfering with the transitivity.

Within my PhD project I furthermore demonstrated, through bombardment of *N. tabacum* BY-2 cells, that the Benyvirus p14s are localized in the nucleolus and in the cytoplasm. Moreover using the FLIM and yeast two-hybrid I proved that the BSBMV p14 forms dimers. As for BNYVV p14, I demonstrated that BSBMV p14 can interact with an RNA sequence required for the long distance movement of the virus, named “coremin” sequence. The 20 nucleotides long “coremin” sequence is present in BNYVV RNA-3 and -5 as well as in the BSBMV RNA-3 and -4. BNYVV RNA-3 “coremin” is part of the “core region” and therefore necessary for the systemic movement of the virus within the plant (Lauber *et al*., 1998) but is also essential for the production and stabilization of the ncRNA-3 which is required as well for long-distance movement (Peltier *et al*., 2012).

Conclusions and prospect

During my PhD program, I developed BNYVV and BSBMV agroinfectious clones useful to investigate Benyviruses biology, protein expression and virus-vector interactions. Agroinfection will widely enhance BNYVV/BSBMV research. Moreover, it represents a starting point to develop an innovative test to assay Rhizomania resistance of sugar beet cultivars in large scale experiments (Delbianco *et al*., 2013).

Agroinfection and *in vitro* transcription have been used to investigate BNYVV/BSBMV RNA-1 and -2 interactions and the behavior of viral chimeras in *C. quinoa* and *N. benthamiana* plants. The chimera StrasBo12 showed normal chlorotic lesions whereas the combination BoStras12 induced severe necrotic lesions that disappeared if a viral replicon expressing BSBMV p14 was added to the inoculum. Moreover, the properties of BSBMV p14 have been investigated. This protein, as well BNYVV p14, is a suppressor of post-transcriptional gene silencing acting downstream to the Dicer proteins without interfering with the transitivity. It is localized in the nucleolus and cytoplam, forms dimers and interact with the "coremin" sequence (Chiba *et al*., 2012).

The results obtained and tools developed by my study will allow new researches about biology, interaction and suppression of post-transcriptional gene silencing of Benyviruses.

The role of p14 and its discovered interaction with RNA-1 in the viral chimera BoStras12 open new hypothesis on molecular mechanisms involved in the pathogenesis of Benyviruses which need to be further investigated. BNYVV/BSBMV chimeras will be therefore tested on *B. macrocarpa* plants, a natural host of Benyviruses.

The characterization of the post-transcriptional gene silencing suppression activity of p14s will be carried on through immunoprecipitation. Moreover, its interaction with the "coremin" sequence, and therefore with the ncRNA-3, has to be further investigated. The mode of action of the ncRNA-3, together with p14, is yet to be discovered but its overproduction could be a way to saturate the silencing machinery of the host, as proposed for the human adenovirus VA-RNA (Andersson *et al*., 2005; Peltier *et al*., 2012).

References

Andersson M. G., Haasnoot P. C., Xu N., Berenjian S., Berkhout B., Akusjarvi G. (2005). Suppression of RNA interference by adenovirus virus-associated RNA. *J. Virol.* 79, 9556–9565.

Chiba S., Hleibieh K., Delbianco A., Klein E., Ratti C., Ziegler-Graff V., Bouzoubaa S., Gilmer D. (2012). The Benyvirus RNA silencing suppressor is essential for long distance movement, requires both Zn-finger domain and NoLS basic residues but not a nucleolar localization for its silencing suppression activity. *Molecular plant-microbe interactions*, Vol.26, No.2, pp.168-181.

D'Alonzo M., Delbianco A., Lanzoni C., Rubies Autonell C., Gilmer D., Ratti C. (2012). *Beet soil-borne mosaic virus* RNA-4 encodes a 32 kDa protein involved in symptom expression and in virus transmission through *Polymyxa betae*. *Virology*, 423, pg.187-194.

Delbianco A., Lanzoni C., Klein E., Rubies Autonell C., Gilmer D., Ratti C. (2013). Agroinoculation of *Beet necrotic yellow vein virus* cDNA clones results in plant systemic infection and efficient *Polymyxa betae* transmission. *Molecular Plant Pathology*, in press.

Gilmer D., Ratti C. (2012). *Benyvirus*. In A.M.Q. King, M.J. Adams, E.B. Carstens, and E.J. Lefkowitz (ed.), Virus taxonomy: classification and nomenclature of viruses: Ninth Report of the International Committee on Taxonomy of Viruses. Elsevier, San Diego, p. 1133-1138.

Grimsley N., Hohn B., Hohn T., Walden R. (1986). Agroinfection, an alternative route for viral infection of plants by using the Ti plasmid. *Proc. Natl. Acad. Sci*. USA 83, 3282-3286.

Lauber E., Guilley H., Tamada T., Richards K.E., Jonard G. (1998). Vascular movement of *Beet necrotic yellow vein virus* in *Beta macrocarpa* is probably dependent on an RNA3 sequence domain rather than a gene product. *Journal of General Virology* 79, 385-393.

Lee L., Telford E.B., Batten J.S., Scholthof K.B., Rush C.M. (2001). Complete nucleotide sequence and genome organization of *Beet soil-borne mosaic virus*, a proposed member of the genus *Benyvirus*. *Arch. Virol.* 146, 2443-2453.

Peltier C., Hleibieh K., Thiel H., Klein E., Bragard C., Gilmer D. (2008). Molecular Biology of the *Beet necrotic yellow vein virus*. *Plant Viruses* 2, 14-24.

Peltier C., Klein E., Hleibieh K, D'Alonzo M., Hammann P., Bouzoubaa S., Ratti C., Gilmer D. (2012). *Beet necrotic yellow vein virus* subgenomic RNA3 is a cleavage product leading to stable non-coding RNA required for long-distance movement. *J. Gen. Virol*. 93 (Pt 5), 1093-102.

Quillet L., Guilley H., Jonard G., Richards K. (1989). In vitro synthesis of biologically active *Beet necrotic yellow vein virus* RNA. *Virology* 172, 293-301.

Ratti C., Hleibieh K., Bianchi L., Schirmer A., Rubies Autonell C., Gilmer D. (2009). *Beet soil-borne mosaic virus* RNA-3 is replicated and encapsidated in the presence of BNYVV RNA-1 and -2 and allows long distance movement in *Beta macrocarpa*. *Virology* 385, 392-399.

PUBLICATIONS

- M. D'Alonzo, **A. Delbianco**, C. Lanzoni, C. Rubies Autonell, D. Gilmer, C. Ratti (2012). *Beet soil borne mosaic virus* RNA-4 encodes a 32 kDa protein involved in symptom expression and in virus transmission through *Polymyxa betae*. *Virology*, 423, pg.187-194.

- S. Chiba*, K. Hleibieh*, **A. Delbianco***, E. Klein, C. Ratti, V. Ziegler-Graff, S. Bouzoubaa, D. Gilmer (2012). The Benyvirus RNA silencing suppressor is essential for long distance movement, requires both Zn-finger domain and NoLS basic residues but not a nucleolar localization for its silencing suppression activity. *Molecular plant-microbe interactions*, Vol.26, No.2, pp.168-181. *Authors contributed equally to this work.

- **A. Delbianco**, M. D'Alonzo, E. Klein, D. Gilmer, C. Rubies Autonell, C. Ratti (2012). Agroinfection: a new tool for *Beet necrotic yellow vein virus* study and for rhizomania-resistance assessment. In *Proceedings of the Eighth Symposium of the International Working Group on Plant Viruses with Fungal Vectors*, Louvain la Neuve (Belgium), 6-8 july 2011, pg.7.

- M. D'Alonzo, **A. Delbianco**, C. Lanzoni, C. Rubies Autonell, D. Gilmer, C. Ratti (2012). Essential role of BSBMV RNA-4's p32 on virus transmission by Polymyxa betae in *Beta vulgaris* plants. In *Proceedings of the Eighth Symposium of the International Working Group on Plant Viruses with Fungal Vectors*, Louvain la Neuve (Belgium), 6-8 july 2011, pg.52.

- **A. Delbianco**, C. Lanzoni, E. Klein, C. Rubies Autonell, D. Gilmer, C. Ratti (2013). Agroinoculation of *Beet necrotic yellow vein virus* cDNA clones results in plant systemic infection and efficient *Polymyxa betae* transmission. *Molecular Plant Pathology,* Vol. 14 (4), pp. 422-428.

SCIENTIFIC CONGRESS COMUNICATION - POSTER

- **A. Delbianco**, E. Klein, M. D'Alonzo, Gilmer, C. Rubies Autonell and C. Ratti. Agroinfection: a new approach for *Beet necrotic yellow vein virus* study. In: Abs. Book of XIIIemes Rencontres de Virologie Végétale, Aussois, 16-20 january 2011, pg.52.

- K. Hleibieh, **A. Delbianco**, A. Flabinus, S. Chiba, E. Klein, D. Scheidecker, C. Ratti, S. Bouzoubaa, V. Ziegler-Graff, D. Gilmer. BNYVV p14 RNA silencing suppressor and viral RNA3 coremin sequence are implicated in the long distance movement." In: Abs. Book of XIVemes Rencontres de Virologie Végétale, Aussois, 13-17 january 2013.

SCIENTIFIC CONGRESS COMUNICATION - ORAL COMUNICATION

- **A. Delbianco**, E. Klein, M. D'Alonzo, D. Gilmer, C. Rubies Autonell, C. Ratti. Agroinfection: a new approach for *Beet necrotic yellow vein virus* study. Séminaire de Microbiologie de Strasbourg, Amphi VLES, IPCB, Strasbourg, 31 march 2011.

- **A. Delbianco**, M. D'Alonzo E. Klein, D. Gilmer, C. Rubies Autonell and C. Ratti. Agroinfection: a new tool for *Beet necrotic yellow vein* virus study and for Rhizomania-resistance assessment. In: Abs. Book of VIIIth International Working Group of Plant Viruses with Fungal Vectors, Louvain La Neuve, 6-8 july 2011, pg.9.

- **A. Delbianco**, M. D'Alonzo, C. Lanzoni, E. Klein, D. Gilmer, C. Rubies Autonell and C. Ratti. Agroinfection: a new approach for *Beet necrotic yellow vein virus* study. In Abs. Book of XVII National Congress of the Italian Society for Phytopathology (SiPAV), Bologna, 12-14 september 2011, pg.15.

- **A. Delbianco**, K. Hleibieh, M. Dall'Ara, C. Rubies Autonell, D. Gilmer, C. Ratti. Characterization of post-trascriptional gene silencing suppressor proteins of *Benyvirus*. In Abs. Book of 11th National Congress of the Italian Society for Virology (SIV), Orvieto, 17-19 september 2012, pg.17.

- **A. Delbianco**, M. Dall'Ara, K. Hleibieh, E. Klein, C. Rubies Autonell, D. Gilmer and C. Ratti. Post-transcriptional gene silencing suppression study of *Beet soil-borne mosaic virus*: characterization of p14 and production of chimeric isolates of Benyviruses. In Abs. Book of XIVemes Rencontres de Virologie Végétale, Aussois, 13-17 january 2013, pg.19.

General Introduction

Before the presentation of the aim of my PhD project, I would like to place the current knowledge and the existing relationships of the main actors used in this research work leading to the most devastating viral disease of sugar beet.

1. Sugar beet

Beta vulgaris ssp. *vulgaris* belongs to the genus *Beta* of the *Chenopodiaceae* family and it descends from *Beta vulgaris* ssp. *maritima* or *Beta maritima*, an halophytic plant adapted to salty environments, native of the Mediterranean area (Francis, 2006). Sugar beet is agriculturally important due to its capability to accumulate a great quantity of sugar in its taproot. It is a biennial plant and its vegetative development is mainly divided into three phases: shoot growth, storage root growth ("tuberization") and sugar storage ("ripening") (Milford, 2006).

Sugar beet has been cropped and eaten as a spinach-like vegetable since ancient historical times, but only in the second part of the eighteen century an industrial crop developed in Europe for sugar production, in order to compete with sugar cane (*Saccharum officinarum*) coming from South American colonies (Francis, 2006).

Nowadays, sugar beet occupies globally a cultivated area of 4,7 million hectares in 51 countries and provides about 25% of world sugar consumption (Rush *et al*., 2006; FAO, 2010). Recent plant breeding has contributed to improve sugar concentration from 12% of the fresh root to the current value of the 20% (Draycott, 2006). In Europe, Germany, France and Ukraine cultivate the widest area but roots production per hectare is quite variable with France, Spain and Belgium obtaining the highest yields (Table I.A, FAO 2010). Recently, the interest in this plant increased thanks to bioethanol production, as a replacement of fossil fuels in transports' sector.

However, sugar beets are susceptible to many pathogens such as nematodes, fungi, bacteria and viruses, attacking crops and leading to the reduction of both taproot size and sugar content (Whitney and Duffus, 1986) (Table I.B). Among viruses, the *Beet necrotic yellow vein virus* and its associated Rhizomania disease is the most pathogenic and worldwide distributed (Stevens *et al*., 2006).

Countries	Area Harvested (Ha)	Production (T)	Yield (T/Ha)
Afghanistan	5.100	15.200	2,98
Albania	2.000	40.000	20,00
Armenia	600	10.000	16,67
Austria	44.841	3.131.670	69,84
Azerbaijan	8.459	251.854	29,77
Belarus	95.529	3.773.390	39,50
Belgium	**59.303**	**4.464.780**	**75,29**
Canada	11.300	508.000	44,96
Chile	16.264	1.420.270	87,33
China	219.000	9.296.000	42,45
Colombia	969	18.979	19,59
Croatia	23.832	1.249.150	52,41
Czech Republic	56.400	3.065.000	54,34
Denmark	39.200	2.356.000	60,10
Ecuador	600	3.900	6,50
Egypt	134.538	7.840.300	58,28
Finland	14.600	542.100	37,13
France	**383.479**	**31.874.800**	**83,12**
Germany	**367.000**	**23.858.400**	**65,01**
Greece	13.200	761.500	57,69
Hungary	13.859	818.941	59,09
Iran	99.486	3.896.820	39,17
Iraq	1.750	18.000	10,29
Italy	62.700	3.550.100	56,62
Japan	62.600	3.090.000	49,36
Kazakhstan	8.800	151.970	17,27
Kyrgyzstan	8.400	139.200	16,57
Lebanon	700	37.000	52,86
Lithuania	15.500	722.500	46,61
Mali	270	3.800	14,07
Morocco	43.200	2.435.910	56,39
Netherlands	70.560	5.280.430	74,84
Pakistan	1.300	53.336	41,03
Poland	199.900	9.822.900	49,14
Portugal	305	7.955	26,08
Republic of Moldova	26.452	837.624	31,67
Romania	21.627	837.895	38,74
Russian Federation	923.800	22.255.900	24,09
Serbia	66.446	3.324.850	50,04
Slovakia	17.932	977.694	54,52
Spain	**44.300**	**3.399.400**	**76,74**
Sweden	37.900	1.973.700	52,08
Switzerland	17.842	1.302.060	72,98
Siria	27.500	1.493.000	54,29
Macedonia	300	8.000	26,67
Turkey	328.651	17.942.100	54,59
Turkmenistan	21.000	234.000	11,14
Ukraine	**492.000**	**13.749.000**	**27,95**
United Kingdom	118.000	6.527.000	55,31
United States of America	467.858	29.060.800	62,11
Venezuela	1.100	20.500	18,64
TOTAL	**4.698.252**	**228.453.678**	**48,63**

Table I.A: World sugar beet production. Area harvested, production and yield in all productive countries of the world (adapted from FAO, 2010).

Disease	Causal agent
Viruses	
Rhizomania	*Beet necrotic yellow vein virus* (BNYVV)
Rhizomania related virus	*Beet soil-borne mosaic virus* (BSBMV)
	Beet soil-borne virus (BSBV)
	Beet virus Q (BVQ)
	Beet oak leaf virus (BOLV)
	Beet black scorch virus (BBSV)
Beet mosaic	*Beet mosaic virus* (BtMV)
Beet yellows	*Beet yellows* (BYV)
	Beet mild yellows (BMYV)
	Beet western yellows (BWYV)
	Beet chlorosis virus (BChV)
Curly top	*Beet curly top virus* (BCTV)
Bacteria	
Bacterial vascular necrosis and rot	*Erwinia carotovora* spp. *betavasculorum*
Bacterial leaf spot or leaf blight	*Pseudomonas syringae*
Yellow wilt	Rickettsia-like organism
Fungi	
Cercospora leaf spot	*Cercospora beticola*
Alternaria leaf blight	*Alternaria alternata, Alternaria brassicae*
Powdery mildew	*Erysiphe betae*
Downy mildew	*Peronospora schachtii* (*farinosa*)
Fusarium yellows / Fusarium root rot	*Fusarium oxysporum* f. sp. *betae*
	Rhizoctonia solani
Root rots	*Pythium* spp.
	Phoma betae
Southern sclerotium root rot	*Sclerotium rolfsii*
Black root / Black leg	*Aphanomyces cochlioides*
Nematodes	
Cyst nematode	*Heterodera schachtii*
Root-knot nematode	*Meloidogyne* spp.

Table I.B: Principal sugar beet diseases.

2. Benyviruses

2.1 The genus

The genus *Benyvirus*, type member *Beet necrotic yellow vein virus* (BNYVV), was accepted by the International Committee of Taxonomy of Viruses (ICTV) in 1997 after a revision of the genus *Furovirus* (Rush, 2003). This genus include the BNYVV, the *Beet soil borne mosaic virus* (BSBMV), *Rice stripe necrosis virus* (RSNV) and the tentative member *Burdock mottle virus* (BdMV) (Gilmer and Ratti, 2012). These viruses have non-enveloped rod-shaped and helically constructed particles. Their multipartite genomes consist of positive and single stranded RNA fragments with 5' m^7G cap, 3' polyadenylated sequence and post translational cleavage of the viral replicase (Hehn *et al*., 1997; Lee *et al*., 2001, Peltier *et al*., 2008).

Benyviruses have limited host ranges. *Beta vulgaris* is the natural host of BNYVV and BSBMV, and experimentally these viruses can infect some species of *Chenopodium* genus that allow local infection only and *Nicotiana benthamiana*, *Spinacia oleracea* and *Beta macrocarpa* where the viruses can move systemically. Both BNYVV and BSBMV are naturally transmitted by the protozoa *Polymyxa betae* and RSNV by *P. graminis* (Tamada, 1975; Lee *et al*., 2001; Rush, 2003).

This genus shares conserved residues within the coat protein and similar viral particle morphology with the *Virgaviridae* family (furo-, peclu-, pomo-, hordei-, tobra- and tobamoviruses). Benyviruses movement strategy involves triple gene block cluster similarly to pomo-, peclu- and hordeiviruses (Verchot *et al*, 2010). Conversely, replication proteins domains display high degree of similarity to those of the *Togaviridae* family and, interestingly, to the human hepatitis virus E (Gilmer and Ratti, 2012). Thereby, benyviruses could be proposed as a distinct family thanks to its particular genome specificities.

2.2 The Rhizomania disease and *Beet necrotic yellow vein virus*

The *Beet necrotic yellow vein virus* has been identified as the causal agent of Rhizomania disease in the early seventies (Tamada and Baba, 1973). In 1959, Antonio

Canova working at the “Istituto di Patologia Vegetale” of the University of Bologna published the first report about a disease affecting sugar beet roots in the Padan Plain of Italy. Later, this syndrome has been named “rizomania” (Canova, 1966), a term composed by the Latin words “rhizo” and “manĭıa” meaning “root madness”, the principal character of this disease (Biancardi, 2005). Since the middle of the twenty century, Rhizomania has been reported in all the sugar beet growing areas all around the world (McGrann *et al*., 2009).

Infected plants show characteristic proliferation of lateral rootlets with consequently stunting of the tap-root and browning of the vascular system. The size of the tap-root can be strongly reduced (Brunt and Richards, 1989; Putz *et al*., 1990). The inefficient nutrient uptake causes yellowing in leaves (Stevens *et al*., 2006). Occasionally, BNYVV spread systemically and leaves show necrosis and yellowing in the leaf veins, these symptoms provided the name for the virus (Tamada and Baba, 1973; Tamada, 2002) (Fig. I.1).

Rhizomania causes severe losses to the sugar beet crop, due to serious decreases in root yield, sugar content and juice purity. The sugar yield reduction can reach the 80% (McGrann *et al*., 2009).

Fig I.1: Rhizomania symptoms caused by BNYVV infection on sugar beet. (A) Yellowing and necrosis on the leaves veins. (B) Lateral rootlet proliferation with browning of the vascular system and constriction of the main root. (C) Symptoms on fields.

The genome of *Beet necrotic yellow vein virus* consists of four to five (+) ssRNAs. RNA-1 and -2 carry “house-keeping” genes involved in virus replication, assembly, cell-to-cell movement and suppression of post-transcriptional gene silencing (Tamada, 1999; Dunoyer *et al*., 2002). These RNAs are necessary and sufficient for viral infection when

inoculated to the leaves of *Beta macrocarpa*, *B. vulgaris*, *Chenopodium quinoa* and *Tetragonia expansa* plants (Koenig *et al*., 1986; Pelsy and Merdinoglu, 1996; Tamada *et al*., 1989). A replicative strain named "Stras12" was obtained extracting viral RNA from leaves of *C. quinoa* and *T. expansa* infected with *in vitro* transcripts of BNYVV RNA-1 and -2 full-length cDNA clones (Quillet *et al*., 1989). However, natural infection requires RNA-3 and -4 encoded proteins directly involved in the pathogenesis and viral transmission through the vector *P. betae*, respectively (Lemaire *et al*., 1988; Koenig *et al*., 1991).

RNA-1 is 6,746 nucleotides (nts) long and encodes for a single open reading frame (ORF) generating a polypeptide of 237 kDa that, after translation, is processed by an autocatalytic cleavage in two proteins of 150 kDa and 66 kDa. The first one contains conserved motifs of methyltransferase (MTR) and helicase (HEL) while the second displays RNA-dependant RNA polymerase (RdRp) motif, which is essential for virus replication (Bouzoubaa *et al*., 1987; Hehn *et al*., 1997). The proteolytic cleavage of the replicase distinguishes *Benyvirus* from all other virus with rod-shaped particles, which have their replication-associated proteins encoded in two ORFs (Gilmer and Ratti, 2012).

RNA-2 (4,609 nts) contains six ORFs. The viral Coat Protein (CP) of 21kDa is encoded by the first ORF and is followed by an in-frame region of 54 kDa read-through (RT) domain. CP and RT are fused in a 75 kDa protein during translation when the internal leaky UAG stop codon is bypassed by ribosomes (Ziegler-Graff *et al*., 1985; Niesbach-Klosgen *et al*., 1990). The N-terminal of this protein is involved in viral assembly whereas the C-terminal is required for viral transmission through the vector *P. betae* (Schmitt *et al*., 1992; Tamada and Kusume, 1991). The three subsequent ORFs show typical motifs of the "triple gene block" (TGB) movement proteins and encode for p42, p13 and p15 proteins that are expressed by the way of two subgenomic RNAs (Gilmer *et al*., 1992). The last ORF is also expressed by a subgenomic RNA and encodes for a cysteine-rich protein of 14kDa, which is a suppressor of post-transcriptional gene silencing (Dunoyer *et al*., 2002; this study: Chiba *et al.*, 2012).

RNA-3 (1,774 nts) encodes the pathogenicity protein p25 and two other proteins of 6.8 kDa (N) and 4.6 kDa. Functions of the last two small proteins are not well documented and the last one has never been detected. Expression of p25 is linked to the

development of the rhizomania symptoms in sugar beet roots and it is able to induce bright yellow local lesions in *C. quinoa* plants and abnormal root branching in transgenic *Arabidopsis thaliana* (Tamada *et al*., 1990; Koenig *et al*.,1991; Tamada *et al*., 1989; Jupin *et al*., 1992; Peltier *et al*., 2010). Variability in different amino acid positions of p25 has been associated with an increased pathogenicity and the capability to overcome rhizomania resistance conferred by the *Rz1* resistance gene derived by the 'Holly' source (Schirmer *et al*., 2005; Acosta-Leal *et al*., 2008; Lewellen *et al*., 1987; Chiba *et al*., 2008; Koenig *et al*., 2009). RNA-3 is required for viral long distance movement in *B. macrocarpa* (Tamada *et al*., 1989). The observation that deletion of nts 1033-1257, named "core region", inhibit the vascular movement, suggested that RNA-3 sequence, rather than RNA-3 encoded protein, was required for BNYVV long distance movement in *B. macrocarpa* (Lauber *et al*., 1998). Ratti *et al*., (2009) identified a stretch of 20 nts named "coremin", inside the "core region", which appears to be responsible of the long distance movement. "Coremin" is also present in BNYVV RNA-5, BSBMV RNA-3 and -4 and in other viral species of the genus *Cucumovirus.* Interestingly, this sequence is present in the 5' leader ORF-less regions of subgenomic CMV RNA-5, BNYVV and BSBMV RNA-3. A recent study demonstrated that BNYVV subRNA-3 is, in fact, a cleavage product leading to stable non-coding RNA (ncRNA-3) required for long distance movement. Mutagenesis revealed the importance of "coremin" sequence both for long distance movement and ncRNA-3 stabilization (Peltier *et al*., 2012).

RNA-4 (1,467 nts) is necessary for the viral transmission through the protozoa *P. betae* (Tamada and Abe, 1989). The encoded protein (p31) is required for efficient transmission and is able to suppress post-transcriptional gene silencing in *N. benthamiana* roots (Andika *et al*., 2005; Rahim *et al*., 2007).

Finally, RNA-5 is present only in some field isolates that appears to be more aggressive (Tamada *et al*., 1996). Such RNA encodes for another pathogenicity protein of 26 kDa that probably acts in a synergistic manner with RNA-3 p25 (Kiguchi *et al*., 1996; Link *et al*., 2005) (Fig. I.2).

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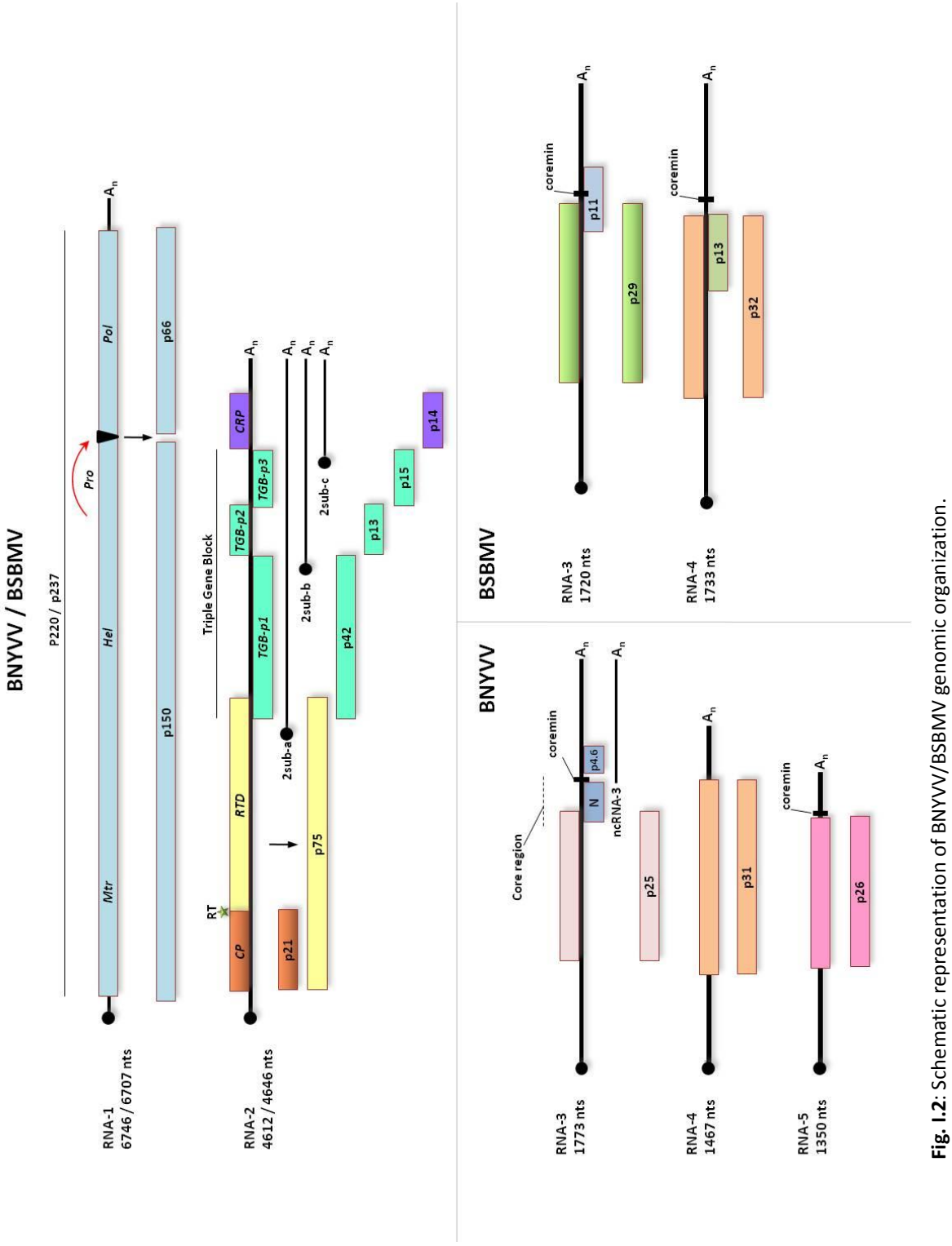

Fig. I.2: Schematic representation of BNYVV/BSBMV genomic organization.

According to restriction length polymorphism, single strain conformation polymorphism patterns and phylogenetic relationships, three different types of BNYVV have been described and named A, B and P (Koenig *et al*., 1995; Kruse *et al*., 1994; Schirmer *et al*., 2005).

A-type is worldwide distributed, while B-type has been found mainly in France, Germany and Japan and it has been reported even in United Kingdom and Sweden (Koenig *et al*., 1995; Miyanishi *et al*., 1999; Lennefors *et al*., 2000). Nucleotide identity score between these two variants is very high, ranging between 96%-99% (Saito *et al*., 1996; Koenig and Lennefors, 2000; Meunier *et al*., 2003).

No pathogenecity differences have been reported between A- and B-type in contrast with P-type that contain RNA-5 and seems to be more aggressive (Heijbroek *et al*., 1999). P-type has been isolated in Pithiviers (France) (Koenig *et al*., 1995) but isolates containing five RNAs have been described also in Japan, United Kingdom and Kazakhstan (Tamada *et al*., 1989; Koenig and Lennefors, 2000; Harju *et al*., 2002;). P variant seems to move more rapidly in plants, resulting in a higher level of infection of tap root compared to A- and B-type, even in partially resistant cultivars (Hejibrowk *et al*., 1999).

2.3 *Beet soil-borne mosaic virus*

Beet soil-borne mosaic virus was discovered in Texas in 1988 as a sugar beet virus morphologically similar but serologically distinct to BNYVV (Liu and Duffus, 1988). BSBMV was completely sequenced by Lee *et al*. (2001) demonstrating that BSBMV and BNYVV have identical genomic organization but sufficient molecular differences to be considered two separated species.

BSBMV is widely distributed only in the United States and up to now it has not been reported in other countries (Rush, 2003; Ratti *et al*., 2009). Sugar beet infected roots often appears asymptomatic whereas leaves show slight distortion, faint general mottling and yellow vein banding, which can progress to chlorosis (Heidel and Rush, 1994).

Rush *et al*. (1994) demonstrated that PCR primers designed on the 3’ end of BNYVV RNAs amplify homologous species of BSBMV RNAs. These PCR products, used as

northern blot probes, weakly hybridized BNYVV RNAs. In contrast, PCR specific primers designed on the 5' end of BNYVV RNAs were not able to amplify BSBMV (Heidel *et al*., 1997). Ratti *et al.* (2009) showed that 5' terminus of BSBMV and BNYVV RNA-3s share common structures.

BSBMV genome consists of four capped and polyadenylated RNAs (Lee *et al*., 2001).

RNA-1 (6,683 nts) contains a single long ORF encoding for a 239kDa protein that share amino acids homology with known viral RNA-dependent RNA polymerases (RdRp) and helicases.

RNA-2 is 4,615 nts long and contains six ORFs. The first ORF encodes for the Coat Protein of 21 kDa and is followed by a leaky UAG stop codon whose suppression leads to the expression of a 74 kDa protein (RT, read-through). These two genes are followed by three ORFs encoding movement proteins showing typical motifs of the Triple Gene Block with predicted masses of 42kDa, 13kDa and 15kDa. The last ORF encodes for a 14kDa cysteine-rich protein similar to BNYVV p14, but so far it has not been deeply investigated (Hehn *et al*., 1995; Lee *et al*., 2001).

RNA-3 (1,730 nts) encodes for a 29 kDa protein that share only the 23% amino acids identity score with the p25 of BNYVV RNA-3 (Ratti *et al*., 2009).

RNA-4 species described by Lee *et al*. (2001) is 1,203 nts long encoding a protein of 13 kDa, considerably smaller than the BNYVV RNA-4 p31. This species could correspond to a shortened form of BSBMV RNA-4 produced after serial mechanical inoculation on *C. quinoa* leaves as already described for BNYVV RNA-3 and -4 by Bouzoubaa *et al*. (1991). Recently, a new species of BSBMV RNA-4 has been characterized which is 1,733 nts long and encodes for a protein of 32 kDa necessary for the viral transmission through the vector *P. betae* (D'Alonzo *et al*., 2012) (Fig. I.2)**.**

2.4 The vector *Polymyxa betae*

BNYVV and BSBMV are both vectored by the plasmodiophorid *Polymyxa betae*. Traditionally, plasmodiophorids have been considered as fungi in phylum *Plasmodiophoromycota*, but recently they have been reclassified within the Protozoa (Braselton, 1995; Rush, 2003).

P. betae is only weakly pathogenic and its host range is restricted to roots of *Chenopodiaceae*, *Amaranthaceae*, *Caryophyllaceae* and *Portulaceae* (Rush, 2003).

Life cycle of *P. betae* includes several stages (Fig. I.3 and I.4). Clusters of thick-walled resting spores, named sporosori, are liberated into the soil during senescence of infected plant roots. Sporosori are able to survive in soil for years, however in the presence of a susceptible host and suitable conditions of temperature and humidity, these resting spores germinate and release primary zoospores. Zoospores encyst in rootlets and inject their cytoplasmic content inside the root cells, inducing the formation of a multinucleated plasmodium. In this phase the viral particles can be transferred to the host or acquired by the vector. The plasmodium can differentiate in a zoosporangium, leading to the production of secondary zoospores that can infect new roots, or in a sporosorus with the production of resting spores (Keskin, 1964; Adams, 1991; Barr and Asher, 1992).

Lubicz *et al*. (2007) observed that BNYVV proteins accumulate inside *P. betae* resting spores and zoospores. Association of viral replication and movement proteins with sporangial and sporogenic stages suggests that the virus resides in the vector more than one life cycle, advancing the hypothesis that *P. betae* may have an additional role as a host. However, in the absence of negative sense viral RNA detection, the replication of benyviruses in their vector is still controversial.

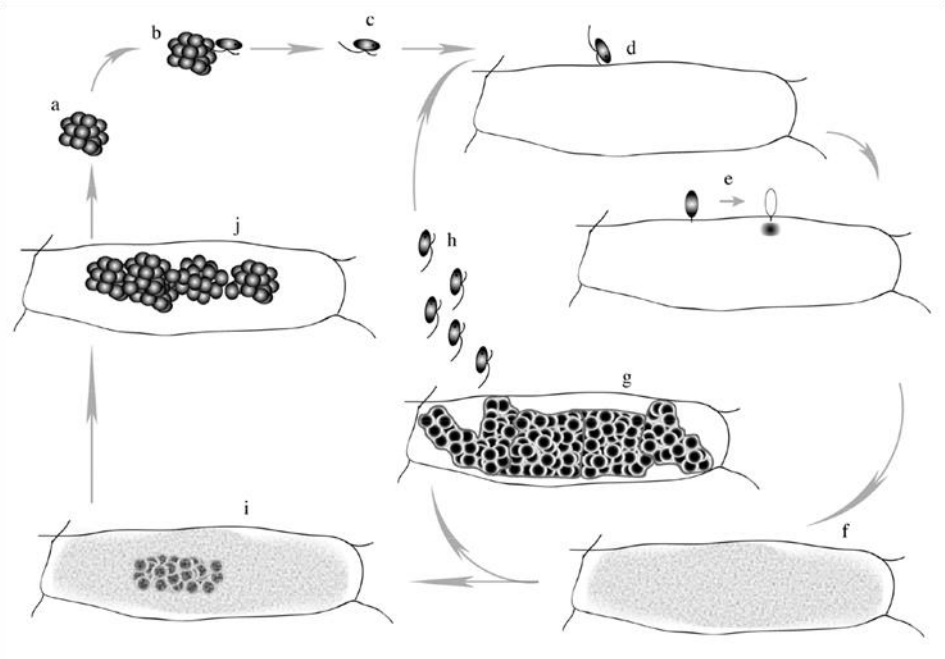

Fig. I.3: Schematic representation of the *P. betae* life cycles and its developing states. (a) sporosore; (b) germinating zoospore; (c) swimming zoospore to a (d) cortical or epidermal cell; (e) the zoospore encyst on the cell and injects its contents through the cell wall and the cellular membrane; (f) developing plasmodium that will tend to a zoosporangium (g) that will issue either (h) the secondary zoospores able to infect new cells or (i) to the sporogenous plasmodium (j) leading to new sporosores. Such sporosores will be further released in soil after root decomposition (adapted from Peltier *et al.*, 2008).

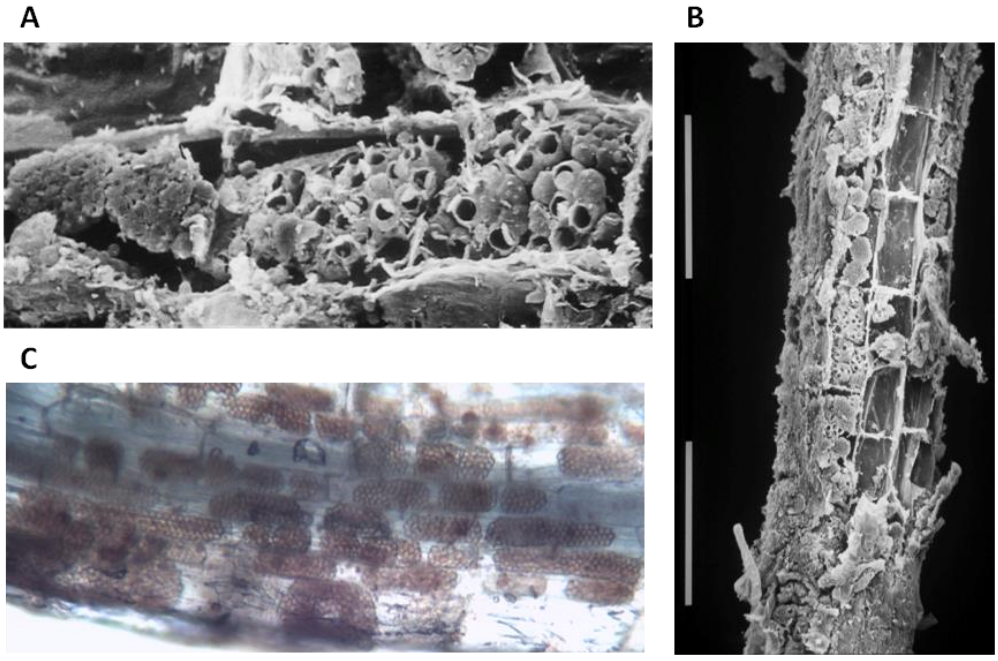

Fig. I.4: (A) and (B) Scanning Electron Microscope and (C) optical microscope images of *P. betae* resting spores in sugar beet cortical root cells.

3. Relationship and interaction between BNYVV and BSBMV

BNYVV and BSBMV have similar host range and particle morphology, present identical genomic organization and share the same vector *P. betae*. Based on the degree of nucleotide and amino acid sequence identity between the two viruses, it has been concluded that BNYVV and BSBMV are distinct species but more closely related to each other than to other multipartite rod-shaped viruses with "fungal" vectors included in the original genus *Furovirus* (Lee *et al*., 2001). Moreover, the first characterization of BSBMV demonstrated that its capsid protein (CP) is serologically distinct to that of BNYVV (Wisler *et al*., 1994).

Sequence alignments shows that BNYVV and BSBMV RNA-1 share 77% nucleotide sequence identity (Lee *et al*., 2001). The large and unique ORF encodes for replication proteins (83% amino acid identity), in contrast with the two ORFs present in other rod-shaped viruses with fungal vectors (Koonin and Doljia, 1993).

BSBMV RNA-2 share the 67% nucleotide sequence identity with the BNYVV RNA-2. The coat protein and the 74 kDa read-through translation product show amino acid sequence identity of 56%, whereas the triple gene block proteins and p14 have 74%, 81%, 65% and 32% amino acid identity, respectively (Lee *et al*., 2001).

BSBMV RNA-3 sequence has 60% identity with the BNYVV RNA-3 and the encoded p29 shared just the 23% amino acid identity with p25. It has been recently reported that BSBMV p29 sequence is closer to BNYVV RNA-5 p26 than to RNA-3 p25 (Ratti *et al*., 2009).

BSBMV and BNYVV RNA-4 share the 47% nucleotide identity score and the encoded proteins of 32 and 31 kDa share a significant amino acid identity of 49,8% (D'Alonzo *et al*., 2012).

Comparisons between 5' and 3' UTRs of all BNYVV and BSBMV RNAs showed highly conserved regions of 7 and 70 nucleotides, respectively (Lee *et al*., 2011). The 5' UTRs of BNYVV and BSBMV RNA-1 and -2 share 92% and 81% nucleotide identity sequence, respectively, in contrast with the 38% and 50% of RNA-3 and -4. The 3'UTRs of RNA-1, -2, -3 and -4 have a nucleotide identity score of 66%, 67%, 79% and 64%, respectively (Lee *et al*., 2001).

Conserved *cis*-acting elements, essential for replication, are present in 5' and 3' UTRs of BNYVV RNA-3 and -4 (Bouzoubaa *et al*., 1991). These conserved regions can fold in double hairpin secondary structure that presumably is recognized by the viral replicase during initiation of minus strand synthesis (Lauber *et al*., 1997). Recently Ratti *et al*. (2009) observed that both BNYVV and BSBMV RNA-3 show strong sequence and structure similarities in 5' and 3' UTRs. In the same work the authors demonstrated that BSBMV RNA-3 can be replicated and encapsidated by BNYVV RNA-1 and -2 and allows long distance movement in *B. macrocarpa*. Moreover expression of different proteins through a BSBMV expression vector (Rep III), containing 5' and 3' UTRs of RNA-3, was possible in the presence of BNYVV RNA-1 and -2. However, Rep III cannot be replicated in the same cell together with BNYVV RNA-3 or its derived replicon leading to the competitive loss of one of the molecules within the local lesion (Ratti *et al*., 2009).

Similarly, BSBMV RNA-4 can be amplified by BNYVV RNA-1 and -2 and can complement BNYVV RNA-4 in virus transmission through the vector *P. beta* in *B. vulgaris* plants (D'Alonzo *et al*., 2012).

In the United States BNYVV and BSBMV are often present in the same field, sometimes in the same plant and therefore interactions such as cross protection have been investigated. Cross protection is a mechanism that occurs when a plant infected by one virus (protecting) is then protected by the infection of a second virus (challenging). This phenomenon usually occurs between two strains of the same virus but sometimes among different viruses.

Mahmood and Rush (1999) showed a high degree of reciprocal cross protection between BNYVV and BSBMV in greenhouse experiments on *Beta vulgaris* seedlings inoculated with protecting virus on roots and with challenging virus on leaves through sap of *C. quinoa* infected leaves. The degree of cross-protection was increased by longer inoculation intervals between the first and the second inoculum. Moreover, RNA of both viruses was detected in doubly infected plants but capsid protein of the BNYVV was undetected by serological tests, suggesting that BSBMV CP is involved in some mechanisms able to avoid superinfection in cross-protection tests.

However, distinct results were obtained using different approaches. Experiments performed with soils naturally infested with *P. betae* zoospores carrying BNYVV and

BSBMV seem to demonstrate that BNYVV is able to suppress BSBMV in mixed infections (Wisler, 2003). When BSBMV was present in mixed infections with BNYVV, its level was strongly reduced, even when BNYVV titer was very low, particularly in Rhizomania-resistant cultivars. Furthermore, the *Rz* allele of rhizomania-resistance does not provides resistance to BSBMV. The significant reduction of BSBMV in the presence of BNYVV may be due to several factors such as competition for host infection sites by virouliferous *P. betae,* BNYVV infected zoospores could be more aggressive or one virus may have a competitive advantages once inside the cell (Wisler, 2003).

However, these studies about BNYVV/BSBMV interactions have been conducted under different experimental conditions that have to be considered (Rush, 2003). Sugar beet plants vortexed in a liquid inoculum become entirely infected, in contrast to infection through *P. betae* that usually remains localized into the roots and rarely goes systemic. With the vortex method, the first virus becomes established and interferes with subsequent infection of a second virus. Whereas, in natural infection through *P. betae* zoospores, the virus with the highest inoculum usually colonize the majority of the roots and it will predominate. Moreover in such experiments the initial inoculum density should be determined and soil temperature must be manipulated in order to obtain repeatable results. In fact, BSBMV usually predominate at temperature <20°C, whereas BNYVV at >25°C (Rush, 2003).

4. Aim of the study

Beet necrotic yellow vein virus and *Beet soil-borne mosaic virus* belong to the *Benyvirus* genus and are both vectored by *P. betae*. These viruses infect roots of *Chenopodiaceae* plants, share the same genomic organization but have sufficient molecular differences to be distinguished in two different species (Lee *et al*., 2001; Peltier *et al*., 2008). However, BSBMV RNA-3 and -4 can be replicated and encapsidated by BNYVV RNA-1 and -2, allowing long distance movement and transmission through the vector (Ratti *et al*., 2009; D'Alonzo *et al*., 2012). In the United States these viruses are frequently present in the same field affecting the same plant but no chimeric forms have been described in nature so far.

The aim of this PhD project is to further investigate molecular interactions between BNYVV and BSBMV and the mechanisms involved in the pathogenesis of these viruses.

One purpose of my thesis was to study the possible synergism or antagonism effect between BNYVV and BSBMV. To perform this study, plant host leaves have to be coinoculated with different ratio of both viruses. BNYVV full-length infectious cDNA clones are available (Quillet *et al*., 1989) as well as full-length cDNA clones of BSBMV RNA-1, -2 (D'Alonzo, 2011), -3 (Ratti *et al*., 2009) and -4 (D'Alonzo *et al*., 2012). Handling of these cDNA clones in order to produce *in vitro* infectious transcripts need sensitive and expensive steps. I decided to develop alternative tools to carry on my experiments so I produced agroclones of BNYVV and BSBMV RNAs, as well as viral replicons allowing the expression of different proteins that are described in Chapter 1.

Chapter 2 is dedicated to the study of the relationship between BNYVV and BSBMV. I checked the capability of BSBMV RNAs to replicate BNYVV RNA-5 and the behavior of artificial viral chimeras between BNYVV and BSBMV RNA-1 and -2 in test plants, such as *Chenopodium quinoa* and *Nicotiana benthamiana*. If BNYVV RNA-1 and -2 are known to support BSBMV RNA-3 and -4 replication to transmission, nothing is known about the properties of chimera exchanging one of the largest RNAs, that could possibly arise from the co-infection described in Chapter 1.

Chapter 3 presents the study about BSBMV p14 demonstrating that it is a suppressor of post-transcriptional gene silencing sharing common features with BNYVV p14 and its implications in viral long distance movement.

Finally, a general discussion and conclusion summarizes the results and present some prospects about Benyviruses research.

References

Acosta-Leal R., Fawley M.W., Rush C.M. (2008). Changes in the intraisolate genetic structure of beet necrotic yellow vein virus populations associated with plant resistance breakdown. Virology 376, 60-68.

Adams M. (1991). Transmission of plant viruses by fungi. Ann. Appl. Biol., 118, 479-92.

Andika I.B., Kondo H., Tamada T. (2005). Evidence that RNA silencing-mediated resistance to beet necrotic yellow vein virus is less effective in roots than in leaves. Molecular plant-microbe interactions, 18, 194-204.

Barr D., Asher M.J.C. (1992). The host range of Polymyxa betae in Britain. Plant Pathol.41, 64-88.

Biancardi E. (2005). Brief history of sugar beet cultivation. In BiancardiE., Campbell L.G., Skaracis G.N. & De Biaggi (eds.) "Genetics and breeding of sugar beet". Science Publishers, Inc., Enfield, NH, USA, 3-9.

Bouzoubaa S., Quillet L., Guilley H. (1987). Nucleotide sequence of beet necrotic yellow vein virus RNA-1. Journal of General Virology, 68, 615-626.

Bouzoubaa S., Niesbach-Klosgen U., Jupin I., Guilley H., Richrds K.E., Honard G. (1991). Shortened forms of beet necrotic yellow vein virus RNA-3 and -4: internal deletions and a subgenomic RNA. Journal of General Virology, 72, 259-266.

Braselton J. (1995). Current status of the plasmodiophorids. Curr. Rev. Microbiol., 21, 263-275.

Brunt A.A., Richards K.E. (1989). Biology and Molecular Biology of Furoviruses. Adv. Virus Research, 36, 1-32.

Canova A. (1959). Appunti di patologie della barbabietola. Informatore fitopatologico, 9, 390-396.

Canova A. (1966). Si studia la rizomania della bietola.Informatore fitopatologico, 16, 235-239.

Chiba S., Miyanishi M., Andika I.B., Kondo H., Tamada T. (2008). Identification of amino acids of the beet necrotic yellov vein virus p25 protein required for induction of the resistance response in leaves of Beta vulgaris plants. Journal of General Virology, 89, 1314-1323.

Chiba S., Hleibieh K., Delbianco A., Klein E., Ratti C., Ziegler-Graff V., Bouzoubaa S., Gilmer D. (2012). The Benyvirus RNA silencing suppressor is essential for long distance movement, requires both Zn-finger domain and NoLS basic residues but not a nucleolar localization for its silencing suppression activity. Molecular plant-microbe interactions, Vol.26, No.2, pp.168-181.

D'Alonzo M., Delbianco A., Lanzoni C., Autonell C.R., Gilmer D., Ratti C. (2012). Beet soil-borne mosaic virus RNA-4 encodes a 32 kDa protein involved in symptom expression and in virus transmission through Polymyxa betae. Virology; 423:187-94.

Draycott A.P. (2006). Introduction. In Sugar Beet, pp.1-8. Edited by A.P. Draycott. Oxford: Blackwell Publishing Ltd.

Dunoyer P., Pfeffer S., Fritsch C., Hemmer O., Voinnet O., Richards K.E. (2002). Identification, subcellular localization and some properties of a cysteine-rich suppressor of gene silencing encoded by peanut clump virus. Plant Journal, 29, 555-567.

FAO (2010). FaoSTAT Agricolture Statistics Database. Web: http://faostat.fao.org/site/567/default.aspx-ancor.

Francis S.A. (2006). Development of sugar beet. In Sugar beet, 9-29. Edited by A.P. Draycott. Oxford: Blackwell Publishing Ltd.

Gilmer D., Bouzoubaa S., Henh A., Guilley H., Richards K.E., Jonard G. (1992). Efficient cell-to-cell movement of bet necrotic yellow vein virus requires 3' proximal genes located on RNA-2. Virology, 189, 40-47.

Gilmer D., Ratti C. (2012). Benyvirus. Pages 1133-1138 in: Virus taxonomy: classification and nomenclature of viruses: Ninth Report of the International Committee on Taxonomy of Viruses.

Harju V.A., Mumford R.A., Blockley A., Boonham N., Clover G.R.G., Weekes R., Henry C.M. (2002). Occurrence in the United Kinfdom of beet necrotiv yellow vein virus isolates which contain RNA5. Plant pathology, 51, 811.

Hehn A., Bouzoubaa S., Bate N., Twell D., Marbach J., Richards K.E., Guilley H., Jonard G. (1995). The small cysteine-rich protein p14 of beet necrotic yellow vein virus regulates accumulation of RNA-2 in cis and coat protein in trans. Virology, 210, 73-81.

Hehn A., Fritsch C., Richards K.E., Guilley H., Jonard G. (1997). Evidence for in vitro and in vivo autocatalytic processing of the primary translation product of beet necrotic yellow vein virus RNA-1 by a papain-like proteinase. Archives of Virology, 142, 1051-1058.

Heidel G.B., Rush C.M. (1994). Distribution of beet necrotic yellow vein virus, beet distorsion mosaic virus and unnamed soilborne sugar beet virus in Texasand New Mexico. Plant disease, 78, 603-606.

Heidel G.B., Rush C.M., Kendall T.L., Lommel S.A., French R.C. (1997). Characteristics of beet soil borne mosaic virus, a furo-like virus infecting sugar beet. Plant disease, 81, 1070-1076.

Heijbroek W., Musters-Van Oorschot P.M.S., Schoone A.H.L. (1999). Variation in pathogenicity and multiplication of beet necrotic yellow vein virus (BNYVV) in relation to the resistance of sugar beet cultivars. European Journal of Plant Pathology, 105, 397-405.

Jupin I., Guilley H., Richards K.E., Jonard G. (1992). Two proteins encoded by beet necrotic yellow vein virus RNA-3 influence symptom phenotype on leaves. EMBO Journal, 11, 479-488.

Kiguchi T., Saito M., Tamada T. (1996). Nucleotide sequence analysis of RNA-5 of five isolates of beet necrotic yellow vein virus and the identity of a deletion mutant. Journal of General Virology, 77 (Pt 4), 575-580.

Keskin, B. (1964). Polymyxa betae n.sp., ein Parasit in den Wurzeln von Beta vulgaris Tournefort, besonders während der Jugendentwicklung der Zuckerrübe. Archives of Microbiology 49, 348-373.

Koenig R., Burgermeister W., Weich H., Sebald W., Kothe C. (1986). Uniform RNA patterns of beet necrotic yellow vein virus in sugar beet roots, but not in leaves from several species. Journal of General Virology, 67, 2043-2046.

Koenig R., Jarausch W., Li Y., Commandeur U., Burgermeister W., Gehrke M., Luddecke P.(1991). Effect of recombinant beet necrotic yellow vein virus with different RNA composition on mechanically inoculated sugar beets. Journal of General Virology, 72, 2243-2246.

Koenig R., Luddecke P., Haeberle A.M. (1995). Detection of beet necrotic yellow vein virus strains, variants and mixed infections by examining single strand conformation polymorphisms of immunocapture RT-PCR products. Journal of General Virology, 76, 2051-2055.

Koenig R., Lennefors B.L. (2000). Genus Benyvirus. In Van Regenmortel MHV, Fauquet CM & Bishop DHL (eds.) Virus Taxonomy. 7th Report of the International Committee on Taxonomy of Viruses. Academic Press, San Diego, CA, pp. 917-922.

Koenig R., Loss S., Specht J., Varrelmann M., Luddecke P., Demi G. (2009). A single U/C nucleotide substitution changing alanine to valine in the beet necrotic yellow vein virus p25 protein promotes increased virus accumulation in roots of mechanically inoculated, partially resistant sugar beet seedlings. Journal of General Virology, 90, 759-763.

Koonin E.V., Dolja V.V. (1993). Evolution and taxonomy of positive-strand RNA viruses: implications of comparative analysis of amino acid sequences. Crit. Rev. Biochem. Mol. Biol., 28, 375-430.

Kruse M., Koenig R., Hoffmann A., Kaufmann A., Commandeur U., Solovyev A.G., Savenkov I., Burgermeister W. (1994). Restriction fragment length polymorphism analysis or reverse transcription-PCR products reveals the existence of two major strain groups of beet necrotic yellow vein virus. Journal og General Virology, 75 (Pt. 8), 1835-1842.

Lauber E., Guilley H., Tamada T., Richards K.E., Jonard G. (1998). Vascular movement of beet necrotic yellow vein virus in Beta macrocarpa is probably dependent on an RNA3 sequence domain rather than a gene product. Journal of General Virology, 79, 385-393.

Lee L., Telford E.B., Batten J.S., Scholthof K.B., Rush C.M. (2001). Complete nucleotide sequence and genome organization of beet soil borne mosaic viru, a proposed member of the genus Benyvirus. Archives of Virology, 146, 2243-2453.

Lemaire O., Merdinoglu D., Valentin P., Putz C., Ziegler-Graff V., Guilley H., Jonard G., Richards K.E. (1988). Effect of beet necrotic yellow vein virus RNA composition on transmission by Polymyxa betae. Virology, 162, 232-235.

Lennefors B.L., Lindsten K., Koenig R. (2000). First record of A and B type beet necrotic yellow vein virus in sugar beets in Sweden. European Journal of Plant Pathology, 106, 199-201.

Lewellen R.T., Skoyen I.O., Erichsen A.W. (1987). Breeding sugarbeet for resistance to rhizomania: evaluation of host-plant reactions and selection for and inheritance of resistance. In Proceedings of the 50th congress of the IIRB. International Institute for Beet Research, Brussels, Belgium, pp.139-156.

Liu H., Duffus J.E. (1988). The occurrence of a complex of viruses associated with rhizomania of sugarbeet. Phytopathology, 1583.

Link D., Schmidlin L., Schirmer A., Klein E., Erhardt M., Geldreich A., Lemaire O., Gilmer D. (2005). Functional characterization of the beet necrotic yellow vein virus RNA-5 encoded p26 protein: evidence for structural pathogenicity determinants. Journal of General Virology, 86, 2115-2125.

Lubicz J.V., Rush C.M., Payton M., Colberg T. (2007). Beet necrotic yellow vein virus accumulates inside resting spores and zoosporangia of its vector Polymyxa betae. Virol. Journal, 4, 37.

Mahmood T., Rush C.M. (1999). Evidence of cross protection between beet soil borne mosaic virus and beeet necrotic yellow vein virus in sugar beet. Plant disease, 83, 521- 526.

McGrann G.R.D., Grimmer M.K., Mutasa-Gottgens E.S., Stevens M. (2009). Progress towards the understanding and control of sugar beet rhizomania disease. Molecular plant pathology 10, 129-141.

Meunier A., Schmit J.F., Stas A., Kutluk N., Bragard C. (2003). Multiplex reverse transcription-PCR for simultaneous detection of Beet necrotic yellow vein virus, Beet soil borne virus, and Beet virus Q and their vector Polymyxa betae Keskin on sugar beet. Applied and Enviromental Microbiology, 69, 2356-2360.

Milford G.F.J. (2006). Plant structure and crop physiology. In Sugar beet, 30-49. Edited by A.P. Draycott. Oxford: Blackwell Publishing Ltd.

Miyanishi M., Kusume T., Saito M., Tamada T. (1999). Evidence for three groups of sequence variants of beet necrotic yellow vein virus RNA5. Archives of Virology, 144, 879-892.

Niesbach-Klosgen U., Guilley H., Jonard G., Richards K.E. (1990). Immunodetection in vivo of beet necrotic yellow vein virus-encoded proteins. Virology, 178, 52-61.

Pelsy F., Merdinoglu D. (1996). Identification and mapping of random amplified polymorphic DNA markers linked to a rhizomania resistance gene in sugar beet (Beta vulgaris L.) by bulked segregant analysis. Plant breeeding, 115, 371-377.

Peltier C., Hleibieh K., Thiel H., Klein E., Bragard C., Gilmer D. (2008). Molecular Biology of the Beet necrotic yellow vein virus. Plant Viruses 2, 14-24.

Peltier C., Schmidlin L., Klein E., Taconnat L., Prinsen E., Erhardt M., Heintz D., Weyens G., Lefebvre M., Renou J.P., Gilmer D. (2010). Expression of beet necrotic yellow vein virus p25 protein induces hormonal changes and a root branching phenotype in Arabidopsis thaliana. Trangenic Res., 20, 443-466.

Peltier C., Klein E., Hleibieh K, D'Alonzo M., Hammann P., Bouzoubaa S., Ratti C., Gilmer D. (2012). Beet necrotic yellow vein virus subgenomic RNA3 is a cleavage product leading to stable non-coding RNA required for long-distance movement. J. Gen. Virol., 93 (Pt 5), 1093-102.

Putz C., Merdinoglu D., Lemaire O., Stocky G., Valentin P. (1990). Beet necrotic yellow vein virus, causal agent of sugar beet rhizomania. Plant Pathology, 69, 247-254.

Quillet L., Guilley H., Jonard G., Richards K.E. (1989). In vitro sysnthesis of biologically active beet necrotic yellow vein virus RNA. Virology, 172, 239-301.

Rahim M.D., Andika I.B., Han C., Kondo H., Tamada T. (2007). RNA4-encoded p31 of beet necrotic yellow vein virus is involved in efficient vector transmission, symptom severity and silencing suppression in roots. Gen Virol ; 88: 1611-1619.

Ratti C., Clover G.R.G., Rubies Autonell C., Harju V.A., Henry C.M. (2005). A multiplex RT-PCR assay capable of distinguishing beet necrotic yellow vein virus types A and B. Journal of Virological Methods, 124, 41-47.

Ratti C., Hleibieh K., Bianchi L., Schirmer A., Rubies Autonell C., Gilmer D. (2009). Beet soil borne mosaic virus RNA-3 is replicated and encapsidated in the presence of BNYVV RNA-1 and -2 and allows long distance movement in Beta macrocarpa. Virology, 385, 392-399.

Rush C.M., French R.C., Heidel G.B. (1994). Differentiation of two closely related furoviruses using the polymerase chain reaction. Phytopathology, 84, 1366-1369.

Rush C.M. (2003). Ecology and epidemiology of benyviruses and plasmodiophorid vectors. Annual Review Phythopathology, 41, 567-592.

Rush C.M., Liu H.Y., Lewellen R.T., Acosta-Leal R. (2006). The continuing saga of rhizomania of sugar beets in the United States. Plant Disease, 90, 4-15.

Saito M., Kiguchi T., Kusume T., Tamada T. (1996). Complete nucleotide sequence of the Japanese isolate S of Beet necrotic yellow vein virus RNA and comparison with European isolates. Arch. Virol., 141, 2163-2175.

Schirmer A., Link D., Cognat V., Moury B., Beuve M., Meunier A., Bragard C., Gilmer D., Lemaire O. (2005). Phylogenetic analysis of isolates of beet necrotic yellow vein virus collected worldwide. Journal of General Virology, 86, 2897-2911.

Schmidlin L., Link D., Mutterer J., Guilley H., Gilmer D. (2005).Use of a Beet necrotic yellow vein virus RNA-5-derived replicon as a new tool for gene expression. Journal of General Virology, 86, 463-467.

Schmitt C., Balmori E., Jonard G., Richards K.E., Guilley H. (1992). In vitro mutagenesis of biologically active transcripts of beet necrotic yellow vein virus RNA-2: evidence that a domain of 75 kDa read-through protein is important for efficient virus assembly. Proceedings of the National Academy of Sciences USA, 89, 5715-5719.

Stevens M., Liu H.Y., Lemaire O. (2006). Virus diseases. In Sugar Beet, pp. 256-285. Edited by A.P. Draycott. Oxford: Blackwelll Publishing Ltd.

Tamada T., Baba T. (1973). Beet necrotic yellow vein virus from rhizomania-affected sugar beet in Japan. Annals of Phytopathologicial Society of Japan, 325, 332.

Tamada T. (1975). Beet necrotic yellow vein virus. CMI/AAB Descriptions of plant viruses 144, pp.4.

Tamada T., Abe H. (1989). Evidence that beet necrotic yellow vein virus RNA-4 is essential for efficient transmission by the fungus Plymyxa betae. Journal of General Virology, 70, 3391-3398.

Tamada T., Shirako Y., Abe H., Saito M., Kiguchi T., Harada T. (1989). Production and pathogenicity of isolates of beet necrotic yellow vein virus with different numbers of RNA components. Journal of Generale Virology, 70, 3399-3409.

Tamada T., Saito M., Kiguchi T., Kususme T. (1990). Effect of isolates of beet necrotic yellow vein virus with different RNA components on the development of rhizomania symptoms. In: Koenig R. (eds.). Proc. Of the first symposium of the international working group on plant viruses with fungal vectors. Eugene Ulmer, Stuttgrart, 41-44.

Tamada T., Kususme T. (1991). Evidence that the 75K readthrough protein of beet necrotic yellow vein virus RNA-2 is essential for transmission by the fungus Polymyxa betae. Journal of General Virology, 72, 1497-1504.

Tamada T., Kusume T., Uchino H., Kiguchi T., Saito M. (1996). Evidence that beet necrotic yellow vein virus RNA-5 is involved in symptom development of sugar beet roots. In Sherwood J.L. &Rush C.M. (eds.). Proceedings of the 3rd symposium of the international working group on plant viruses with fungal vectors, pp. 49-52.

Tamada T. (1999). Beyviruses. In Webster RG & Granoff A (eds.) Encyclopedia of Virology (2nd edn). Academic Press, London, UK, 154-160.

Tamada T. (2002). Beet necrotic yellow vein virus. CMI/AAB Description of plant viruses, No. 391. Wemmesbourne: Association of Applied Biologists.

Verchot-Lubicz J., Torrance L., Solovyev A.G., Morozov S.Y., Jackson A.O., and Gilmer D. (2010). Varied movement strategies employed by triple gene block-encoding viruses. Mol. Plant-Microbe Interact., 23, 1231-1247.

Whitney E.D., Duffus J.E. (1986). Biotic diseases and disorders – disease development. In Compendium of beet diseases and insects. Edited by E.D. Whitney and j.E. Duffus, St. Paul, The American Phytopathological Society.

Wisler G.C., Lewellen R.T., Sears J.L., Wasson J.W., Liu H.Y., Wintermantel W.M. (2003). Interactions between beet necrotic yellow vein virus and beet soilborne mosaic virus in sugar beet. Plant disease, 87, 1170-1175.

Ziegler-Graff V., Richards K., Guilley H., Jonard G., Putz C. (1985). Cell-free translation of beet necrotic yellow vein virus: read-through of the coat protein cistron. Journal of General Virology, 66, 2079-2087.

Chapter 1

Construction of BNYVV and BSBMV agroinfectious clones

1. Introduction

1.1 The reverse genetic approach

Genetic studies began in the 1860s with Gregor Mendel, a scientist and Augustinian monk, who demonstrated that inheritance of visible traits in pea plants follows precise schemes, now referred to as the laws of Mendelian inheritance. Mendel suggested the existence of genes and although his work was first rejected and rediscovered only at the beginning of the 20th century, he is now considered the founder of modern genetics (Griffiths *et al*., 2000).

The following discovery of DNA structure by Watson J. and Crick F. in the 1950s (Watson and Crick, 1953), the development of sequencing in the 1970s (Sanger *et al*., 1977) and polymerase chain reaction technology in the 1980s (Mullis and Faloona, 1987), greatly accelerated the accumulation of information and knowledge about genetics.

Two different approaches can be used to investigate a gene function, generally referred to as “forward” and “reverse” genetic. Forward genetic studies start from observable natural or induced phenotypic variation and aims to determine the sequence involved.

Reverse genetics works in the opposite direction: the gene sequence is known, but its exact function is uncertain or unknown. Therefore the nucleotide sequence is modified and the subsequent phenotype is observed and measured. The phenotypic analysis of variants allows associating the sequence variation to the morpho-physiological variation (Tierney and Lamour, 2005).

The reverse genetic approach is widely used in virology as it makes possible direct identification of viral gene function. Molecular mechanisms involved in the pathogenesis of RNA plant viruses are mainly investigated by the use of two techniques: *in vitro* transcription and agroinfection.

1.2 *In vitro* transcription

Studies about RNA viruses have exclusively relied upon cDNA intermediates from which biologically active RNA molecules were generated. Production of cDNA clones is therefore an essential step to develop a reverse genetic system which allows genetic manipulation and dissection of gene function. *In vitro* transcription of viral RNAs from full-length cDNA clones have been first reported for poliovirus using a bacterial phage promoter (Racaniello and Baltimore, 1981).

Full-length cDNA of viral genomic RNA is currently obtained through reverse transcription and polymerase chain reaction (RT-PCR). Amplicons are then cloned in a bacterial plasmid, flanked at the 5' end with a transcription promoter (T7, T3 or Sp6) that is recognized by the appropriate bacteriophage RNA polymerase. These clones are then linearized at the 3' end and used to produce *in vitro* run-off transcripts which can be used to infect plants or plant cell protoplasts (Fig. 1.1).

Within Benyviruses' studies this approach has been used to produce full-length cDNA clones of BNYVV RNA-1 to -5 (Quillet *et al*., 1989; Ziegler-Graff *et al*., 1988; Link *et al*., 2005) and viral replicons derived vectors able to express different proteins (Erhardt *et al*., 2000; Schmidlin *et al*., 2005). *In vitro* transcripts have been employed in different test plants such as *C. quinoa*, *N. benthamiana*, *B. macrocarpa* and *T. expansa* to investigate the role of viral RNAs and to characterize proteins involved in virus pathogenecity (Jupin *et al*., 1992), cell-to-cell movement (Gilmer *et al*., 1992), systemic movement (Lauber *et al*., 1998) and transmission through the vector (Rahim *et al*., 2007).

More recently, *in vitro* transcripts from BSBMV RNAs cDNA clones have been used to investigate relationship with BNYVV and to characterize proteins and sequences involved in long distance movement and natural transmission of the virus (Ratti *et al*., 2009; D'Alonzo, 2011; D'Alonzo *et al*., 2012).

In vitro transcription is a useful technique. However, it can be expensive and time consuming to perform. The use of cDNA clones for *in vivo* experiments requires to perform multiple and sensitive steps. Once the construction has been obtained, it has to be multiplied in *E. coli* cell cultures and then extracted with appropriate procedure. A large amount of plasmid has to be linearized by digestion with the appropriate

restriction enzyme followed by a clean-up procedure, such as phenol-extraction and ethanol precipitation. The linearized DNA is then used for *in vitro* transcription that sometimes, especially for long sequences, results in transcripts that appear not infectious. For all these reasons this system presents limitations when applied to large scale experiments and therefore researchers are often looking for an alternative technique.

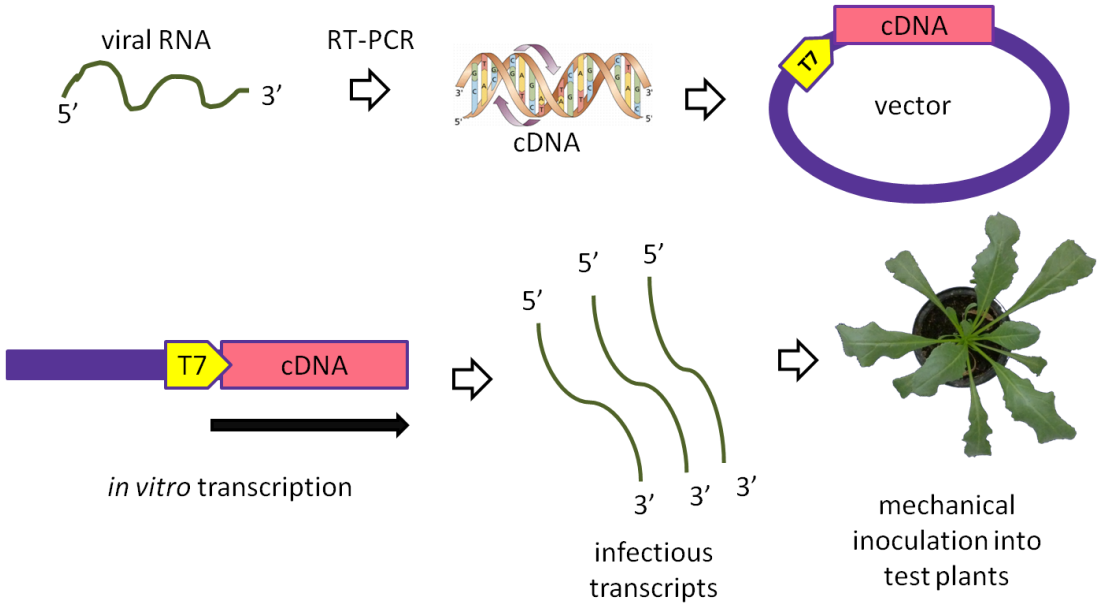

Fig. 1.1: Schematic representation of *in vitro* transcription. Viral RNA has to be retro-transcribed in cDNA and then inserted in a vector under the control of transcription promoter, such as T7. This clone has to be linearized and *in vitro* transcribed in order to obtain infectious transcripts that are subsequently inoculated into test plants.

1.3 Agroinfection

Agrobacterium tumefaciens is a phytopathogenic, soil-living, Gram-negative and rod-shaped bacteria responsible of the disease named crown gall. Its ability to induce this neoplastic disease is associated to the presence of a large plasmid of about 200 kb known as Ti-plasmid (tumor-inducing) (Escobar and Dandekar, 2003). The factors required for tumor formation reside in a region of the Ti-plasmid named T-DNA (transferred DNA) that is imported into plant cells and integrated into the host chromosomal DNA, resulting in a genetic manipulation of the host. The T-DNA region is defined and delimited by highly homologous, directly repeated 25-28 bp border sequences (Wang *et al*., 1984; Zupan *et al*., 2000). In addition the Ti-plasmid possesses the *vir* region that contains at least eight operons (*virA*, *virB*, *virC*, *virD*, *virE*, *virF*, *virG*

and *virH*) encoding for virulence proteins that control T-DNA transfer and integration (Păcurar *et al*., 2011).

The expression of T-DNA-encoded bacterial genes, in the host cell, results in the production of enzymes that catalyze the synthesis of plant hormones responsible of tumor growth and formation of a novel aminoacid-sugar termed as opines (Pitzschke and Hirt, 2010). The outcome is the alteration of the plant secondary metabolism resulting in abnormal cell proliferation and synthesis of nutritive compounds used by *A. tumefaciens* as carbon and nitrogen source (Păcurar *et al*., 2011). The tumor cells generated by *A. tumefaciens* do not require the continuous presence of the bacteria for proliferation (White and Braun, 1942), indicating that the plant cells have been transformed genetically.

The capability of *A. tumefaciens* to transfer and integrate its T-DNA in the host genome has been exploited to develop a technique for plant or plant tissue transformation. Wild type T-DNA has been modified in order to obtain a "disarmed" Ti plasmid in which an extraneous DNA fragment can be inserted and then transferred from *A. tumefaciens* cells to the plant genome (Hoekema *et al*., 1983). The large dimension (about 150 kb) of the final plasmid represented a serious limitation to the use of this system. To overcome this problem the binary vector system was developed, based on the observation that the transfer process of T-DNA is active also when virulence genes and T-DNA are located on separate plasmids in the same *A. tumefaciens* cell (Hoekema *et al*., 1983). The system is therefore composed by two plasmids: the binary vector and the helper Ti-plasmid. The binary vector carries the DNA fragment of interest between the right and left border sequence that will produce the T-DNA, while the helper Ti-plasmid allows the expression of the virulence genes required for the T-DNA transfer (Păcurar *et al*., 2011). Both plasmids usually carry distinct selectable markers, such as resistance to kanamycin and rifampicin antibiotics. Once the T-DNA is transferred in a plant cell, a double strand intermediate is formed and is integrated randomly in the host genome (Gietl *et al*., 1987). Pluripotency of plant cells allows the regeneration of modified plant expressing the sequence of interest under the control of a constitutive, inducible or tissue specific promoter (Benfey *et al*., 1989).

Agroinoculation (Grimsley *et al*., 1986) consist in plant tissue infiltration with a suspension of *A. tumefaciens* cells containing a binary plasmid carrying a plant-

functional promoter and cDNA of a viral RNA. These elements are transferred to the plant cell genome from which, after transcription, biologically active viral RNAs can be generated and initiate infection (Fig. 1.2).

As for *in vitro* transcription, limitation exists, particularly when cytoplasmic replicating viral RNAs are forced to have a nuclear step where splicing could affect the integrity of genomic RNAs thanks to cryptic introns.

Nevertheless, agroinfection appears to be a useful alternative to *in vitro* transcription since it is less expensive and more reproducible strategy to infect plants. We therefore decided to develop BNYVV and BSBMV RNAs agroclones. BNYVV cDNA clones of the four genomic RNAs and replicons have been placed under the control of 35S promoter. The biological properties of these constructions are described in the article published in *Molecular Plant Pathology*.

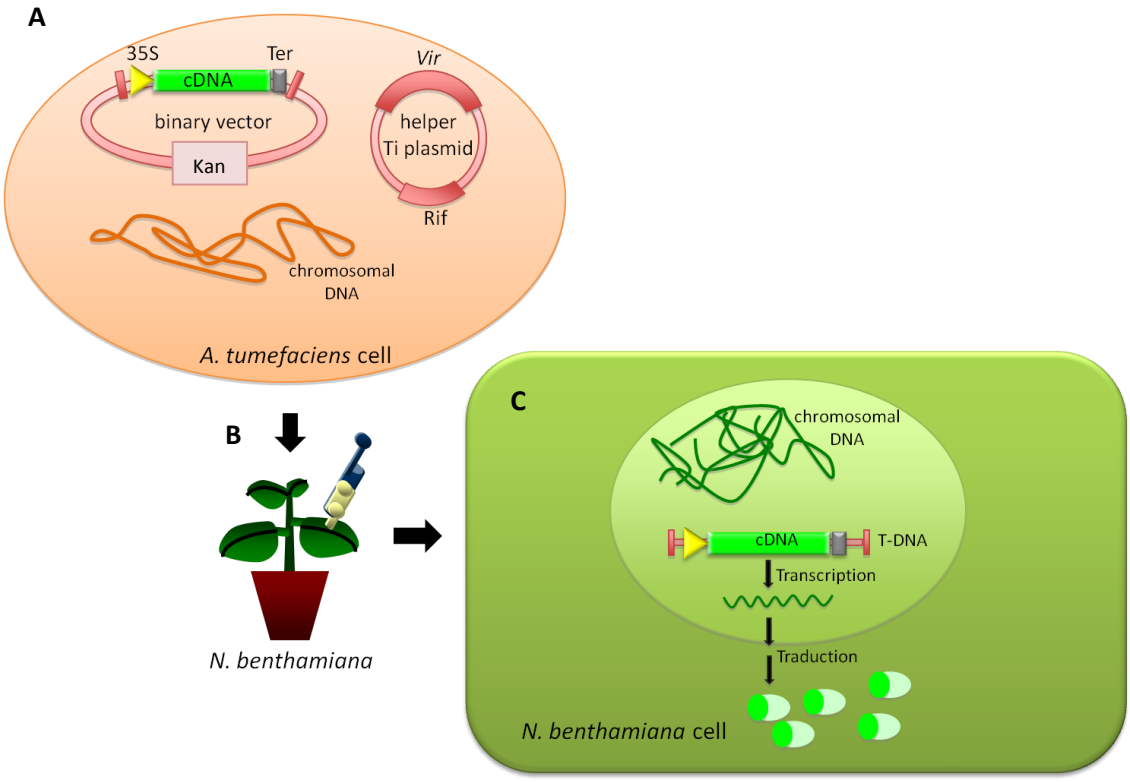

Fig. 1.2: Schematic representation of agroinfection. (A) *A. tumefaciens* cells carry a binary system composed by two plasmids: the helper Ti plasmid and the binary vector. The helper Ti plasmid carries the *Vir* genes and the rifampicin selectable marker. The binary vector carries the cDNA of interest, as a T-DNA, between the right and left border sequence and under the control of the 35S promoter. (B) A suspension of transformed A. *tumefaciens* cells is infiltrated into *N. benthamiana* leaves. (C) The 35S promoter and the cDNA are transferred to the plant cell genome from which, after transcription, biologically active viral RNAs can be generated and initiate infection.

2. Construction of BSBMV agroclones

2.1 Construction of agroclones

AgroBS-1

Full-length cDNA of BSBMV RNA-1 has been previously cloned in the vector pUC19 under the T7 promoter (D'Alonzo, 2011), giving rise to the clone EUB11. The cDNA of BSBMV RNA-1 has been amplified from this available clone using Pfu Ultra II Fusion Hotstart Polymerase (Agilent Technologies, Santa Clara, CA) and BSBMV1AgroF and BSBMV1AgroR primers. The amplicon has been directly ligated in the pJL89 binary vector, previously digested with *Stu*I and *Sma*I restriction enzymes, placing the viral sequence under the control of the *Cauliflower mosaic virus* 35S promoter (Lindbo *et al*., 2007; Crivelli *et al.*, 2011). No recombinant colonies have been obtained when the ligated product has been introduced into *A. tumefaciens* cells (strain C58C1) by electroporation.

A strategy to reduce the amplicon size inserted in pJL89 plasmid has been therefore followed. A fragment of 3300 bp, including 5' and 3' UTRs of BSBMV RNA-1 and the complete sequence of the vector pUC19, has been amplified with primers BSBMV1R13*Nco*I and BSBMV1F12*Stu*I (Fig. 1.3A). After self-ligation a new clone has been obtained in *E. coli* cells (strain MC1022)(Fig. 1.3B) and successively employed to amplify a fragment of 590 bp corresponding to BSBMV RNA-1 5' and 3' UTRs, using BSBMV1AgroF and BSBMV1AgroR primers (Fig. 1.3B and C). This amplicon has been ligated in the pJL89 vector and successfully transformed into *A. tumefaciens* cells (strain C58C1) (Fig. 1.3D). This plasmid has been digested with *Nco*I and *Stu*I enzymes to introduce the missing BSBMV RNA-1 *Nco*I/*Stu*I fragment from EUB11 (Fig. 1.3E). After ligation and transformation, the resulting plasmid AgroBS-1 corresponds to the full-length cDNA clone of BSBMV RNA-1 inserted in the pJL89 binary vector (Fig. 1.3F).

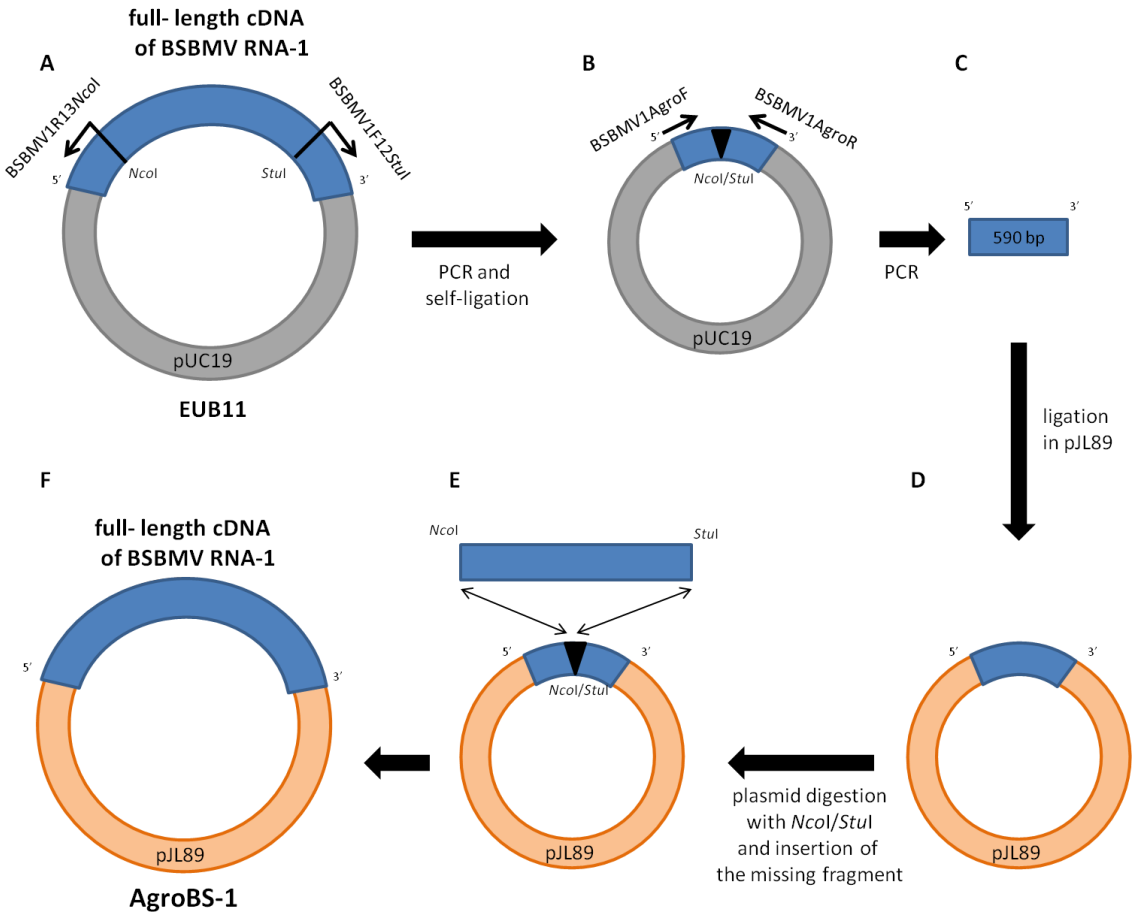

Fig. 1.3: Schematic representation of the strategy used to construct the clone AgroBS-1. (A) Amplification of 5' and 3' UTRs of the cDNA of BSBMV RNA-1 and pUC vector. (B) Self-ligation of the amplicon. (C) Amplification of 5' and 3' UTRs of the cDNA of BSBMV RNA-1 (590 bp). (D) Ligation of the amplicon in the pJL89 binary vector. (E) Insertion of the missing fragment within the 5' and 3' UTRs. (F) The resulting clone composed by the full-length cDNA of BSBMV RNA-1 inserted in the pJL89 binary vector.

AgroBS-2

Full-length cDNA of BSBMV RNA-2 has been amplified using Pfu Ultra II Fusion Hotstart Polymerase (Agilent Technologies, Santa Clara, CA) and primers BSBMV2AgroF and BSBMVAgroR from the available clone EUB22 (D'Alonzo, 2011), carrying the cDNA sequence of BSBMV RNA-2 in the vector pUC19. Due to its unsuccessful insertion in the binary vector pJL89, a fragment of 3000bp, corresponding to the vector pUC19 and 5' and 3' UTRs of BSBMV RNA-2, has been amplified using the primers BSBMVR2*Mlu*I and BSBMVF2*Stu*I to adopt a similar strategy as to RNA-1. This fragment has been self-ligated and transformed in *E. coli* cells. Primers BSBMV2AgroF and BSBMV2AgroR have been used to amplify a fragment of 412 bp corresponding to the 5 and 3' UTRs which has been successful ligated in the pJL89 vector and transformed in *A. tumefaciens* cells. The new clone has been digested using *Mlu*I and *Stu*I enzymes and then ligated with

the fragment of BSBMV RNA-2 *Mlu*I/*Stu*I obtained from the clone EUB22. The plasmid containing the full-length sequence of BSBMV RNA-2 has been named AgroBS-2 and multiplied in *A. tumefaciens* cells.

AgroBS-3

The clone pUC31 (Ratti *et al*., 2009) has been used as a source to amplify the complete cDNA sequence of BSBMV RNA-3 (1720 bp) using the primer pair BSBMV3AgroF / BSBMV3AgroR and the Pfu Ultra II Fusion Hotstart Polymerase (Agilent Technologies, Santa Clara, CA). Direct ligation in the pJL89 binary vector, previously digested *Stu*I/*Sma*I, successful originated the AgroBS-3 clone maintained *A. tumefaciens* cells (strain C58C1).

AgroBS-4

Full-length cDNA of BSBMV RNA-4, present in the clone pUC47 (D'Alonzo *et al*., 2012), has been amplified using primers BSBMV4AgroF and BSBMV4AgroR and FideliTaq PCR MasterMix (Fermentas) or Pfu Ultra II Fusion Hotstart Polymerase (Agilent Technologies, Santa Clara, CA). All tentatives to insert it directly or in successive steps in the binary vector pJL89 were unsuccessful.

The agroclone of BSBMV RNA-4 is therefore not available.

2.2 Agroinfection of *N. benthamiana* and *B. macrocarpa* with BSBMV agroclones

Agroclones of BSBMV RNA-1, -2 and -3 have been tested through agroinoculation of *N. benthamiana* and *B. macrocarpa* plants. *A. tumefaciens* cells carrying BSBMV agroclones were grown overnight at 28°C in 5 ml LB supplemented with 100 µg/ml kanamycin and 50 µg/ml rifampicin. The bacteria were collected by centrifugation and resuspended in MA buffer (10mM MgCl2, 200 µM Acetosyringone) adjusting OD600 to 0.6 for *N. benthamiana* and 0.3 for *B. macrocarpa*. Each agroclone cell line was mixed in equal amount with others and left at room temperature for 3-4 hours before leaf agroinfiltration of 3-weeks old plants.

Typical chlorotic symptoms appeared 7 days after agroinfection in the infiltrated leaves of both *N*. *benthamiana* and *B. macrocarpa*. However, no systemic symptoms have

been observed 15 days after agroinfection and even later, suggesting that the virus was not able to move from the initial point of infection.

Northern and western blot analysis evidenced the presence of BSBMV RNA-1, -2 and -3 and CP protein in the agroinoculated leaves but not in the upper leaves of the plant (Fig. 1.4A and B), confirming the observation that the RNAs didn't move systemically into the plant in contrast with the ability of wild type BSBMV to move at long distance in both *N*. *benthamiana* and *B. macrocarpa* hosts. In order to better investigate this unexpected result the presence of viral particles on symptomatic tissues was investigated through a Transmission Electron Microscope (TEM). No virions were observed on agroinoculated leaves of *N*. *benthamiana* or *B. macrocarpa*, as well on *C. quinoa* leaves inoculated with *in vitro* transcripts of EUB11 and EUB22 clones, demonstrating that both AgroBS-2 and EUB22 clones are not fully functional in one or more of their components necessary for the encapsidation of the viral RNAs.

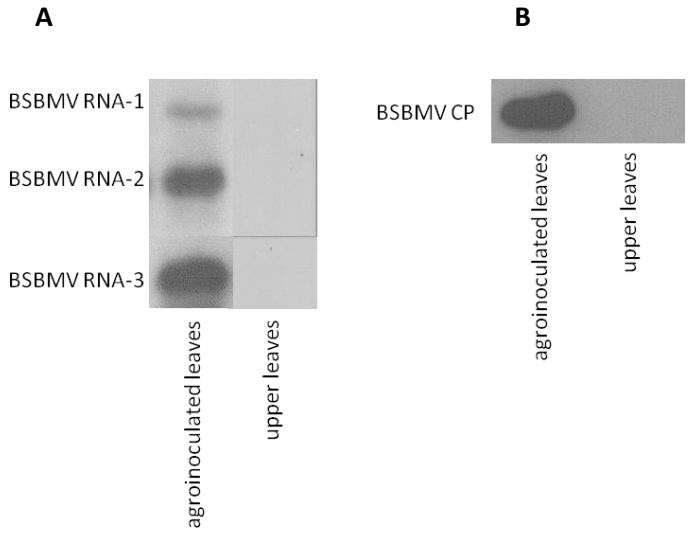

Fig. 1.4: (A) northern and (B) western blot analysis of agroinoculated and upper leaves of *N. benthamiana* plant agroinfected with AgroBS-1, -2 and -3. BSBMV RNAs and CP are detected only in the agroinoculated leaves, confirming the observation that the virus doesn't move sistemically in the infected plant.

2.3 Discussion

So far, molecular studies of *Beet soil borne mosaic virus* have been carried out using *in vitro* transcription of full-length cDNA clones of viral RNAs (Ratti *et al*., 2009; D'Alonzo *et al*., 2012). Agroinfection (Grimsley *et al*., 1986) is highly reproducible and represents

an economic technique; therefore it is an effective alternative to infect plants with the viral RNA of interest and to perform reverse genetics studies about phytoviruses.

In order to extend the biotechnological tools available to perform experiments regarding *Beet soil-borne mosaic virus* biology, molecular interaction with *Beet necrotic yellow vein virus* and their natural hosts, we developed BNYVV and BSBMV agroinfectious clones. Production of these clones has been tricky and time consuming, such as the previous cloning of BSBMV genomes in T7 vectors used for *in vitro* transcription (Ratti *et al*., 2009; D'Alonzo, 2011; D'Alonzo *et al*., 2012). To date, just the production of BSBMV RNA-4 agroclone failed and requires further attempts. Complete sequences of cDNA of BSBMV RNA-3 have been directly ligated in the pJL89 binary vector and transformed in *A. tumefaciens* cells, obtaining the clone named AgroBS-3. Construction of BSBMV RNA-1 and -2 sequences under the 35S promoter has been complex due to the instability of the viral cDNA inserted in a bacterial plasmid vector, a limitation observed for a number of plus-sense RNA viruses (Miyanishi *et al*., 2002). Cloning problems could be due to the presence of strong secondary structures of the sequence or to the production of viral proteins from cryptic promoters elements that results in a cytotoxic effects for bacteria cells (Miyanishi *et al*., 2002). Many strategies have been adopted to overcome instability problems of bacterial vectors containing viral cDNAs as reported by Rice *et al*. (1989) who successfully generated infectious yellow fever virus RNA from a pair of cDNA clones ligated *in vitro* before RNA transcription.

The largest single stranded and positive sense RNA genome present in nature, about 30 Kb, of the transmissible gastroenteritis virus (TGEV, genus *Alphacoronavirus*) has been cloned through systematic assembly of cDNA subclones (Yount *et al*., 2000). TGEV genome has been cloned also exploiting bacterial artificial chromosome (BAC), a low copy number plasmid (Almazàn *et al*., 2000).

Insertion of introns into cloned cDNA of *Pea seed-borne mosaic virus* (genus *Potyvirus*) facilitated plasmid amplification in *E. coli*. Multiple stop codons in the inserted introns interrupt the open reading frame of the cDNA, thereby terminating undesired translation of viral protein in *E. coli*, whereas intron splicing in eukaryotic cells reestablish the viral genome sequence (Johansen, 1996). Such approach could be adapted for the production of the RNA-4 BSBMV clone.

Functionality of BSBMV agroclones has been tested on *N. benthamiana* and *B. macrocarpa* plants. Agroinoculated leaves showed typical symptoms and northern and western blot analysis confirmed the presence of viral RNAs and CP protein, respectively. However, the virus didn't seem to move systemically into the host since no symptoms appeared in the upper leaves of the systemic hosts tested. Northern and western blot analysis didn't allow the detection of the viral components in the not inoculated leaves. Surprisingly viral particles could not be observed by TEM in agroinoculated leaves suggesting that BSBMV RNAs are able to move cell-to-cell without encapsidation as does BNYVV (Quillet *et al.*, 1989).

Initially, full-length cDNA of BSBMV RNA-2 was amplified from the available clone EUB22 (D'Alonzo, 2011) and then inserted in the vector pJL89 giving rise to the agroclone AgroBS-2. Obtaining the clone EUB22 has been tricky and complicated, due to multiple steps required. The cDNA of BSBMV RNA-2 have been obtained subcloning three different amplicons in the vector pUC19. This clone was not infectious when its *in vitro* transcript was inoculated onto *C. quinoa* leaves together with the transcript of EUB11 (cDNA of BSBMV RNA-1). Sequence analysis showed few nucleotide substitutions in the TGB proteins that have been corrected through PCR site-directed mutagenesis. The subsequent mechanical inoculation of *C. quinoa* leaves with *in vitro* transcripts of EUB11 and EUB22 induced chlorotic lesions proving the clone's infectivity. However, the presence of viral particles had not been verified before the subcloning in pJL89 (D'Alonzo, 2011). Thus, both the infectious clone EUB22 and the agroclone AgroBS-2 seem to carry one or more mutations that prevent the viral encapsisation.

Sequence analysis, compared with published sequence (Lee *et al*., 2001), showed two point mutations in the Read-Through domain: a guanine mutated in adenine ($G^{1567}A$) and a thymine mutated in adenine ($T^{2000}A$). These mutations result in two aminoacid substitutions, a methionine replaced by a valine and a glutamine replaced by a leucine. CP-RT domain of BNYVV is essential to initiate particle formation (Schmitt *et al*., 1992; Tamada *et al*., 1996).

In order to obtain an infectious clone of BSBMV RNA-2, and also a functional agroclone, PCR site-directed mutagenesis will be performed to restore the correct sequences. Meanwhile, even the possible implication of p14 will be investigated

through mechanical inoculation of EUB11 and EUB22 added to a viral replicon expressing BNYVV p14 since recent experiments evidenced the role of the benyvirus silencing suppressor in the long distance movement of BNYVV (see Chapter 3 and Chiba *et al*., 2012).

As stated before, the strategies adopted to create BSBMV RNAs agroinfectious clones were also applied for BNYVV RNAs and replicons cDNA sequences. The obtained results are presented in the following article accepted for publication in *Molecular Plant Pathology*.

3. Agroinoculation of *Beet necrotic yellow vein virus* cDNA clones results in plant systemic infection and efficient *Polymyxa betae* transmission

Molecular Plant Pathology

MOLECULAR PLANT PATHOLOGY (2013) **14**(4), 422–428 DOI: 10.1111/mpp.12018

Technical advance

Agroinoculation of *Beet necrotic yellow vein virus* cDNA clones results in plant systemic infection and efficient *Polymyxa betae* transmission

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SUMMARY

Agroinoculation is a quick and easy method for the infection of plants with viruses. This method involves the infiltration of tissue with a suspension of *Agrobacterium tumefaciens* carrying binary plasmids harbouring full-length cDNA copies of viral genome components. When transferred into host cells, transcription of the cDNA produces RNA copies of the viral genome that initiate infection. We produced full-length cDNA corresponding to *Beet necrotic yellow vein virus* (BNYVV) RNAs and derived replicon vectors expressing viral and fluorescent proteins in pJL89 binary plasmid under the control of the *Cauliflower mosaic virus* 35S promoter. We infected *Nicotiana benthamiana* and *Beta macrocarpa* plants with BNYVV by leaf agroinfiltration of combinations of agrobacteria carrying full-length cDNA clones of BNYVV RNAs. We validated the ability of agroclones to reproduce a complete viral cycle, from replication to cell-to-cell and systemic movement and, finally, plant-to-plant transmission by its plasmodiophorid vector. We also showed successful root agroinfection of *B. vulgaris*, a new tool for the assay of resistance to rhizomania, the sugar beet disease caused by BNYVV.

Beet necrotic yellow vein virus (BNYVV) (Tamada and Baba, 1973), the leading infectious agent that affects sugar beet, is a member of the *Benyvirus* genus, together with *Beet soil-borne mosaic virus* (BSBMV), and both are transmitted through the soil from the plasmodiophorid *Polymyxa betae* (Gilmer and Ratti, 2012). BNYVV is the causal agent of rhizomania (Canova, 1959), a disease widespread in all the sugar beet-growing areas of Europe, Asia and America, which causes abnormal rootlet proliferation and losses of sugar yields.

The BNYVV genome consists of four plus-sense 5′ capped and 3′ polyadenylated RNAs (Tamada, 1999). The RNA-1 and RNA-2 carry 'house-keeping' genes involved in virus replication, assembly, cell-to-cell movement and suppression of post-transcriptional gene silencing (PTGS) (Chiba *et al*., 2013; Dunoyer *et al*., 2002; Tamada, 1999). The RNA-3 and RNA-4 play important roles in pathogenesis and vector transmission, respectively, for the efficient production of typical rhizomania symptoms, long-distance movement and vector propagation (Koenig *et al*., 1991; Lemaire *et al*., 1988; Peltier *et al*., 2012). Sequence analysis of a number of isolates revealed the existence of two types of BNYVV: A type (found in most European countries, Iran, North America, China and Japan) and B type (present in France, Belgium, Germany, Sweden, the Netherlands, Iran, China, UK and Lithuania) (Lennefors *et al*., 2000; Miyanishi *et al*., 1999; Ratti *et al*., 2005; Sohi and Maleki, 2004). Additional isolates, characterized by the presence of RNA-5 and typically more aggressive, were identified in France, Japan, China, Kazakhstan and the UK (Harju *et al*., 2002; Koenig and Lennefors, 2000; Kruse *et al*., 1994; Li *et al*., 1998; Tamada *et al*., 1989; Ward *et al*., 2007).

The control of rhizomania disease relies on resistant sugar beet cultivars. Two proximal, but distinct, loci have been mapped on chromosome III of *Beta vulgaris*, represented by alleles *Rz1*, *Rz4* and *Rz5* and alleles *Rz2* and *Rz3* at the first and second locus, respectively (McGrann *et al*., 2009). However, the identified loci confer only a partial resistance to rhizomania, reducing viral replication and titre, and new isolates of BNYVV able to cause significant yield penalties on resistant cultivars have recently evolved (McGrann *et al*., 2009). New approaches to study virus–host–vector interactions therefore need to be explored in order to find alternative strategies to control the virus.

Up to now, reverse genetics for this virus has relied on full-length cDNA clones of all BNYVV RNAs and replicons based on RNA-3 and RNA-5 (D'Alonzo *et al*., 2012; Quillet *et al*., 1989; Ratti *et al*., 2009) under the control of the bacteriophage T7 promoter for *in vitro* transcription. The use of these clones for *in vivo* experiments requires the performance of multiple, expensive and sensitive steps. In particular, clones containing a full-length DNA copy of viral RNA must be linearized and transcribed *in vitro* in order to

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obtain infectious transcripts that need to be mechanically inoculated onto a test plant, such as *Nicotiana benthamiana* or *Chenopodium quinoa*.

Full-length infectious clones of BNYVV RNA-3 and RNA-4, under the control of the *Cauliflower mosaic virus* 35S promoter, have been obtained by Koenig *et al*. (1991) and combined with BNYVV helper strain, containing RNA-1 and RNA-2 species, therefore skipping the *in vitro* transcription step for these two components. Field isolates must be used to investigate or assess Benyviruses–plant–vector interactions and rhizomania resistance (Bornemann and Varrelmann, 2011; Koenig *et al*., 1991). These systems are expensive and time-consuming, and present serious limitations when applied to massive experiments.

A powerful alternative to these methods is agroinoculation (Grimsley *et al*., 1986), a less expensive and more reproducible strategy for the infection of plants with transcripts obtained *in vivo*, leading to virus agroinfection. Agroinfection involves plant tissue infiltration with a suspension of a combination of *Agrobacterium tumefaciens* cell clones carrying binary plasmids, each harbouring a full-length cDNA copy of a viral genome component. A plant functional promoter and DNA copy of the RNA viral genome are transferred as T-DNA from *A. tumefaciens* into plant cells from which, after *in vivo* transcription, biologically active viral RNAs are generated and can initiate infection.

Agroinfection is extensively used for the study of insect-transmitted viruses that cannot be mechanically inoculated into test plants, a serious obstacle for the study of such pathogens. This is the case for *Turnip yellows virus* (TuYV) and *Beet mild yellowing virus* (BMYV), both transmitted by aphids for which agroinfectious clones have been produced and successfully used for the infection of *N. benthamiana* plants (Leiser *et al*., 1992; Stephan and Maiss, 2006), and for the whitefly-transmitted Geminivirus *Tomato yellow leaf curl virus* (Navot *et al*., 1991) and the Crinivirus *Lettuce chlorosis virus* (Chen *et al*., 2012).

Previous work has shown that two virus species affecting sugar beet and difficult or impossible to transmit through mechanical inoculation can overcome such a barrier through agroinfiltration: *Beet curly top virus* (BCTV), transmitted by leafhoppers (Briddon *et al*., 1989), and the aphid-transmitted *Beet yellows virus* (BYV) for which mechanical transmission is inefficient (Chiba *et al*., 2006; Polak and Klir, 1969; Russell, 1963).

Here, we describe the production of agroinfectious clones of BNYVV B-type RNAs able to infect *N. benthamiana* and *B. macrocarpa* plants and to reproduce a complete viral life cycle. Moreover, direct agroinoculation of sugar beet roots is possible and could be used to screen for rhizomania resistance in different cultivars.

Full-length cDNA copies of BNYVV RNA-1 to RNA-5 have been cloned previously under the T7 promoter giving rise to pB15, pB214, pB35, pB45 and pB55 clones, respectively (Link *et al*., 2005; Quillet *et al*., 1989; Ziegler-Graff *et al*., 1988), as well as to viral replicons Rep3GFP (Erhardt *et al*., 2000), Rep5GFP (Schmidlin *et al*., 2005), Rep3BNYVV-p26HA (Link *et al*., 2005), Rep3BSBMV-p29HA and Rep5BNYVV-p25HA (A. Delbianco, unpublished data) expressing a tagged version of p29 and p25 usually expressed by BSBMV RNA-3 and BNYVV RNA-5, respectively. Expression vectors Rep3 and Rep5, containing minimal 5′ and 3′ untranslated regions (UTRs) of BNYVV RNA-3 and RNA-5, respectively, are replicated in the presence of BNYVV RNA-1 and RNA-2 and can be used to express foreign proteins (Bleykasten-Grosshans *et al*., 1997; Schmidlin *et al*., 2005).

Complete cDNA sequences of viral and replicon RNAs were introduced downstream of the *Cauliflower mosaic virus* double 35S promoter in binary vector pJL89 (Crivelli *et al*., 2011; Lindbo, 2007).

The cDNAs of BNYVV RNA-2 (4612 nucleotides), RNA-3 (1773 nucleotides), RNA-4 (1467 nucleotides) and RNA-5 (1350 nucleotides) and replicons expressing different proteins were amplified from the available clones using Pfu Ultra II Fusion Hotstart Polymerase (Agilent Technologies, Santa Clara, CA, USA) and ligated directly into the pJL89 vector digested with *Stu*I/*Sma*I, and then introduced into *A. tumefaciens* cells (strain C58C1) by electroporation, leading to AgroBN-2, AgroBN-3, AgroBN-4, AgroBN-5, AgroRep3GFP, AgroRep3p26HA, AgroRep3p29HA, AgroRep5GFP and AgroRep5p25HA clones (Fig. 1a).

We could not perform cloning of the full-length cDNA amplicon of BNYVV RNA-1 (6746 nucleotides) with the same one-step approach: in fact, we removed from pB15 *Sph*I/*Sph*I (1995–3742 nucleotides) and *Mlu*I/*Mlu*I (4125–5396 nucleotides) fragments, obtaining the pB15-*Sph*I-*Mlu*I clone (Fig. 1b). This cDNA sequence corresponding to partial BNYVV RNA-1 was amplified from pB15-*Sph*I-*Mlu*I and introduced into the pJL89-*Sph*I plasmid (pJL89 from which the *Sph*I restriction site has been eliminated through Klenow treatment). The two fragments, previously removed from the BNYVV RNA-1 sequence, were introduced back into the aforesaid vector in two steps to reconstitute the full-length RNA-1 sequence. All intermediate plasmids were cloned in *Escherichia coli* cells (strain MC1022) and the complete cDNA of RNA-1 in pJL89 (named AgroBN-1) was then introduced into *A. tumefaciens* cells (strain C58C1).

For leaf agroinfiltration, *A. tumefaciens* cells carrying clones of BNYVV RNA-1 to RNA-5 were grown overnight at 28 °C in 5 mL of Luria–Bertani (LB) medium supplemented with 100 μg/mL kanamycin and 50 μg/mL rifampicin. The bacteria were collected by centrifugation and resuspended in MA buffer (10 mM $MgCl_2$, 200 μM acetosyringone), adjusting the optical density at 600 nm (OD_{600} nm) to 0.6 for *N. benthamiana* and 0.3 for *B. macrocarpa*. Each agroclone cell line was mixed in equal amounts with the others and left at room temperature for 3–4 h before leaf agroinfiltration of 3-week-old plants.

Local symptoms appeared 1 week after agroinfiltration as chlorotic spots, whereas systemic symptoms appeared 2 weeks later,

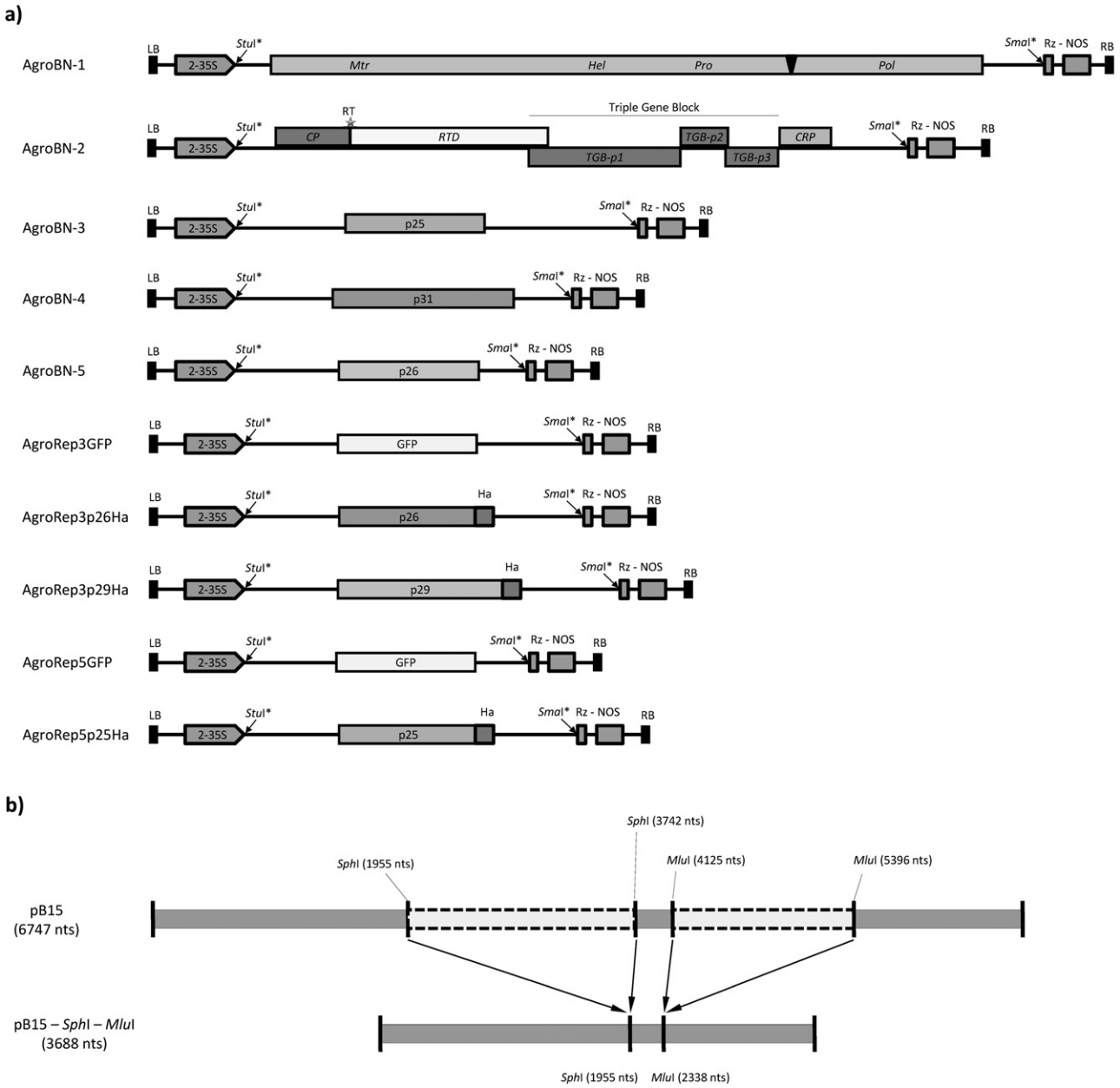

Fig. 1 (a) *Beet necrotic yellow vein virus* (BNYVV) constructs built and used in this study. Each clone has the same pJL89-derived backbone. Double *Stu*I/*Sma*I (*) restriction digestion allows the insertion of blunt-end polymerase chain reaction (PCR) fragments between the double 35S promoter (2-35S) and the hepatitis delta virus ribozyme (Rz) followed by a Nos terminator. LB and RB represent the left and right borders of the T-DNA. Mtr, methyltransferase; Hel, helicase; Pro, protease; Pol, RNA polymerase; RT, readthrough; RTD, readthrough domain; star, suppressible UAG stop codon; CRP, cysteine-rich protein; GFP, green fluorescent protein; Ha, haemagglutinin tag. (b) Schematic representation of pB15 and pB15–*Sph*I–*Mlu*I clones used for the production of AgroBN-1. Broken lines represent segments between restriction sites *Sph*I and *Mlu*I removed from BNYVV RNA-1 clone (pB15) in order to facilitate cloning in the pJL89-*Sph*I plasmid.

showing leaf distortion and providing evidence for the efficient long-distance movement of the virus within the plant (Fig. 2a,b). *Beta macrocarpa*-infiltrated leaves also displayed local lesions (Fig. 2c) and the systemic infection appeared to be particularly evident, as all leaves were completely distorted and the plant remained stunted, dying 1 month later (Fig. 2d, middle and right plants). Both local and systemic symptoms were identical to those obtained when plants were infected with *in vitro* transcripts or wild-type BNYVV isolate (data not shown).

To ensure that the expression occurred efficiently in the infiltrated area, we used a replicon expressing the green fluorescent protein (GFP) only on viral replication (Link *et al*., 2005). *Nicotiana*

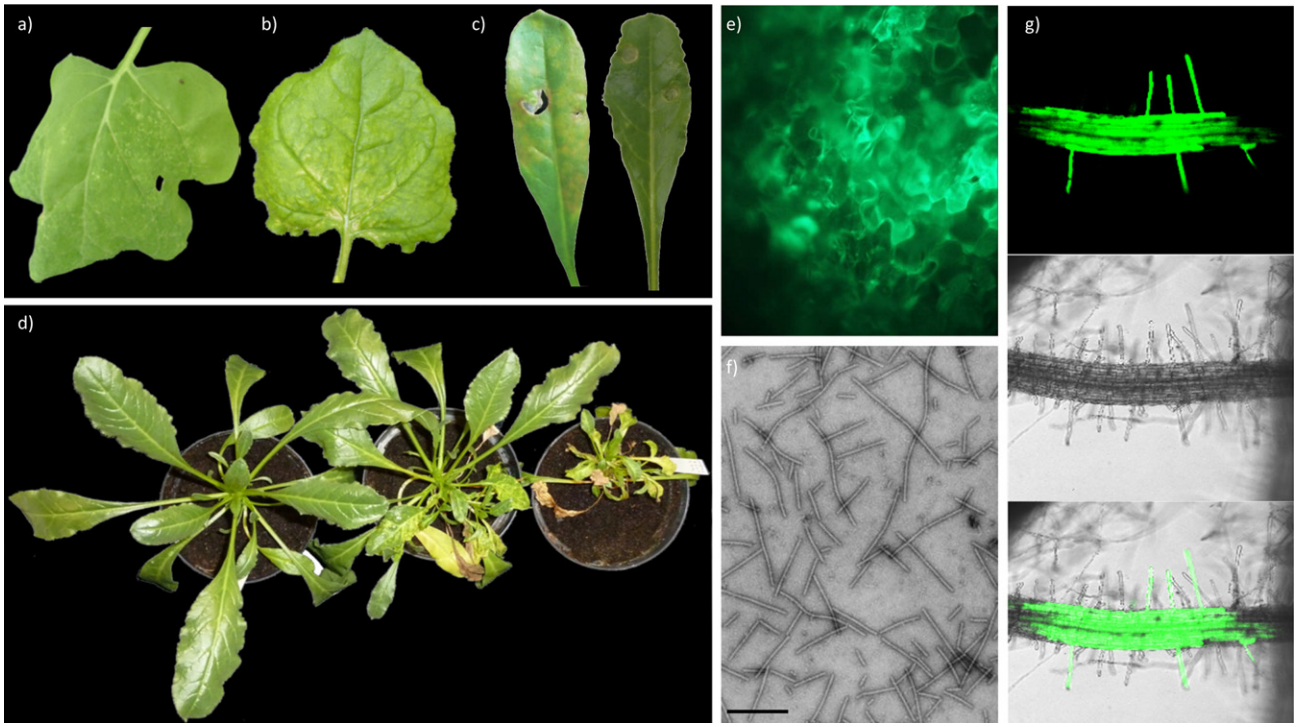

Fig. 2 Symptom expression, particle detection and reporter gene expression obtained by agroinfiltration of *Agrobacterium tumefaciens* cells carrying AgroBN-1 to AgroBN-5 clones (a, b, c, d and f) or AgroBN-1 to AgroBN-4 and AgroRep5GFP clones (e and g). (a) Local symptoms in agroinfiltrated leaf of *Nicotiana benthamiana* at 7 days post-inoculation (dpi). (b) Symptoms in systemically infected leaf of *N. benthamiana* at 14 dpi. (c) Local chlorotic spots in leaf of *Beta macrocarpa* agroinfiltrated with AgroBN-1 to AgroBN-5 clones (left) and symptomless leaf of *B. macrocarpa* agroinfiltrated with empty pJL89 binary vector (right) at 7 dpi. (d) Systemic symptom development in *B. macrocarpa*: nonagroinfected plant on the left, infected plant in the middle, showing some distorted leaves (14 dpi), and infected plant with totally distorted leaves on the right (30 dpi). (e) Agroclone-mediated green fluorescent protein (GFP) expression in *N. benthamiana* cells. (f) *Beet necrotic yellow vein virus* (BNYVV) particles observed in *B. macrocarpa* roots at transmission electron microscopy (TEM) by immunosorbent electron microscopy (ISEM) (14 dpi). Bar represents 200 nm. (g) Agroclone-mediated GFP expression in agroinfected roots of *B. macrocarpa*, visualized by confocal microscopy (14 dpi).

benthamiana leaves agroinfected with AgroBN-1 to AgroBN-4 and AgroRep5GFP were observed using a Nikon E800 microscope (Nikon, Tokyo, Japan) equipped with a Nikon DXM1200 camera. Replicon-mediated GFP expression was observed in cells of the infiltrated area (Fig. 2e). To test for the completion of a full infection cycle, the leaves and roots of the inoculated plants were collected and used for transmission electronic microscopy (TEM) imaging employing the immunosorbent electron microscopy (ISEM) technique. Characteristic rod-shaped particles were visualized in all tissues, demonstrating that encapsidation had occurred and the virus was able to spread in the entire plant (Fig 2f).

To complete our analyses, total RNAs were extracted from leaves showing local symptoms (Fig. 3a) and further analysed by Northern blot to confirm the presence of BNYVV RNA-1 to RNA-5. All RNA species were detected using specific probes, as described previously (Link *et al*., 2005; Schmidlin *et al*., 2005), demonstrating the replication and systemic movement of all the viral RNAs. Similarly, expression of the coat protein (CP) and the proteins encoded by agroclones carrying Replicons 3 or 5 was shown by their respective immunodetection using specific antibodies after Western blotting of total proteins from infiltrated or systemic tissues, as described by Link *et al*. (2005). BNYVV CP, haemagglutinin (HA)-tagged BNYVV p25 (and p26, not shown) and BSBMV p29 were detected using anti-HA antibody, whereas GFP was detected using a specific polyclonal antibody (Fig. 3b).

During the natural life cycle of *P. betae*, the zoospores infect the rootlets of the host plants, injecting their cytoplasmic content into the root cell and inducing plasmodium development. In this phase, the plasmodium acquires the virus from the infected plant and sequentially differentiates in a zoosporangium or in a cystosorus, leading to the production of secondary viruliferous zoospores able to infect new rootlets (Adams, 1991; Keskin, 1964).

To investigate the aptitude of 35S-driven RNAs to be transmitted by the plasmodiophorid vector, leaves of 3-week-old *B. macrocarpa* plants were infected with the agroclones of BNYVV RNA-1 to RNA-5 and the pots were successively infested with aviruliferous *P. betae* zoospores. The systemic infection arose 3 weeks after agroinfection and the presence of *P. betae* cystosori and BNYVV particles in the roots was confirmed by light microscopy and TEM

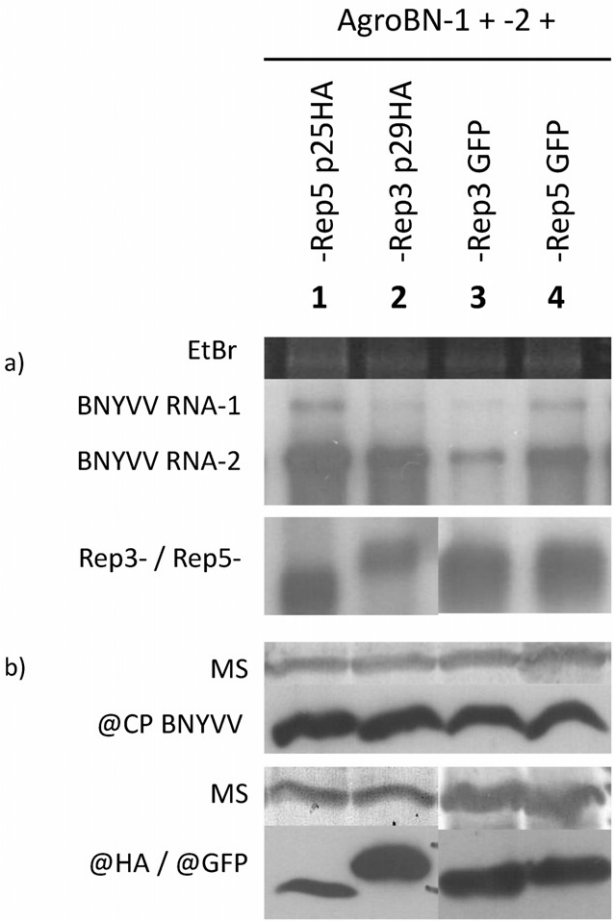

Fig. 3 Northern (a) and Western (b) blot analysis of *Nicotiana benthamiana* agroinoculated leaves: 1, AgroBN-1 + AgroBN-2 + AgroRep5p25Ha; 2, AgroBN-1 + AgroBN-2 + AgroRep3p29Ha; 3, AgroBN-1 + AgroBN-2 + AgroRep3GFP; 4, AgroBN-1 + AgroBN-2 + AgroRep5GFP. The equal loading of the Northern blot was visualized by ethidium bromide (EtBr) staining. Different RNAs were detected using complementary riboprobes. Immunodetection of virally expressed protein was performed on equally loaded gel as shown with membrane staining (MS).

observations (data not shown), respectively. Roots suspected to be infected by viruliferous *P. betae* cystosori were then collected, air dried and crumbled into pots carrying *B. vulgaris* seeds. After 2 weeks, viral particles were observed in the new seedling roots, and the root samples were analysed by reverse transcription-polymerase chain reaction (RT-PCR), Northern blot and Western blot (data not shown), demonstrating BNYVV transmission to new plants through the vector *P. betae*. Transmission occurred in all the 25 plants tested, leading to a 100% efficiency. BNYVV agroclones therefore represent a new tool to establish virus infection from cDNA, generating viral RNAs able to replicate, encapsidate, be acquired and transmitted by the vector to new host plants. Moreover, the behaviour of viral progeny was consistent with that observed using BNYVV wild-type isolate.

The capability of BNYVV agroclones to infect *B. vulgaris* roots was investigated in order to evaluate the possibility to develop a fast and reproducible rhizomania resistance test protocol, easily and economically applicable to a large number of plants. To date, resistance tests have been performed by growing sugar beet plants in soil infested by different BNYVV isolates. Alternatively, mechanically inoculated *C. quinoa* leaves, on which the virus is multiplied, can be used as a viral source to perform root inoculation in different experiments and resistance tests (Bornemann and Varrelmann, 2011; Koenig *et al*., 1991; Tamada *et al*., 1989). In our experiments, 10-day-old *B. vulgaris* plants were agroinoculated directly by vortexing the roots for 1 min in MA buffer containing carborundum (30 mg/mL) and a mixture of *A. tumefaciens* cells carrying AgroBN-1 to AgroBN-4 and AgroRep5GFP. Plants were successively planted in sterile sand and roots were analysed after 3 weeks. We demonstrated successful infection of *B. vulgaris* roots through BNYVV RNA and CP detection, viral particle observation (data not shown) and GFP expression visualization by confocal laser scanning microscopy with an LSM510 Zeiss laser scanning confocal microscope (Carl Zeiss Imaging Solutions GmbH, Gottingen, Germany) (Fig. 2g).

Using BNYVV agroclones, we were able to perform successful infection of *B. macrocarpa*, *B. vulgaris* and *N. benthamiana* hosts. Such a method represents an important achievement for studying BNYVV and opens up new possibilities for research studies. To our knowledge, this is the first time that agroinfection has been applied to a multipartite virus composed of five RNAs, although it has already been applied to bipartite viruses, such as *Lettuce infectious yellows virus* (Wang *et al*., 2009) and *Lettuce chlorosis virus* (Chen *et al*., 2012), and to the *Cucumber mosaic virus*, *Ourmia melon virus* and *Brome mosaic virus* with a tripartite genome (Crivelli *et al*., 2011; Kwon and Rao, 2012; Yao *et al*., 2011).

The agroinfectious clones described in this work represent a new tool for the verification of synergistic or antagonistic effects, as well as cross-protection, between BNYVV and other viruses affecting sugar beet, such as BSBMV. Previous studies have shown a high degree of reciprocal cross-protection in sugar beet mechanically inoculated with fresh sap of *C. quinoa* infected with BNYVV and BSBMV, but, when plants are grown in soil infested with viruliferous *P. betae* zoospores, it seems that BNYVV may suppress BSBMV, probably by competition for replicative or movement proteins inside the host cells (Mahmood and Rush, 1999; Rush, 2003; Wisler *et al*., 2003). A major limitation of these methods is the difficulty in providing equal inoculum density and the changing environmental conditions, such as the soil temperature, which can influence vector efficiency (Rush, 2003). The production of BSBMV agroclones is in progress and, combined with BNYVV agroclones, will represent a new complementary tool to advance benyvirus interaction studies. Moreover, as the infection can be established with a precise viral inoculum density, synergistic or antagonistic effects could be tested directly starting from root infection.

Viral or foreign proteins can also be produced by these agroclones, when they are combined with the use of replicon vectors Rep3 or Rep5 (Bleykasten-Grosshans *et al*., 1997; Schmidlin *et al*., 2005), with the purpose to test their expression and/or their effects on the plant in the viral context. This approach represents an alternative method to perform experiments *in planta* aimed at studying interactions between different viruses, protein expression and virus–vector interaction or virus resistance. Finally, agroinfection of sugar beet roots represents an innovative test to assay rhizomania resistance of sugar beet cultivars in large-scale experiments characterized by homogeny of viral infection pressure ensured by the precise quantification of *A. tumefaciens* cells. This represents a quick and economic way to infect plants, and therefore will widely enhance BNYVV research.

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REFERENCES

Adams, M. (1991) Transmission of plant viruses by fungi. *Ann. Appl. Biol.* **118**, 479–492.

Bleykasten-Grosshans, C., Guilley, H., Bouzoubaa, S., Richards, K.E. and Jonard, G. (1997) Independent expression of the first two triple gene block proteins of *Beet necrotic yellow vein virus* complements virus defective in the corresponding gene but expression of the third protein inhibits viral cell-to-cell movement. *Mol. Plant–Microbe Interact.* **10**, 240–246.

Bornemann, K. and Varrelmann, M. (2011) Analysis of the resistance-breaking ability of different *Beet necrotic yellow vein virus* isolates loaded into a single *Polymyxa betae* population in soil. *Phyopathology,* **101**, 718–724.

Briddon, R.W., Watts, J., Markham, P.G. and Stanley, J. (1989) The coat protein of *Beet curly top virus* is essential for infectivity. *Virology,* **172**, 628–633.

Canova, A. (1959) On the pathology of sugarbeet. *Inf. Fitopatol.* **9**, 390–396.

Chen, A.Y.S., Pavitrin, A. and Ng, J.C.K. (2012) Agroinoculation of the cloned infectious cDNAs of *Lettuce chlorosis virus* results in systemic plant infection and production of whitefly transmissible virions. *Virus Res.* **169**, 310–315.

Chiba, M., Reed, J.C., Prokhnevsky, A.I., Chapman, E.J., Mawassi, M., Koonin, E.V., Carrington, J.C. and Dolja, V.V. (2006) Diverse suppressors of RNA silencing enhance agroinfection by a viral replicon. *Virology,* **346**, 7–14.

Chiba, S., Hleibieh, K., Delbianco, A., Klein, E., Ratti, C., Ziegler-Graff, V., Bouzoubaa, S. and Gilmer, D. (2013) The benyvirus RNA silencing suppressor is essential for long-distance movement, requires both Zn-finger and NoLS basic residues but not a nucleolar localization for its silencing suppression activity. *Mol. Plant–Microbe Interact.* **26**, 168–181.

Crivelli, G., Ciuffo, M., Genre, A., Masenga, V. and Turina, M. (2011) Reverse genetic analysis of ourmiaviruses reveals the nucleolar localization of the coat protein in *Nicotiana benthamiana* and unusual requirements for virion formation. *J. Virol.* **85**, 5091–5104.

D'Alonzo, M., Delbianco, A., Lanzoni, C., Rubies Autonell, C., Gilmer, D. and Ratti, C. (2012) *Beet soil-borne mosaic virus* RNA-4 encodes a 32 kDa protein involved in symptom expression and in virus transmission through *Polymyxa betae*. *Virology,* **423**, 187–194.

Dunoyer, P., Pfeffer, S., Fritsch, C., Hemmer, O., Voinnet, O. and Richards, K.E. (2002) Identification, subcellular localization and some properties of a cysteine -rich suppressor of gene silencing encoded by *Peanut clump virus*. *Plant J.* **29**, 555–567.

Erhardt, M., Morant, M., Ritzenthaler, C., Stussi-Garaud, C., Guilley, H., Richards, K., Jonard, G., Bouzoubaa, S. and Gilmer, D. (2000) p42 movement protein of *Beet necrotic yellow vein virus* is targeted by the movement proteins P13 and P15 to punctate bodies associated with plasmodesmata. *Mol. Plant–Microbe Interact.* **13**, 520–528.

Gilmer, D. and Ratti, C. (2012) *Benyvirus*. In: *Virus Taxonomy: Classification and Nomenclature of Viruses. Ninth Report of the International Committee on Taxonomy of Viruses* (King, A.M.Q., Adams, M.J., Carstens, E.B. and Lefkowitz, E.J., eds), pp. 1133–1138. San Diego, CA: Elsevier.

Grimsley, N., Hohn, B., Hohn, T. and Walden, R. (1986) Agroinfection, an alternative route for viral infection of plants by using the Ti plasmid. *Proc. Natl. Acad. Sci. USA,* **83**, 3282–3286.

Harju, V.A., Mumford, R.A., Blockley, A., Boonham, N., Clover, G.R.G., Weekes, R. and Henry, C.M. (2002) Occurrence in the United Kingdom of *Beet necrotic yellow vein virus* isolates which contain RNA5. *Plant Pathol.* **51**, 811.

Keskin, B. (1964) *Polymyxa betae* n.sp., ein Parasit in den Wurzeln von *Beta vulgaris* Tournefort, besonders während der Jugendentwicklung der Zuckerrübe. *Arch. Microbiol.* **49**, 348–373.

Koenig, R. and Lennefors, B.L. (2000) Molecular analyses of European A, B and P type sources of *Beet necrotic yellow vein virus* and detection of the rare P type in Kazakhstan. *Arch. Virol.* **145**, 1561–1570.

Koenig, R., Jarausch, W., Li, Y., Commandeur, U., Burgermeister, W., Gehrke, M. and Lüddeke, P. (1991) Effect of recombinant *Beet necrotic yellow vein virus* with different RNA compositions on mechanically inoculated sugarbeets. *J. Gen. Virol.* **72**, 2243–2246.

Kruse, M., Koenig, R., Hoffmann, A., Kaufmann, A., Commandeur, U., Solovyev, A.J., Savenko, I. and Burgermeister, W. (1994) RFLP analysis of RT-PCR products reveals the existence of two major strain groups of *Beet necrotic yellow vein virus*. *J. Gen. Virol.* **75**, 1835–1842.

Kwon, S.J. and Rao, A.L.N. (2012) Emergence of distinct *Brome mosaic virus* recombinants is determined by the polarity of the inoculum RNA. *J. Virol.* **89**, 5204–5220.

Leiser, R.M., Ziegler-Graff, V., Reutenauer, A., Herrbach, E., Lemaire, O., Guilley, H., Richards, K. and Jonard, G. (1992) Agroinfection as an alternative to insects for infecting plants with *Beet western yellows* luteovirus. *Proc. Natl. Acad. Sci. USA,* **89**, 9136–9140.

Lemaire, O., Merdinoglu, D., Valentin, P., Putz, C., Ziegler-Graff, V., Guiney, H., Jonard, G. and Richards, K. (1988) Effect of *Beet necrotic yellow vein virus* RNA composition on transmission by *Polymyxa betae*. *Virology,* **162**, 232–235.

Lennefors, B.L., Lindsten, K. and Koenig, R. (2000) First report of A and B type *Beet necrotic yellow vein virus* in sugar beets in Sweden. *Eur. J. Plant Pathol.* **106**, 199–201.

Li, M., Liu, T., Wang, B., Han, C., Li, D. and Yu, J. (1998) Phylogenetic analysis of *Beet necrotic yellow vein virus* isolates from China. *Virus Genes,* **36**, 429–432.

Lindbo, J.A. (2007) High-efficiency protein expression in plants from agroinfection-compatible Tobacco mosaic virus expression vectors. *BMC Biotechnol.* **7**, 52.

Link, D., Schmidlin, L., Schirmer, A., Klein, E., Erhardt, M., Geldreich, A., Lemaire, O. and Gilmer, D. (2005) Functional characterization of the *Beet necrotic yellow vein virus* RNA-5-encoded p26 protein: evidence for structural pathogenicity determinants. *J. Gen. Virol.* **86**, 2115–2125.

Mahmood, T. and Rush, C. (1999) Evidence of cross-protection between *Beet soil-borne mosaic virus* and *Beet necrotic yellow vein virus* in sugar beet. *Plant Dis.* **83**, 521–526.

McGrann, G.R.D., Grimmer, M.K., Mutasa-Göttgens, E.S. and Stevens, M. (2009) Progress towards the understanding and control of sugar beet rhizomania disease. *Mol. Plant Pathol.* **10**, 129–141.

Miyanishi, M., Kusume, T., Saito, M. and Tamada, T. (1999) Evidence for three groups of sequence variants of *Beet necrotic yellow vein virus* RNA5. *Arch. Virol.* **144**, 879–892.

Navot, N., Pichersky, E., Zeidan, M., Zamir, D. and Czosnek, H. (1991) *Tomato yellow leaf curl virus:* a whitefly-transmitted geminivirus with a single genomic component. *Virology,* **185**, 151–161.

Peltier, C., Klein, E., Hleibieh, K., D'Alonzo, M., Hammann, P., Bouzoubaa, S., Ratti, C. and Gilmer, D. (2012) *Beet necrotic yellow vein virus* subgenomic RNA3 is a cleavage product leading to stable non-coding RNA required for long-distance movement. *J. Gen. Virol.* **93**, 1093–1102.

Polak, J. and Klir, O. (1969) Mechanical transmission of *Beet yellow virus* to *Chenopodium quinoa* and *Chenopodium foliosum*. *Biol. Plant.* **11**, 366–369.

Quillet, L., Guilley, H., Jonard, G. and Richards, K. (1989) In vitro synthesis of biologically active *Beet necrotic yellow vein virus* RNA. *Virology,* **172**, 293–301.

Ratti, C., Clover, G.R.G., Rubies Autonell, C., Harju, V.A. and Henry, C.M. (2005) A multiplex RT-PCR assay capable of distinguishing *Beet necrotic yellow vein virus* types A and B. *J. Virol. Methods,* **124**, 41–47.

Ratti, C., Hleibieh, K., Bianchi, L., Schirmer, A., Rubies Autonell, C. and Gilmer, D. (2009) *Beet soil-borne mosaic virus* RNA-3 is replicated and encapsidated in the presence of BNYVV RNA-1 and -2 and allows long distance movement in *Beta macrocarpa*. *Virology,* **385**, 392–399.

Rush, C. (2003) Ecology and epidemiology of *Benyvirus* and plasmodiophorid vectors. *Annu. Rev. Phytopathol.* **41**, 567–592.

Russell, G.E. (1963) Isolation of individual strains of *Beet yellows virus*. *Nature,* **197**, 623–624.

Schmidlin, L., Link, D., Mutterer, J., Guilley, H. and Gilmer, D. (2005) Use of a *Beet necrotic yellow vein virus* RNA-5-derived replicon as a new tool for gene expression. *J. Gen. Virol.* **86**, 463–467.

Sohi, H. and Maleki, M. (2004) Evidence for presence of types A and B of *Beet necrotic yellow vein virus* (BNYVV) in Iran. *Virus Genes,* **29**, 353–358.

Stephan, D. and Maiss, E. (2006) Biological properties of *Beet mild yellowing virus* derived from a full-length cDNA clone. *J. Gen. Virol.* **87**, 445–449.

Tamada, T. (1999) *Benyviruses*. In: *Encyclopedia of Virology*, Vol. II, 2nd edn. (Webster, R. and Granoff, A., eds), pp. 154–160. New York: Academic Press.

Tamada, T. and Baba, T. (1973) *Beet necrotic yellow vein virus* from rhizomania-affected sugar beet in Japan. *Ann. Phytopathol. Soc. Jpn.* **39**, 325–332.

Tamada, T., Shirako, Y., Abe, H., Saito, M., Kigushi, T. and Harada, T. (1989) Production and pathogenicity of isolates of *Beet necrotic yellow vein virus* with different numbers of RNA components. *J. Gen. Virol.* **70**, 3399–3409.

Wang, J., Turina, M., Stewart, L.R., Lindbo, J.A. and Falk, B.W. (2009) Agroinoculation of the Crinivirus, *Lettuce infectious yellows virus*, for systemic plant infection. *Virology,* **392**, 131–136.

Ward, L., Koenig, R., Budge, G., Garrido, C., McGrath, C., Stubbley, H. and Boonham, N. (2007) Occurrence of two different types of RNA-5-containing *Beet necrotic yellow vein virus* in the UK. *Arch. Virol.* **152**, 59–73.

Wisler, G.C., Lewellen, R.T., Sears, J.L., Wasson, J.W., Liu, H.Y. and Wintermantel, W.M. (2003) Interactions between *Beet necrotic yellow vein virus* and *Beet soilborne mosaic virus* in sugar beet. *Plant Dis.* **87**, 1170–1175.

Yao, M., Zhang, T., Tian, Z., Wang, Y. and Tao, X. (2011) Construction of Agrobacterium-mediated *Cucumber mosaic virus* infectious cDNA clones and 2b deletion viral vector. *Sci. Agric. Sin.* **44**, 3060–3068.

Ziegler-Graff, V., Bouzoubaa, S., Jupin, I., Guilley, H., Jonard, G. and Richards, K. (1988) Biologically active transcripts of *Beet necrotic yellow vein virus* RNA-3 and RNA-4. *J. Gen. Virol.* **69**, 2347–2357.

References

Almazan F., Gonzalez J.M., Penzes Z., Izeta A., Calvo E., Plana-Duran J., Enjuanes L. (2000). Engineering the largest rNA virus genome as an infectious bacterial artificial chromosome. PNAS, Vol. 97, N. 10, p. 5516-5521.

Benfey P. N., Ren L., Chua N.H. (1989). The CaMV 35S enhancer contains at least two domains which can confer different developmental and tissue-specific expression patterns. The EMBO Journal, vol. 8, no.8, pp. 2195-2202.

Briddon, R.W., Watts, J., Markham, P.G. & Stanley J. (1989). The coat protein of Beet curly top virus is essential for infectivity. Virology 172, 628-633.

Chiba S., Hleibieh K., Delbianco A., Klein E., Ratti C., Ziegler-Graff V., Bouzoubaa S.E., and Gilmer D. (2012). The benyvirus RNA silencing suppressor is essential for long-distance movement, requires both Zn-finger and NoLS basic residues but not a nucleolar localization for its silencing suppression activity. Mol Plant-Microbe Interact., Vol. 26, No.2, pp.168-181.

D'Alonzo M. (2011). Reverse genetic studies of Benyvirus – Polymyxa betae molecular interaction: role of the RNA-4 encoded protein in virus transmission. PhD thesis Universiry of Strasbourg and University of Bologna.

D'Alonzo, M., Delbianco, A., Lanzoni, C., Rubies Autonell, C., Gilmer, D. & Ratti, C. (2012). Beet soil-borne mosaic virus RNA-4 encodes a 32 kDa protein involved in symptom expression and in virus transmission through Polymyxa betae. Virology 423, 187–194.

Erhardt, M., Morant, M., Ritzenthaler, C., Stussi-Garaud, C., Guilley, H., Richards, K., Jonard, G., Bouzoubaa, S. & Gilmer, D. (2000). p42 movement protein of Beet necrotic yellow vein virus is targeted by the movement proteins P13 and P15 to punctate bodies associated with plasmodesmata. Mol Plant Microbe Interact 13, 520-528.

Escobar M.A., Dandekar A.M. (2003). Agrobacterium tumefaciens as an agent of disease. Trends in Plant Science, Vol. 8, No.8.

Gietl C., Koukolikova-Nicola Z., Hohn B. (1987). Mobilization of T-DNA from Agrobacterium to plant cells involves a protein that binds single-stranded DNA. Proc. Natl. Acad. Sci. USA, vol. 84, pp. 9006-9010.

Gilmer D., Bouzoubaa S., Hehn A., Guilley H., Richards K.E., Jonard G. (1992). Efficient cell to cell movement of beet necrotic yellow vein virus requires 3' proximal genes located on RNA2. Virology 189, 40-47.

Griffiths A.J.F., Miller J.H., Suzuki D.T., Lewontin R.C., Gelbart W.M. (2000). An introduction to genetic analysis. 7ed. New York: W.H Freeman and Company.

Grimsley, N., Hohn, B., Hohn, T. & Walden, R. (1986). Agroinfection, an alternative route for viral infection of plants by using the Ti plasmid. Proc. Natl. Acad. Sci. USA 83, 3282-3286.

Hoekema A., Hirsch P.R., Hooykaas P.J.J., Schilperoort R.A. (1983). A binary plant vector strategy based on separation of vir- and T-region of the Agrobacterium tumefaciens Ti-plasmid. Nature 303, 179–180.

Johansen E.E. (1996). Intron insertion facilitates amplification of clones virus cDNA in *Escherichia coli* while biological activity is reestablished after transcription *in vivo*. *Proc. Natl. Acad. Sci. USA*, Vol. 93, pp. 12400-12405.

Jupin I., Guilley H., Richards K.E., Jonard G. (1992). Two proteins encoded by beet necrotic yellow vein virus RNA3 influence symptom phenotype on leaves. EMBO Journal 11, 479-488.

Lauber E., Guilley H., Tamada T., Richards K.E., Jonard G. (1998). Vascular movement of beet necrotic yellow vein virus in Beta macrocarpa is probably dependent on an RNA3 sequence domain rather than a gene product. Journal of General Virology 79 (Pt. 2), 385-393.

Leiser, R. M., Ziegler-Graff, V., Reutenauer, A., Herrbach, E., Lemaire, O., Guilley, H., Richards, K. & Jonard, G. (1992). Agroinfection as an alternative to insects for infecting plants with Beet western yellows luteovirus. Proc. Natl. Acad. Sci. USA 89, 9136-9140.

Link, D., Schmidlin, L., Schirmer, A., Klein, E., Erhardt, M., Geldreich, A., Lemaire, O. & Gilmer, D. (2005). Functional characterization of the Beet necrotic yellow vein virus RNA-5-encoded p26 protein: evidence for structural pathogenicity determinants. J. Gen. Virol. 86, 2115–2125.

Mahmood, T. & Rush, C. (1999). Evidence of cross-protection between Beet soilborne mosaic virus and Beet necrotic yellow vein virus in sugar beet. Plant Dis. 83, 521–526.

Miyanishi M., Roh S.H., Yamamiya A., Ohsato S., Shirako Y. (2002). Reassortment between genetically distinct Japanese and US strains of Soil-borne mosaic virus: RNA1 from Japanese strain and RNA2 from a US strain make a pseudorecombinant virus.

Mullis K.B., Faloona F.A. (1987). Specific synthesis of DNA in vitro via a polymerase-catalyzed chain reaction. Methods in Enzymology, 155, 335-350.

Păcurar D.I., Thordal-Christensen H., Păcurar M.L., Pamfil D., Botez C., Bellini C. (2011). Agrobacterium tumefaciens: from crown gall tumors to genetic transformation. Physiological and Molecular Plant Pathology, 76, 76-81.

Pitzschke A., Hirt H. (2010). New insights into an old story: Agrobacterium-induced tumor formation in plants by plant transformation. The EMBO Journal, 29, 1021-1032.

Quillet, L., Guilley, H., Jonard, G. & Richards, K., (1989). In vitro synthesis of biologically active Beet necrotic yellow vein virus RNA. Virology 172, 293–301.

Racaniello V.R., Baltimore D. (1981). Molecular cloning of poliovirus cDNA and determination of the complete nucleotide sequence of the viral genome. Proceedings of the National Academy of Sciences of the United States of America, 78, 4887-4891.

Rahim M.D., Andika I.B., Han C., Kondo H., Tamada T. (2007). RNA-4 encoded p31of beet necrotic yellow vein virus is involved in efficient vector transmission, symptom severity and silencing suppression in roots. Journal of General Virology 88, 1611-1619.

Ratti, C., Hleibieh, K., Bianchi, L., Schirmer, A., Rubies Autonell, C., & Gilmer, D. (2009). Beet soil-borne mosaic virus RNA-3 is replicated and encapsidated in the presence of BNYVV RNA-1 and -2 and allows long distance movement in Beta macrocarpa. Virology 385, 392–399.

Rice C.M., Grakoui A., Galler R., Chambers T.J. (1989). Transcription of infectious yellow fever RNA from full-length cDNA templates produced by in vitro ligation. New Biol., 1, 285-296.

Rush, C. (2003). Ecology and epidemiology of Benyvirus and plasmodiophoridvectors. Annual review of phytopathology 41, 567-592.

Sanger F., Nicklen S., Coulson A.R. (1977). DNA sequencing with chain-terminating inhibitors. Proceeedings of the National Academy of Science ,74 (12), 5463-5467.

Schmidlin, L., Link, D., Mutterer, J., Guilley, H. & Gilmer, D. (2005). Use of a Beet necrotic yellow vein virus RNA-5-derived replicon as a new tool for gene expression. J. Gen. Virol. 86, 463–467.

Schmitt C., Balmori E., Jonard G., Richards K.E., Guilley H. (1992). In vitro mutagenesis of biologically active transcripts of beet necrotic yellow vein virus RNA 2: evidence that a domain of the 75-kDa readthrough protein is important for efficient virus assembly. Proc Natl Acad Sci USA, 89, 5715-5719.

Stephan, D. & Maiss, E. (2006). Biological properties of Beet mild yellowing virus derived from a full-length cDNA clone. Journal of General Virology 87, 445–449.

Tamada T., Schmitt C., Saito M., Guilley H., Richards K., Jonard G. (1996). High resolution analysis of the readthrough domain of beet necrotic yellow vein virus readthrough protein: a KTER motif is important for efficient transmission of the virus by Polymyxa betae. J. Gen. Virol., 77, 1359-1367.

Tierney M.B., Lamour K.H. (2005). An introduction to reverse genetic tools for investigating gene function. The plant health instructor, 10, 1094/PHI-A-2005-1025-01.

Torrance L., Wright K.M., Crutzen F., Cowan G.H., Lukhovitskaya N.I., Bragard C., Savenkov E.I. (2011). Unusual features of pomoviral RNA movement. Front Microbiol, 2, 259.

Wang K., Herrera-Estrella L., Van Montagu M., Zambryski P. (1984). Right 25 bp terminus sequence of the nopaline T-DNAis essential for and determines direction of DNA transfer from Agrobacterium to the plant genome. Cell, 38:455-62.

Watson J.D., Crick F.H.C. (1953). A structure of deoxyribose nucleic acid. Nature, 171, 737-738.

White P.R., Braun A.C. (1942). A cancerous neoplasm of plants: autonomous bacteria-free crown in gall tissue. Cancer Res., 2, 597-617.

Yount B., Curtis K.M., Baric R.S. (2000). Strategy for systemic assembly of large RNA and DNA genomes: transmissible gastroenteritis virus model. Journal of Virology, Vol. 74, No.22, p.10600-10611.

Ziegler-Graff, V., Bouzoubaa, S., Jupin, I., Guilley, H., Jonard, G. & Richards, K. (1988). Biologically active transcripts of Beet necrotic yellow vein virus RNA-3 and RNA-4. J. Gen. Virol. 69, 2347-2357.

Zupan J., Muth T.R., Draper O., Zambryski P. (2000). The transfer of DNA from Agrobacterium tumefaciens into plants: a feast of fundamental insights. Plant J., 23, 11-28.

Chapter 2

Production of chimeric isolates of BNYVV and BSBMV

Chapter 3

Characterization of *Benyvirus* p14s

Within the study of molecular mechanisms involved in the pathogenesis of beet soil-borne viruses, we investigated the role of Benyvirus p14s. According to the results presented in Chapter 2, p14s seem to be involved in the incompatible relationship of BoStras12 chimera leading to the induction of necrotic lesions in the hosts *C. quinoa* and *N. benthamiana*. While BNYVV p14 has already been identified as a suppressor of post-transcriptional gene silencing (VSR, viral suppressor of RNA silencing), BSBMV p14 has been suspected to possess a similar function on the basis of sequence similarity with BNYVV p14. However, since proteins sharing sequence similarities could exhibit or not VSR activity (Kozlowska-Makulska *et al*., 2010), it appeared necessary to characterize BSBMV p14 function.

Studies about BNYVV p14 demonstrated that this cysteine-rich protein (CRP) is able to bind zinc-ions *in vitro* (Niesbach-Klosgen *et al*., 1990) and point mutations in its sequence inhibit the accumulation of viral RNAs in protoplasts (Hehn *et al*., 1995). Later, BNYVV p14 has been described as a viral suppressor of RNA silencing (Dunoyer *et al*., 2002).

Part of the experiments performed and results obtained about characterization of Benyviruses p14s have been reported in the paper I co-authored in the *Molecular Plant-Microbe Interactions* journal, which is part of the present chapter.

1. Introduction

RNA silencing refers to the related processes of post-transcriptional gene silencing (PTGS) in plants (Lindbo *et al*., 1993), RNA interference (RNAi) in animals (Fire *et al*., 1998) and quelling in fungi (Romano and Macino, 1992). This mechanism has been discovered accidentally in petunia flowers in 1990. Attempts to obtain purple flowers overexpressing the chalcone synthase (CHS) by introducing a CHS transgene in the plant, resulted in the suppression of anthocyanin biosynthesis and the production of white petals (Napoli *et al*., 1990). RNA silencing is involved in the regulation of gene expression, the maintenance of genome integrity, stress response and pathogen defense. The unifying principle of RNA silencing is the inactivation of a target RNA by either degradation in a sequence-specific manner or translational inhibition (Fig. 3.1).

This inactivation is triggered by the presence of double stranded RNA (dsRNA) molecules that derive from pairing of anti-sense transcripts, hairpin structures, replication intermediates of RNA viruses, nuclear transgene and high abundance of aberrant transcripts (Carrington *et al.*, 2001).

The dsRNA are processed into small RNAs (sRNA) of 21-24 nucleotides in size by RNAse III-type enzymes called Dicers (Hamilton and Baulcombe 1999; Bernstein *et al*., 2001; Elbashir *et al*., 2001). One of the two sRNA strands joins the RNA-induced silencing complex (RISC) which contains an Argonaute (Ago) protein with a slicer activity (Diaz-Pendon and Ding, 2008). The RISC can either cleave RNA molecules homologous to the incorporated sRNA (Baumberger and Baulcombe, 2005), inhibit RNA translation (Brodersen *et al*., 2008) or affect methylation of target DNA (Ekwall, 2004). The first two mechanisms are referred to as post-transcriptional gene silencing (PTGS), whereas DNA methylation leads to transcriptional extinction of the target gene and therefore is named transcriptional gene silencing (TGS).

In plants, the silencing signal can be amplified through RNA-dependent RNA polymerases (RDRs) that produce secondary sRNAs corresponding to sequences upstream and downstream of the primary targeted region. This mechanism is called transitivity (Himber *et al*., 2003). Moreover, RNA silencing produces mobile RNA signals that can be transported cell to cell through plasmodesmata and at long distance through the phloem (Voinnet, 2005). PTGS appears as an innate defense mechanism against viral infection since double-stranded RNA molecules are produced during replication of RNA viruses (Waterhouse *et al*., 1998). As a counterstrategy, these pathogens have evolved proteins acting as viral suppressors of RNA silencing (VSR) (Voinnet, 2001; Li and Ding, 2006). Over 30 VSRs have been identified from RNA and DNA viruses and available data suggest that each plant virus encodes at least one VSR (Li and Ding, 2006).

According to Diaz-Pendon and Ding (2008), VSRs can be divided into three broadly defined families. VSRs of the first family, such as potyviral HC-Pro and tobamoviral p126, act in the early stages of infection to suppress intracellular antiviral silencing induced in the first infected cells before cell-to-cell movement. Their expression therefore enhances virus accumulation in the inoculated protoplasts. Suppression of

antiviral silencing induced by VSRs of the second family, such as potexviral p25, is required for cell-to-cell movement of the virus but has no apparent effect on viral accumulation in the inoculated protoplasts. Most of the known VSRs belong to the third family, such as cucumoviral 2b, tombusviral p19 and carmoviral p38, which facilitate virus long distance movement and/or intensify disease symptoms but are not essential for viral replication and cell-to-cell movement.

VSRs vary greatly in their sequence and structure and can target different steps of the PTGS pathway (Voinnet *et al*., 1999; Burgyan and Havelda, 2011). There is evidence that siRNA sequestration is the most common mode of action of RNA silencing suppressors (Lakatos *et al*., 2006; Merai *et al*., 2006; Csorba *et al*., 2007; Ding and Voinnet, 2007). The capsid protein of *Turnip crinckle virus*, p38, is able to bind dsRNA and also to inhibit the activity of Dicer proteins (Qu *et al*., 2003; Deleris *et al*., 2006). The protein P0 of *Beet western yellow virus* contains a F-box domain and target AGO proteins degradation (Bortolamiol *et al*., 2007; Baumberger *et al*., 2007; Derrien *et al*., 2012) preventing RISC assembly but doesn't have an RNA-binding activity (Zhang *et al*., 2006; Csorba *et al*., 2010). The 2b protein of *Cucumber mosaic virus* was among the first viral silencing suppressors protein identified (Brigneti *et al*., 1998). It has been shown to directly interact with the AGO proteins reducing their slicer activity and it is also able to binds small interfering RNA *in vivo* (Zhang *et al*., 2006; Hamera *et al*., 2012). Both CMV 2b and p19 of *Tomato bushy stunt virus* (TBSV) facilitate long-distance movement and enhance disease severity (Diaz-Pendon and Ding, 2008). Further functional and structural studies have shown that TBSV p19 has a high affinity for short dsRNAs and suppresses RNA silencing by sequestering duplex siRNAs and therefore preventing their incorporation into RISC (Lakatos *et al*., 2004; 2006). Beside its role in potyvirus aphid-mediated transmission, genome amplification, polyprotein processing and long distance movement, the multifunctional helper component-proteinase (HC-Pro) that binds siRNA prevents the RISC assembly (Merai *et al*., 2006; Shiboleth *et al*., 2007; Diaz-Pendon and Ding, 2008). The *Potato virus X* p25 is encoded by the first gene of the "triple gene block" and thus is involved in PVX movement (Verchot *et al*., 1998). The suppressor activity of p25 is required for cell-to-cell

movement and it also induces AGO degradation through the proteasome pathway (Bayne *et al*., 2005; Chiu *et al*., 2010).

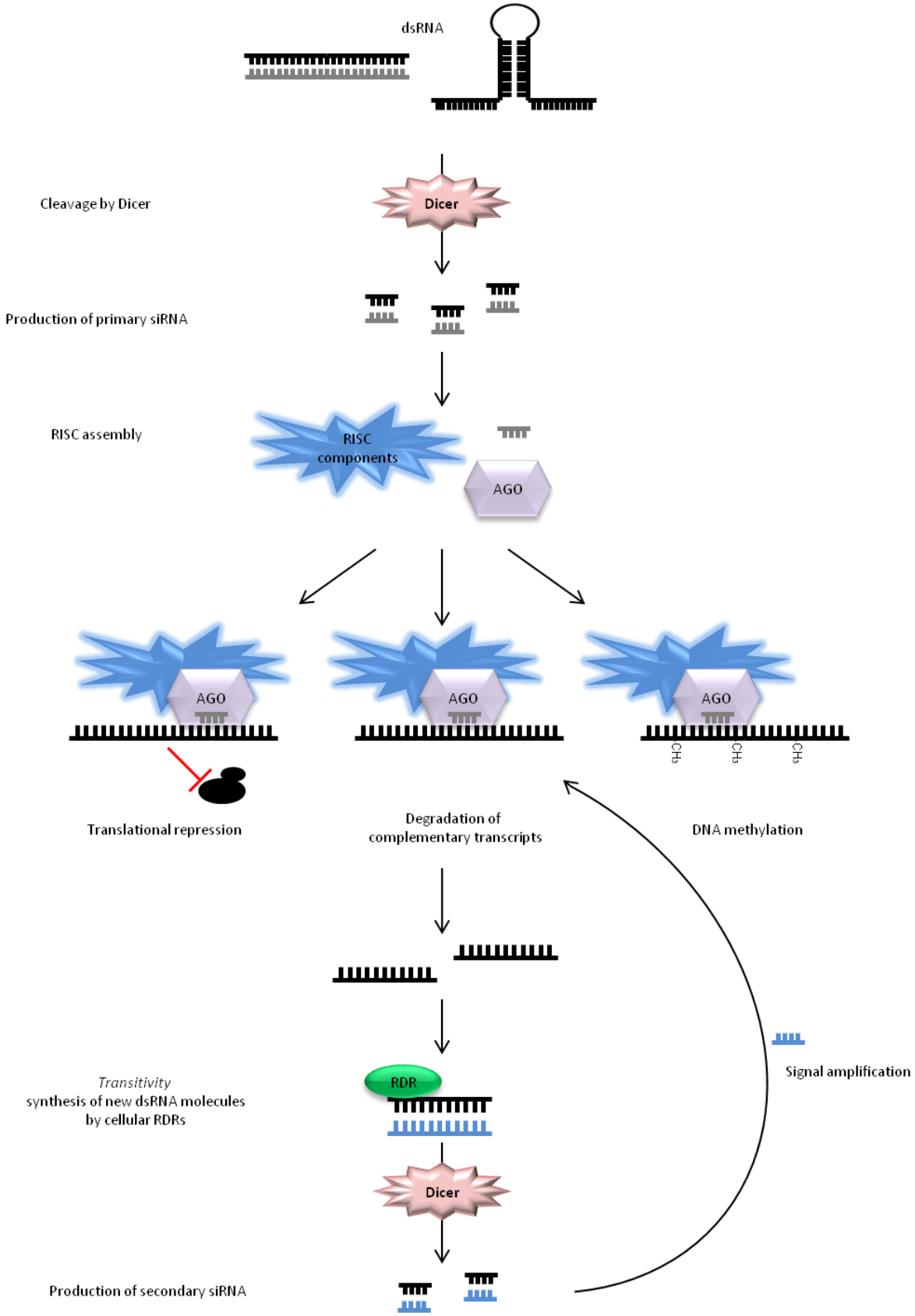

Fig. 3.1: Simplified model of RNA silencing in plants. AGO: Argonaute protein; RISC: RNA-induced silencig complex; RDR: RNA-dependent RNA polymerases.

2. The Benyvirus RNA silencing suppressor is essential for long distance movement, requires both Zn-finger domain and NoLS basic residues but not a nucleolar localization for its silencing suppression activity

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e-Xtra*

The Benyvirus RNA Silencing Suppressor Is Essential for Long-Distance Movement, Requires Both Zinc-Finger and NoLS Basic Residues but Not a Nucleolar Localization for Its Silencing-Suppression Activity

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The RNA silencing-suppression properties of *Beet necrotic yellow vein virus* (BNYVV) and *Beet soil-borne mosaic virus* (BSBMV) cysteine-rich p14 proteins have been investigated. Suppression of RNA silencing activities were made evident using viral infection of silenced *Nicotiana benthamiana* 16C, *N. benthamiana* agroinfiltrated with green fluorescent protein (GFP), and GF-FG hairpin triggers supplemented with viral suppressor of RNA silencing (VSR) constructs or using complementation of a silencing-suppressor-defective BNYVV virus in *Chenopodium quinoa*. Northern blot analyses of small-interfering RNAs (siRNAs) in agroinfiltration tests revealed reduced amounts of siRNA, especially secondary siRNA, suggesting that benyvirus VSR act downstream of the siRNA production. Using confocal laser-scanning microscopy imaging of infected protoplasts expressing functional p14 protein fused to an enhanced GFP reporter, we showed that benyvirus p14 accumulated in the nucleolus and the cytoplasm independently of other viral factors. Site-directed mutagenesis showed the importance of the nucleolar localization signal embedded in a C4 zinc-finger domain in the VSR function and intrinsic stability of the p14 protein. Conversely, RNA silencing suppression appeared independent of the nucleolar localization of the protein, and a correlation between BNYVV VSR expression and long-distance movement was established.

During host infection, viruses face plant antiviral defense, particularly the innate response targeting double-stranded RNA arising from viral RNA genome replication. Such an antiviral mechanism, known as RNA interference (RNAi) or post-transcriptional gene silencing (PTGS), is widely distributed among eukaryotes (Ding 2010; Ding and Voinnet 2007; Voinnet 2001, 2005, 2008). This extensively explored mechanism provides detailed characterization of the pathways involving the cleavage of double-stranded RNA by dicer-like proteins (Deleris et al. 2006; Moissiard and Voinnet 2006), the loading of small-interfering RNAs (siRNAs) into ARGONAUTE complexes and their slicer activity (Azevedo et al. 2010; Duan et al. 2012; Ruiz-Ferrer and Voinnet 2007), as well as transitivity provided by endogenous RNA-dependent RNA polymerases (Moissiard et al. 2007). Many if not all steps of this defense mechanism are targets of viral elements, known as viral suppressors of RNA silencing (VSR) (Li and Ding, 2006), that tend to inhibit or inactivate one or more of the silencing machinery actors (Burgyan and Havelda 2011). Thus, VSR provide important insight in the understanding of RNA silencing and are used as powerful molecular probes to elucidate some biochemical silencing steps (Voinnet 2005). Due to their wide structural diversity and primary functions, VSR identification per se is difficult even when they were already described as pathogenicity factors (Brigneti et al. 1998). VSR described thus far belong to structural proteins (e.g., *Carmovirus* p38) or to nonstructural proteins involved in replication (e.g., *Tobamovirus* spp.), movement (e.g., *Potexvirus* spp.), vector transmission (e.g., *Potyvirus* spp. HC-Pro) or to yet unidentified primary function (e.g., *Polerovirus* spp. P0). Subcellular localizations of VSR have been shown to vary greatly because some accumulate in either the cytoplasm, peroxisomes, nucleus, or nucleolus. Some experiments have provided a link between VSR activities and the subcellular localization of the proteins but these remain poorly documented. The subcellular localization of a protein could affect functions, by regulating either its concentration or degradation; therefore, it appears essential to study the fate of a VSR during the infection cycle and correlate its activity with its subcellular targeting.

Benyviruses belong to class IV of the Baltimore classification (Baltimore 1971) and consist of positive-stranded multipartite RNA viruses transmitted by the protozoa *Polymyxa betae* (Gilmer and Ratti 2012). Within the genus *Benyvirus*, *Beet necrotic yellow vein virus* (BNYVV) and *Beet soil-borne mosaic virus* (BSBMV) share common properties but are distinct species, while the closely related bipartite *Burdock mottle virus* (BdMoV) and *Rice stripe necrosis virus* (RSNV) (Lozano and Morales 2009) are tentative members of the *Benyvirus* genus. Out of the five BNYVV RNAs, RNA1 and 2 are carried by all BNYVV strains isolated from fields (Chiba et al. 2011;

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*The *e*-Xtra logo stands for "electronic extra" and indicates one supplementary figure is published online.

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Schirmer et al. 2005) and are sufficient to establish an infection on some mechanical hosts, indicating that housekeeping functions are all present within these two components (Peltier et al. 2008). In such conditions, smaller RNAs (RNA3, RNA4, and RNA5) are dispensable and have been engineered as expression vectors for protein of interest (Schmidlin et al. 2005), although they have beneficial roles in natural infection (Peltier et al. 2008).

Genetic, biochemical, and viral complementation screens are commonly used to identify and characterize VSR (Li and Ding 2006). Such descriptions of silencing suppressors include complementation of VSR defective viruses, infection of silenced plants, as well as patch test experiments (Angell and Baulcombe 1997; Brigneti et al. 2004; Ratcliff et al. 2001; Voinnet et al. 2003). Such tools were used to screen for silencing suppressor activity of BNYVV and confirmed the VSR function of BNYVV p14 (Andika et al. 2012; Dunoyer et al. 2002; Guilley et al. 2009; Kozlowska-Makulska et al. 2010; Zhang et al. 2005). Moreover, they identified BdMoV p13 as a VSR (Guilley et al. 2009).

In this article, we highlight the properties of benyvirus p14 proteins. We show that BNYVV and BSBMV p14 proteins act downstream of the initial steps of the silencing response. P14 VSR are zinc-finger (Znf) cysteine-rich proteins (CRP) that are addressed to the nucleolus of infected cells by the presence of basic amino acids embedded in the Znf (Niesbach-Klosgen et al. 1990). Sequence motives required for nucleolar targeting, dimer formation, and cysteine residues essential to the Znf structure folding of BNYVV p14 protein have been identified. Our work also shows that the ability of BNYVV to suppress the RNAi defense mechanism is not related to the p14 nucleolar localization. Finally, we demonstrate that the p14-silencing-suppressor function is essential for an efficient systemic spread of the virus in two experimental host plants.

RESULTS

Benyvirus p14 CRP are VSR.

Viral infection of green fluorescent protein (GFP)-silenced *Nicotiana benthamiana* 16C plants, that initially express constitutively the *GFP* gene under the 35S promoter, revealed restoration of GFP expression in stem and mesophyll tissues of *Peanut clump virus* (PCV)-infected plants (Dunoyer et al. 2002) whereas BNYVV-infected plants displayed only limited reappearance of the fluorescence (Fig. 1A, middle panel): GFP mRNA was detected mainly in the stem and leaf veins (Fig. 1A, right panel, lanes s and v; GFP) although BNYVV RNA1 and 2 were found in all *N. benthamiana* tissues (Fig. 1A, right panel, lanes s, v, and m; RNA1 and RNA2). Conversely, no fluorescence was present on mock-inoculated silenced plants where GFP mRNA was at the detection limit when compared with nonsilenced 16C plants. Earlier studies showed that the CRP expressed from an RNA2-derived subgenomic RNA displays VSR activity (Dunoyer et al. 2002; Gilmer et al. 1992; Koonin et al. 1991).

P14-deficient BNYVV can be complemented by other VSR proteins and, in particular, by the p15 CRP of PCV (Guilley et al. 2009) and by BSBMV p14 expressed from a replicon vector (data not shown), suggesting similar functions for both benyvirus VSR proteins. Sequence comparisons of benyvirus CRP using the MAFFT software (Katoh and Toh 2008) allowed us to define conserved cysteine residues (Fig. 1B, blue boxes). Using the NoD algorithm (Scott et al. 2011), a putative nucleolus localization signal (NoLS) was predicted between residues 66 and 90 (Fig. 1B, underlined in red). No such NoLS motif was identified on BSBMV p14 and the two *Benyvirus* tentative members BdMoV and RSNV CRP. We compared the silencing-suppression activities of BNYVV and BSBMV p14 proteins (p14-BN and p14-BS, respectively) to that of the known polerovirus P0 VSR (Fig. 1C) by agroinfiltration tests using *N. benthamiana* 16C plants. Co-infiltration of bacteria carrying the GFP-silencing trigger construct and an empty binary vector resulted in the extinction of GFP expression. Moreover, the appearance of specific GFP siRNAs in the patched area 4 days postinfiltration indicated the induction of the GFP mRNA silencing (Fig. 1C, Ø). When the *Turnip yellows virus* (TuYV) P0 VSR was co-expressed with the GFP construct, the fluorescence intensity of the patches markedly increased (not shown); this was correlated to a strong increase of the GFP mRNA and a decrease of GFP siRNAs accumulation (Fig. 1C, P0). When benyvirus VSR were expressed, the fluorescence of the patches became brighter (not shown) and a reduction of GFP siRNAs was observed (Fig. 1C, p14-BN and p14-BS). The amount of GFP mRNA was efficiently increased with p14-BS and only slightly increased with p14-BN. However, when the influenza hemagglutinin A epitope (HA) tag was added to the N-terminus of the p14-BS protein, the VSR activity was reduced (Fig. 1C, compare p14-BS and HA:p14-BS). The silencing-suppression effect of both benyvirus CRP diminished after 4 days, while that of P0 VSR was maintained even after 7 days (data not shown).

Benyvirus p14 CRP act downstream of primary siRNA production.

We then conducted similar experiments on *N. benthamiana* wild-type (wt) plants infiltrated with pBin-GFP and VSR together with a GF-FG hairpin trigger (Himber et al. 2003) corresponding to the 5′ part of the GFP mRNA. We analyzed the production of primary and secondary "GF" siRNAs as well as secondary "P" siRNAs produced by the transitivity pathway (Himber et al. 2003; Moissiard et al. 2007). In the control experiment, *Turnip crinkle virus* (TCV) P38 VSR expression dramatically reduced the accumulation of both primary and secondary siRNAs and increased that of GFP (Fig. 1D, P38-TCV), as previously reported. However, we were unable to show any increase in the accumulation of GFP mRNA (Azevedo et al. 2010; Deleris et al. 2006). The use of TuYV P0 VSR induced the accumulation of both the GFP mRNA and GFP protein. As expected, P0 suppressor activity did not affect the primary siRNA production but reduced secondary siRNA accumulation (Bortolamiol et al. 2007). When benyvirus CRP were expressed in the presence of the hairpin trigger, GFP messenger and GFP protein levels increased compared with those of the negative control experiment but were lower than those produced by P0 (Fig. 1D). GF siRNAs were reduced in the presence of BNYVV CRP but were comparable with those produced in the absence of VSR when BSBMV CRP was used. A reduction of secondary P siRNAs was observed using both benyvirus CRP, suggesting that p14 CRP did not fully inhibit the transitivity mechanism per se. Again, the HA-tagged BSBMV p14 exhibited less efficient VSR activity compared with the wt p14BS because more GF siRNAs were detected for similar loading (Fig. 1D). A comparable effect of an HA tag was described for tombusvirus p19 that lost 50% of its suppression activity (Dunoyer et al. 2004).

BNYVV p14 CRP is a cytoplasmic and nucleolar protein.

In order to investigate the subcellular localization of the BNYVV p14 CRP, its sequence was fused to that of enhanced (E)GFP (Fig. 2A) in the BNYVV rep0 replicon vector (Guilley et al. 2009; Jupin et al. 1990; Schmidlin et al. 2005) to produce in vitro transcripts. These transcripts were co-inoculated to tobacco BY-2 protoplasts with BNYVV RNA1 and either an RNA2 deficient in p14 expression (RNA2Δp14) or a wt

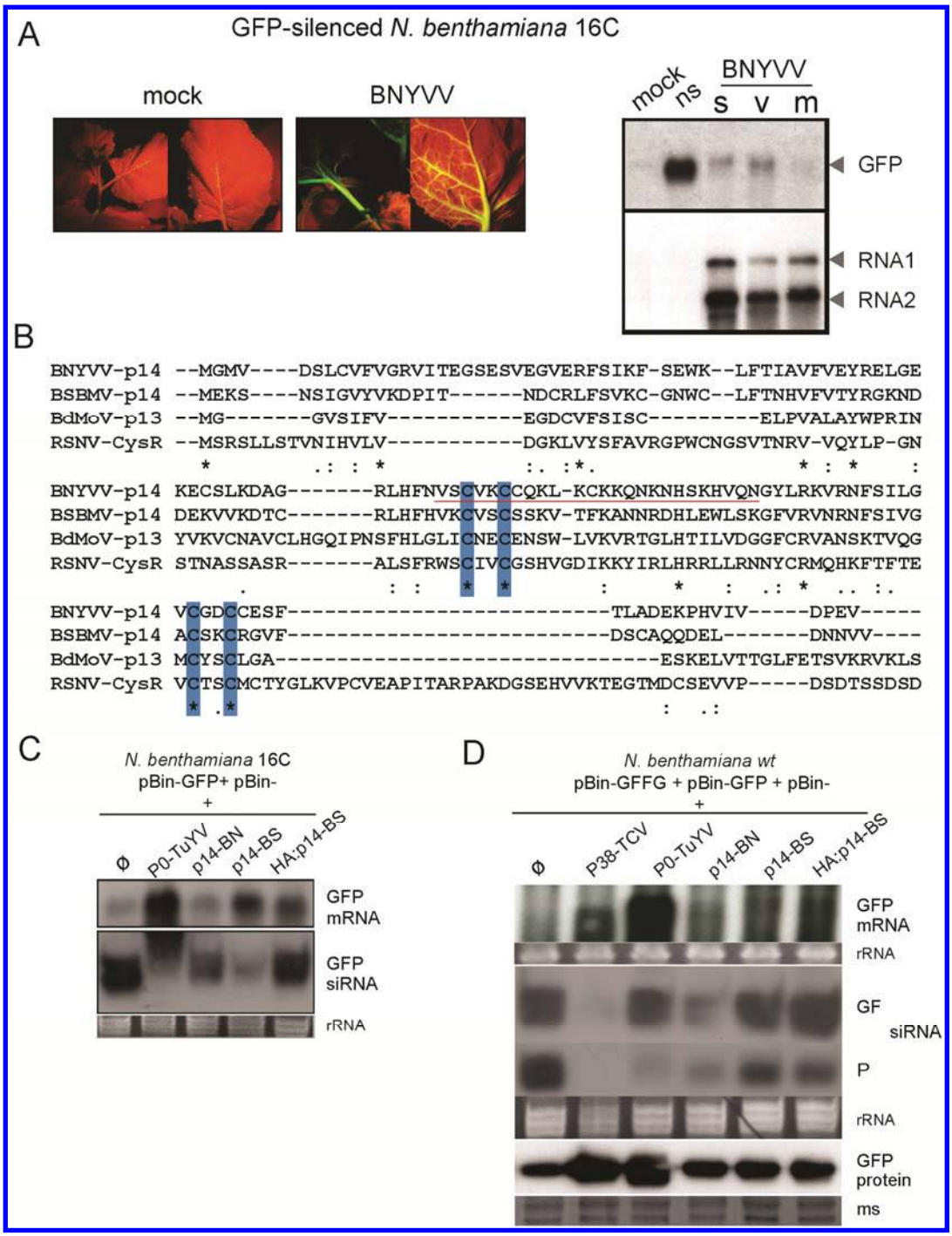

Fig. 1. Benyviruses encode cysteine-rich protein (CRP) RNA silencing suppressor. **A,** Viral suppression of established green fluorescent protein (GFP) mRNA silencing. GFP-expressing transgenic *Nicotiana benthamiana* 16C plants were agroinfiltrated with pBin-GFP to trigger RNA silencing and challenged with *Beet necrotic yellow vein virus* (BNYVV) infection (BNYVV, middle panel) or mock inoculated (mock, left panel). Photographs were taken 14 days postinoculation (dpi) under UV light in darkness (left and middle panels). Viral RNA1, RNA2. and GFP mRNA from infected plants were extracted from stem (s), vein (v), and mesophyll (m) tissues and detected with specific probes (right panel). Mesophyll tissues from nonsilenced (ns) and mock-inoculated silenced (mock) plants were analyzed in parallel. **B,** Alignment of BNYVV and *Beet soil-borne mosaic virus* (BSBMV) benyvirus CRP with those of *Burdock mottle virus* (BdMoV) and RSNV tentative members. Amino acid sequences of BNYVV-p14 (X04197), BSBMV-p14 (NC_003503), BdMoV-p13 (Dr. Hideki Kondo, *personal communication*), and RSNV-CysR (EU099845) were analyzed with MAFFT software (v.6). Blue boxes indicate conserved cystein residues, which are presumably required for the zinc-finger (Znf) structure (this article). Basic amino acid rich region, representing the BNYVV-p14 nucleolar localization signal detected by NoD software in the loop of the Znf, is underlined in red. **C,** Identification of viral suppressor of RNA silencing (VSR) activity by patch test. *N. benthamiana* 16C leaves were co-infiltrated with *Agrobacterium* spp. carrying pBin-GFP together with bacteria carrying pBin61 empty vector (Ø) or expressing *Turnip yellows virus* (TuYV) P0, BNYVV p14 (p14BN), or BSBMV p14 without or with an hemagglutinin A epitope (HA) tag (p14BS/HAp14BS). Northern blot analyses were conducted on high molecular (GFP mRNA) and low molecular (GFP small-interfering [si]RNA) weight RNAs extracted from infiltrated leaves using a GFP-specific RNA probe. **D,** Analysis of primary and secondary siRNA production. *N. benthamiana* leaves were co-infiltrated with three *Agrobacterium* mixtures, one carrying pBin-GFP, the second carrying pBin-GF-FG trigger, and the third carrying empty or VSR-expressing vectors. Northern blot analyses were conducted on RNAs extracted from infiltrated leaves. Primary siRNAs were detected using a specific probe corresponding to a sequence used as a trigger (GF) whereas secondary siRNA were detected with a probe specific to the 3′ part of the GFP messenger absent from the trigger (P). Western blot analysis of GFP was performed in parallel. Equal loadings were checked by visualization of ethidium bromide–stained total RNAs (rRNA) or membrane staining (ms).

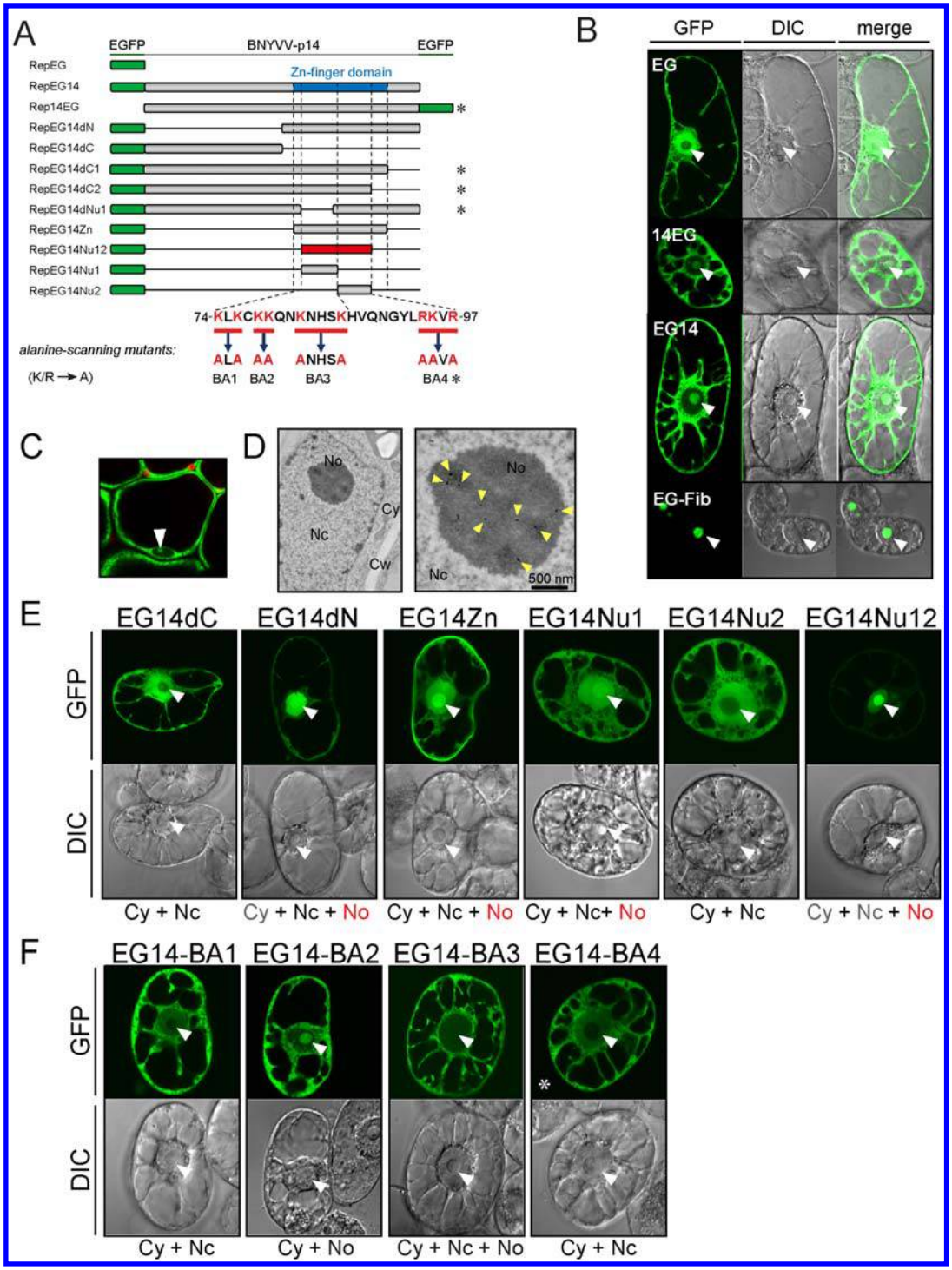

Fig. 2. Nucleolar localization signal (NoLS) is present in *Beet necrotic yellow vein virus* (BNYVV) p14 protein. **A,** Drawings of the enhanced green fluorescent protein (EGFP)-p14 fusion genes (EG14 and 14EG) cloned into the RNA3-based replicon Rep0. The green box represents the EGFP sequence whereas gray boxes correspond to the p14 sequence and its derivatives; blue and red boxes highlight the zinc-finger (Znf) domain and the basic amino acid rich sequence, respectively, displayed at the bottom of the panel (amino acids 74 to 97). The nature and the position of the mutations introduced are detailed below. Lysine or arginine residues of the NoLS were substituted with alanine residues (shown in red letters). **B,** Subcellular localization of EGFP (EG), p14-EGFP (14EG), EGFP-p14 (EG14), and EGFP-fibrillarin fusion protein (EG-Fib) in tobacco BY-2-infected protoplasts 24 h after inoculation with replicon constructs supplemented with RNA1 and RNA2. GFP fluorescence was observed under confocal laser-scanning microscopy (GFP). Differential interference contrast (DIC) images of the same cells and images merged with GFP panels (merge) are presented. White arrowheads indicate the nucleolus (B, C, E, F, and G). **C,** EGFP-p14 protein localizes similarly in mesophyll cells. RNA1+2+repEG14 were inoculated to *Chenopodium quinoa* leaf and observed 7 days postinoculation. Red dots correspond to chloroplast auto fluorescence. **D,** Immuno-labeling of nucleolar p14 in BNYVV-infected *C. quinoa* leaf cell. Ultrathin sections of BNYVV-infected *C. quinoa* leaves were treated with gold-labeled anti-p14 antiserum and subjected to electron microscopic observation. Left panel displays the nucleus (Nc) of a BNYVV-infected cell where cytoplasm (Cy) and cell wall (Cw) appear. In the enlarged right panel, yellow arrowheads indicate the presence of specific gold particles in the nucleolus compartment (No). **E,** Subcellular localization of the EGFP fused to specific domains of p14. The strong retention of EG14Nu12 fusion protein in the nucleolus demonstrates the presence of an NoLS at amino acid position 74 to 97, as shown in A. **F,** Point mutation analyses of NoLS p14 mutants. Lysine or arginine residues of the NoLS were replaced with alanine residues in the EG14 fusion sequence (see A). Subcellular localization profiles were analyzed as for B. Asterisks (A and F) indicate weak fluorescence of the constructs that rendered statistical analyses difficult for localization investigations.

RNA2. Protoplasts were observed 24 h postinoculation (hpi) under confocal laser-scanning microscopy (CLSM), as described previously (Erhardt et al. 2000). The unfused EGFP protein expressed from the replicon was distributed in the cytoplasm and nuclear compartments without reaching the nucleolus (Fig. 2B, EG). In contrast, when p14 was fused to the C-terminus of the EGFP sequence (Fig. 2A, RepEG14), the protein accumulated in the cytoplasm where it was produced and, in addition, clearly labeled the nucleolus of the infected cells (Fig. 2B, EG14). Similar results were obtained with p14 fused to the N terminus of the EGFP sequence (Fig. 2A, rep14EG; and B, 14EG) but with lower fluorescence intensities. Nucleolus labeling was visualized in parallel by the use of an EGFP-fibrillarin fusion protein (Fig. 2B, EG-Fib). Nucleolar localization was also found within *Chenopodium quinoa* leaves infected with RNA1+2+RepEG14 (Fig. 2C) and confirmed by immuno-gold labeling of the wt p14 in the nucleolus of infected cells (Fig. 2D) which was absent in noninfected cells (data not shown). EGFP-p14 fusion was chosen for further analyses in order to maintain identical translation contexts for mutagenesis experiments and efficient detection of the fluorescent cells. Using deletion mutants of the BNYVV p14 (Fig. 2A), we were able to assign the nuclear or nucleolar targeting properties to the C-terminus part of the protein (Fig. 2E, compare EG14dN and EG14dC). Furthermore, we demonstrated the existence of an NoLS within the Znf domain (Fig. 2A, RepEG14Zn) between amino acid residues 74 and 97, because the fusion protein accumulated in the nucleus and, particularly, in the nucleolus of infected cells (Fig. 2A and E, EG14Zn and EG14Nu12). The NoLS sequence was further separated into two domains (Fig. 2A, EG14Nu1 and EG14Nu2). EG14Nu1 targeted both the nucleus and nucleolus whereas EG14Nu2 was solely addressed to the nucleus (Fig. 2E, EG14Nu1 and EG14Nu2), demonstrating the requirement of the entire Nu12 domain for nucleolar targeting. In this experiment, constructs labeled with an asterisk (Fig. 2A) showed either weak fluorescence or undetectable proteins in Western blotting experiments (data not shown) and, therefore, were considered with caution for data interpretation even if their localization validated our results (e.g., EG14dNu1 was not detected in the nucleolus; data not shown).

Alanine replacement of basic residues within domain 74-97 was performed to produce p14-BA1 to p14-BA4 mutants in the RepEG14 context (Fig. 2A). All constructs were detected in infected cells; however, only EG14BA2 mutant ($KK^{78-79}AA$) behaved like EG14 (compare Fig. 2B, EG14 to F, EG14BA2) whereas the EG14BA3 mutant ($K^{82}A$-$K^{86}A$) was able to reach both the nucleus and the nucleolus; however, the fluorescence distribution appeared distinct from EG14. EG14BA1 ($K^{74}A$-$K^{76}A$) and EG14BA4 ($RK^{94-95}AA$-$R^{97}A$) mutant proteins were detected in the nucleus but no longer in the nucleolus (Fig. 2F, EG14BA1 and EG14BA4). We further analyzed the effect of single basic residue replacement on EG14 nucleolar localization. $K^{70}A$, $K^{74}A$, $K^{76}A$, $R^{94}A$, $K^{95}A$, and $K^{97}A$ individual substitutions were introduced within EG14 and subjected to CLSM observation. Only EG14$K^{97}A$ was restricted to the cytoplasm and nucleus whereas all other single mutants behaved like wt p14 (data not shown). To limit the occurrence of reversion of such single amino-acid changes during replication cycles and to take advantages of their varied localizations, p14BA1 to p14BA4 were chosen for subsequent analyses. New analyses were performed in an RNA2Δp14 background and gave the same localizations as those obtained in the presence of wt RNA2 (data not shown). Because no relocalization of the mutants was observed in the presence of wt p14 in protoplast infections, we concluded that the localizations of EGFP-p14BA1 to p14BA4 are intrinsic to the proteins.

BNYVV p14 nucleolus targeting requires a functional Znf domain.

Because the NoLS is embedded in the putative Znf domain, an alanine scanning of the BNYVV p14 cysteine residues (C/A) was conducted. Nine single mutants ($C^{8}A$, $C^{54}A$, $C^{68}A$, $C^{71}A$, $C^{72}A$, $C^{77}A$, $C^{105}A$, $C^{108}A$, and $C^{109}A$) were fused to EGFP in the RepEG14 context (Fig. 3A). Protoplasts were infected with wt RNA1 and RNA2 supplemented with the different RepEG14(C/A) mutants. EG14$C^{8}A$, EG14$C^{54}A$, EG14$C^{72}A$, EG14$C^{77}A$, and EG14$C^{109}A$ mutants behaved like EG14, localizing to the cytoplasm and the nucleolus of the infected cells (Fig. 3B). Conversely, EG14$C^{68}A$, EG14$C^{71}A$, EG14$C^{105}A$, and EG14$C^{108}A$ mutants lost their nucleolus targeting and showed weak fluorescence (Fig. 3B, asterisk in the corner of the picture). The same transcript combinations were rub inoculated to *C. quinoa* leaves. Chlorotic local lesions appeared 7 days dpi and viral components were analyzed (Fig. 3C) using Western blot (WB) and Northern blot (NB). No significant difference in viral RNA accumulation was observed (Fig. 3C, NB) and RNA2-encoded coat protein (CP) and wt p14 accumulations were comparable with the control (Fig. 3C, WB, middle and lower panels). In contrast, only EG14 mutant proteins that were still able to enter the nucleolus were easily detected on WB whereas mutants EG14$C^{68}A$, EG14$C^{71}A$, EG14$C^{105}A$, and EG14$C^{108}A$ were almost undetectable (Fig. 3C, WB).

In order to test the functionality of the above C/A substitution mutants, in vitro transcripts of each mutant were inoculated together with RNA1 and RNA2Δp14 on *C. quinoa* leaves. After 7 days, small necrotic lesions appeared on leaves inoculated with RNA1+2-Δp14 alone. Chlorotic fluorescent local spots were observed when wt EG14 or one of the five C/A mutant proteins that were still able to reach the nucleolus was expressed from the replicon RNA (data not shown), indicating their functional VSR activity (Guilley et al. 2009; Kozlowska-Makulska et al. 2010). NB and WB analyses were performed on infected tissues and the results confirmed the effective complementation provided by the EG14 proteins, with a slightly lower efficiency observed for EG14$C^{54}A$. None of the mutants affected at cysteine residues 68, 71, 105, and 108 was able to complement RNA2Δp14 and failed to form local lesions due to the lack of a functional VSR, as indicated by the absence of viral amplification detection (Fig. 3D).

Nucleolar localization is not essential for silencing suppression, but intact NoLS is required for protein stability and VSR efficiency.

Having demonstrated the essential role of the Znf for both the nucleolar addressing and the stability of the p14 protein, we checked whether the basic rich residues of the NoLS sequence were involved in the VSR activity of the protein. For this purpose, NoLS mutants were expressed in the viral context either by replacing the RNA2 p14 sequence with the mutated sequence or by providing the mutated sequence in fusion or not with the EGFP sequence via a replicon vector. When RNA1 and RNA2 mutants were inoculated to protoplasts, 1+2-BA3 RNAs accumulation was comparable with that of the wt, with a decrease of RNA1 amount, whereas other RNA2 mutants were replicated but accumulated at higher levels than the p14-defective mutant (Fig. 4A, compare RNA2 accumulation and rRNA load). When the same transcript combinations were inoculated onto *C. quinoa* leaves, only RNA2-BA1, RNA2-BA2, and RNA2-BA3 mutants induced the formation of chlorotic local lesions at a necrotic center 7 days postinoculation (dpi) (Fig. 4B, bottom lane, phenotype Cn). Conversely, RNA2Δp14 and RNA2-BA4 mutants induced small necrotic lesions (Fig. 4B, phenotype Sn) in which viral RNAs and proteins were below the limit of detection (Fig. 4B, left panel, NB

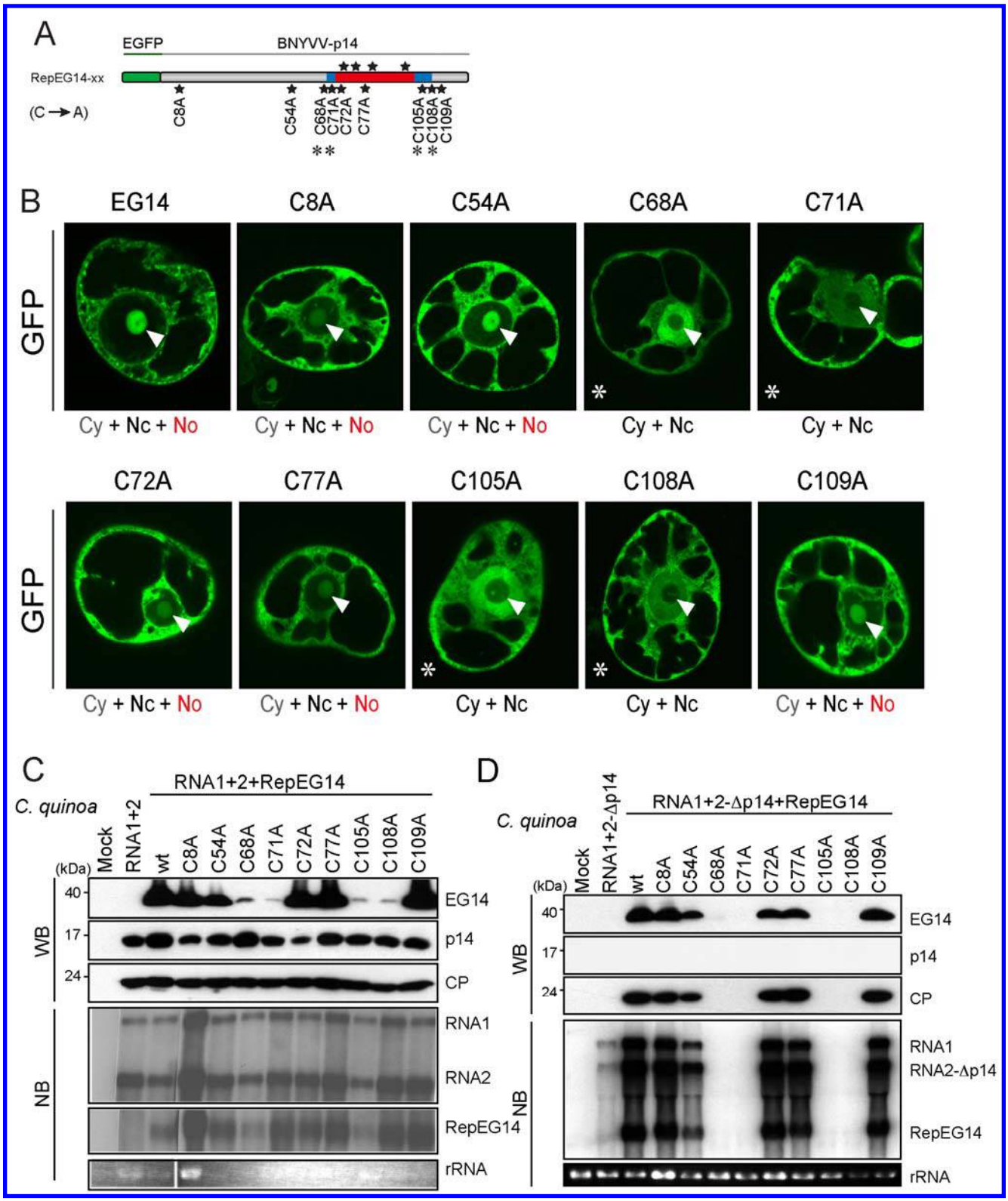

Fig. 3. Identification of the essential cysteine residues for p14 zinc-finger (Znf) folding. **A,** Drawings of the enhanced green fluorescent protein (EGFP)-p14 fusion genes. The green box represents the EGFP sequence whereas gray boxes correspond to the p14 sequence; blue and red boxes highlight the Znf domain and the nucleolar localization signal, respectively. The nature and the position of the mutations introduced are detailed. The nine cysteine residues were independently mutated into alanine in the EG14 fusion sequence and their position is indicated by stars. **B,** Four out of nine cysteine residues are essential for p14 stability and its proper localization. GFP fluorescence was observed under confocal laser-scanning microscopy (GFP). Asterisks indicate weak fluorescence of the constructs (also highlighted on A) that lost their nucleolar localization. **C,** Molecular analyses of C/A substitution mutant fate in the viral context. In vitro transcripts of RepEG14 were inoculated to *Chenopodium quinoa* leaves together with RNA1 and RNA2. Total proteins and RNAs were extracted from local lesions. The coat protein (CP), p14 protein, and EGFP-p14 fusion protein (EG14) were specifically immunodetected by Western blotting (upper panels). Viral RNA1 and -2 and replicon EG14 were detected using specific antisense riboprobes (lower panels). Ethidium bromide staining of rRNA estimated loading of the gel. **D,** Molecular analyses of C/A substitution mutant fate in the absence of wild-type (wt) p14. In vitro transcripts of RepEG14 variants were inoculated to *C. quinoa* leaves together with RNA1 and RNA2Δp14 and analyzed as in C.

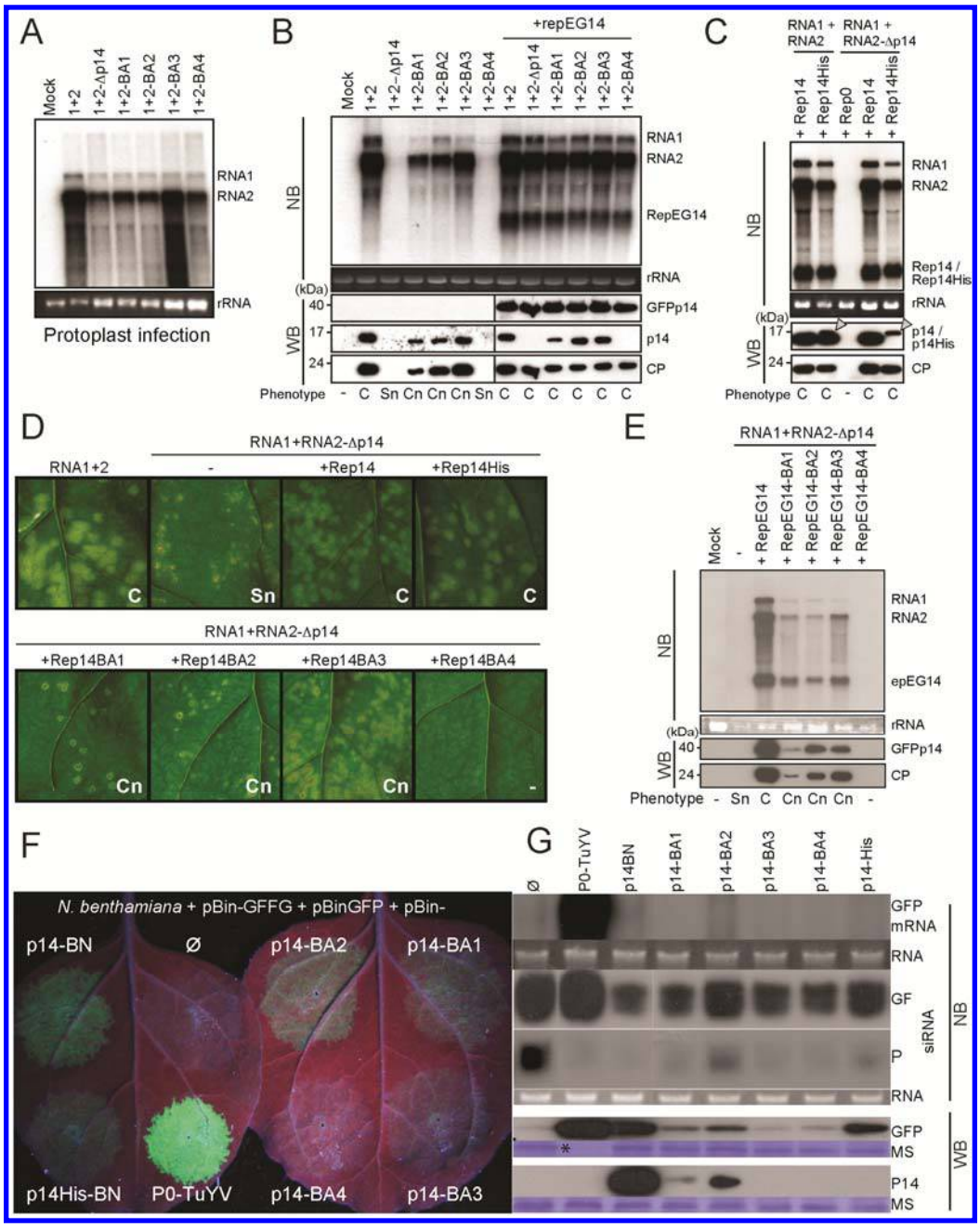

Fig. 4. Viability of p14 nucleolar localization signal (NoLS) mutant viruses and viral suppressor of RNA silencing (VSR) activity. **A,** Replication of NoLS mutants at the single-cell level. *Chenopodium quinoa* protoplasts were infected with *Beet necrotic yellow vein virus* (BNYVV) RNA1 and p14-NoLS-mutated RNA2. RNAs extracted 24 h postinoculation were subjected to Northern blot analysis using BNYVV-specific RNA probes. **B,** Virus accumulation in local lesions on *C. quinoa* leaves. RNA1 and RNA2 variants (wild type [wt]: 2; p14 defective: 2-Δp14; p14-NoLS mutants: 2-BA1 to 2-BA4) were inoculated to *C. quinoa* leaves alone or in the presence of RepEG14. Observed phenotypes of local lesions 7 days postinoculation (dpi) that were harvested are detailed at the bottom of the panels (C: chlorotic lesion; Cn: C with necrosis in the center; Sn: small necrotic lesion; –: no symptom; see D for pictures). Northern analysis was conducted using BNYVV specific riboprobes. Western blot analyses of enhanced green fluorescent protein (EGFP)-p14, p14, and coat protein (CP) in the infected foci are presented. **C,** A 10-fold less accumulation of histidine-tagged p14 (p14His) was sufficient to provide VSR function. In vitro synthesized RNA1 and RNA2 or RNA2-Δp14, with or without a replicon carrying p14His$_6$-tagged protein sequence (Rep14His), were inoculated to *C. quinoa* leaves. Total RNA and protein samples were analyzed as for B. Arrows indicate the size-elevated position of the His$_6$-tagged p14 protein. **D,** Complementation of p14-defective BNYVV with replicon expressing NoLS mutants. Comparative analysis of the *C. quinoa* local lesion phenotypes obtained 7 dpi with wt RNA1+2, RNA1+2Δp14 alone, or supplemented with replicon carrying wt-p14, His-tagged, or NoLS-mutated variants. A chlorotic local lesion phenotype implies complete complementation while Cn represents partial complementation of p14 deficient function. **E,** The p14 protein properties are not influenced by fusion with the EGFP sequence. Viral RNA and protein accumulation levels were analyzed 7 dpi from *C. quinoa* local lesions infected by RNA1+2Δp14 supplemented with RepEG14 or NoLS mutants. P14 mutants behaved similarly when fused or not to EGFP. All RNA and protein species are specified on the right of each panel. **F,** Mutations within NoLS affect VSR activity. *Nicotiana benthamiana* leaves were infiltrated with *Agrobacterium* spp. containing the *GFP* gene and GF-FG trigger together with empty binary vector (Ø) or binary vector expressing the VSR (p14-BN, p14His-BN, P0- *Turnip yellows virus* [TuYV], and p14BA mutants). Leaves were photographed under UV light 4 days postinfiltratino. **G,** Molecular analyses of GFP mRNA and primary and secondary small-interfering (si)RNAs within the patched areas. Experiments were conducted as in Figure 1D. Primary siRNAs were detected using a specific probe corresponding to sequence used as a trigger (GF) whereas secondary siRNAs were detected with a probe specific to the 3′ part of the GFP messenger absent from the trigger (P). Western blot analyses of GFP and p14 protein were performed in parallel using specific antibodies. Equal loading was checked by visualization of ethidium-bromide-stained total RNAs (rRNA) or membrane staining (ms), except for the P0-TuYV sample, which was subjected to a one-fifth dilution for GFP detection and is labeled with an asterisk.

and WB). Viral RNA1 and RNA2 as well as CP and p14 proteins were detected from 1+2-BA1-, 1+2-BA2-, and 1+2-BA3-inoculated samples, hence confirming the efficient viral amplification provided by the attenuated VSR activity of p14-NoLS mutants. The accumulations of viral RNAs and proteins were lower than that of the wt (Fig. 4B, left). This was not due to a *cis* effect of the mutations present on p14 open reading frame because all mutants were efficiently amplified when they were supplemented with a RepEG14 (Fig. 4B, right panel). In the presence of EG14 protein, the necrotic center disappeared, giving rise to full chlorotic lesions and RNA accumulation was restored in the infection foci (Fig. 4B, right). Here again, WB evidenced the instability of the p14BA4 mutant and a lower stability of p14BA1 protein as compared with wt (Fig. 4B, right, WB).

To distinguish between the effect of the NoLS mutation per se and p14 stability in VSR function, we used a C-terminal histidine-tagged p14 (p14His), which was functionally active in silencing suppression in patch test experiments (discussed below). p14His accumulated approximately 10-fold lower than the wt p14 but still efficiently complemented the p14-defective virus on *C. quinoa* leaves (Fig. 4C, CP levels; and D, chlorotic spots). Interestingly, when the empty replicon vector (Rep0) was added to inoculums deficient in p14 synthesis, no lesion appeared on the leaves (Fig. 4, compare B, 1+2-Δp14 and C, 1+2-Δp14 + Rep0), indicating a defective interfering effect of the viral vector in the absence of a functional VSR, a feature observed previously for Znf-deficient p14 mutants (Fig. 3D). To further benefit from this effect, BNYVV RNA1+2-Δp14 in vitro transcripts were inoculated on *C. quinoa* leaves together with replicon vectors expressing wt or mutated p14 proteins. Local lesions clearly appeared on the leaves 7 dpi (Fig. 4D) when p14-BA1, p14-BA2, and p14-BA3 proteins were expressed, indicating that these p14 mutants were able to complement RNA2Δp14. The complementation was more efficient for the BA3 mutant, because larger local lesions with a small necrotic center appeared, than for BA1 and BA2, because smaller chlorotic lesions were produced and harbored a necrotic center. The same results were obtained with EG14 protein expressed via the replicon because higher viral RNA amounts were found for BA1 and BA3 compared with BA2 (Fig. 4E, NB, compare RNA2, repEG14, and rRNA loads). Again, CP accumulation in BA1 was lower than in BA2 and BA3 (Fig. 4E, WB), as previously observed (Fig. 4B). This suggested that EG14 protein behaves similarly to p14 proteins in terms of complementation, symptom expression, and protein stability; these properties also apply for fused and non-fused mutants. Taken together, the partial complementation of the p14-defective virus provided by p14-BA1 to p14-BA3 supports an effect of the introduced mutations per se on the VSR activity.

To support these observations, the four NoLS mutants p14-BA1 to p14-BA4 were expressed via *Agrobacterium tumefaciens* in *N. benthamiana* 16C plants, as described before. The resulting fluorescence was comparable among the p14 mutants, rendering the interpretation difficult (Supplementary Fig. S1). Interestingly, when the same experiment was monitored 4 days postinfiltration in wt *N. benthamiana* in the presence of the pBin-GFP and the GF-FG-silencing trigger, a silencing suppression of the GFP comparable with p14His was detected for BA1 and BA2 mutants (Fig. 4F). No fluorescence was noticed for BA3 and BA4 mutants (Fig. 4F). NB detection patterns of GFP mRNA and siRNAs and protein content analyses were conducted on equally loaded RNAs and proteins (Fig. 4G). The obtained data confirmed the results presented in Figure 1 for TuYV-P0 and p14-BN. Immunodetection of the GFP within the patches reflected the low fluorescence observed for p14-BA1 and p14-BA2 mutants. Unexpectedly, GFP mRNA detection was poor for all samples expressing BNYVV VSR variants, thus rendering the silencing-suppression interpretations difficult in regard to fluorescence intensities. We noticed, however, that GFP protein levels were related to the detection level of the p14 proteins, except for p14His, which was not detected in the patches (Fig. 4G, WB). Finally, we were unable to correlate the accumulation of the GFP protein to lowered siRNAs accumulation except for wt p14. Surprisingly, the GF and P siRNA accumulations for p14-BA1, p14-BA3, and p14-BA4 were comparable with those of wt p14 in the patches (Fig. 4G, NB). Conversely, the p14His effect was comparable with p14-BA2 in term of siRNAs outcome, suggesting lower VSR effects as higher levels of siRNA were monitored (Fig. 4G), and P secondary siRNAs were detected for BA2 and p14His samples. The function of wt p14 appeared comparable between viral and patch experiments. However, the mutations introduced in the NoLS affected properties of p14 that apparently were not linked directly to its VSR activity per se; in particular, p14-BA3, which provided a significant complementation in the viral context but was unable to suppress the silencing of the *GFP* in the patches, suggesting its stabilization by another viral component.

P14 CRP self-interact.

When BNYVV p14 protein was produced in infected *C. quinoa* plants and was analyzed by WB using BNYVV-p14-specific antibodies, high molecular weight proteins corresponding to twice the size of the protein were detected. Because such a signal was not always retrieved, we performed sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) followed by Western blotting of total proteins from infected tissues that were heat denatured at 95°C for 0 to 120 min. In such conditions, the CP was detected in all samples (Fig. 5A, CP) whereas p14 amounts decreased in relation to the time of heat denaturation and had completely disappeared after 30 min of heating (Fig. 5A, p14). A band corresponding to the expected dimer form of p14 protein was detected in non-heated samples and at a lower level in the 2-min-treated samples but disappeared after extended treatments (Fig. 5A, p14-dimer). To confirm the existence of p14 self-interaction, we used the yeast two-hybrid system.

The wt p14 and NoLS mutants were fused to the DNA binding domain (BD) and activating domain (AD) of the GAL4 transcription factor in pGBKT7 and pGADT7 vectors, respectively. Yeast strains AH109 and Y187 were transformed with recombinant plasmids. After selection and mating, diploids were selected on SD-WL minimal media. Yeasts carrying vectors were then plated onto SD-WLH and analyzed for the expression of the BD-p14 and AD-p14 fusion proteins (Fig. 5B, upper panel). All p14 proteins were detected in both fusion forms, with the exception of ΔNoLS and BA2 mutants detected only in fusion with AD and BD, respectively (Fig. 5B, lower panel). Nevertheless, when yeast diploids were plated onto SD-WLH, the *His3* gene expression revealed p14 and p14BA2 self-interactions as well as p14 and p14BA2 interactions and, thus, the presence of AD-BA2 protein expression. A slower growth suggested weaker interactions between wt p14 and p14BA3 and between p14BA2 and p14BA3. BSBMV-p14 (p14BS) self-interaction was analyzed in the same way. Yeasts were transformed with the BD- and AD-p14BS fusion constructs and selected on SD-WL media, then challenged for *His3* gene expression (Fig. 5C, left panel). As for BNYVV, BSBMV p14 was interacting with itself (Fig. 5C, -WLH). However, although both proteins possess comparable domains (Fig. 1B), they were unable to interact with each other. The same results were obtained regardless of the yeast strains used.

To confirm p14BS self-interaction, FRET-FLIM experiments were designed. First, the subcellular localization of p14BS was determined under CLSM using tobacco BY-2 cells transfected with pCK plasmids expressing the p14BS sequence fused to EGFP (Fig. 5D, pCK-GFPp14BS), monomeric red fluorescent protein (mRFP) (Fig. 5D, pCK-RFPp14BS), or both (Fig. 5D, upper panel). As for BNYVV CRP, BSBMV p14 was present in the cytoplasm and the nucleolus even though no NoLS was predicted with NoD algorithm. Cells expressing both p14BS fusion proteins or the EGFP and RFP-p14BS (not shown) were analyzed by FRET-FLIM. A FRET efficiency of 9.25 ± 2 (12 cells measured) was obtained, indicating an interaction between the EGFP-p14BS donor and the RFP-p14BS acceptor.

P14 is necessary for long-distance movement.

Previous reports demonstrated cell-to-cell movement and VSR complementation of BNYVV by TMV movement protein (Lauber et al. 1998) and unrelated viral VSR (Guilley et al. 2009; Kozlowska-Makulska et al. 2010), indicating that p14

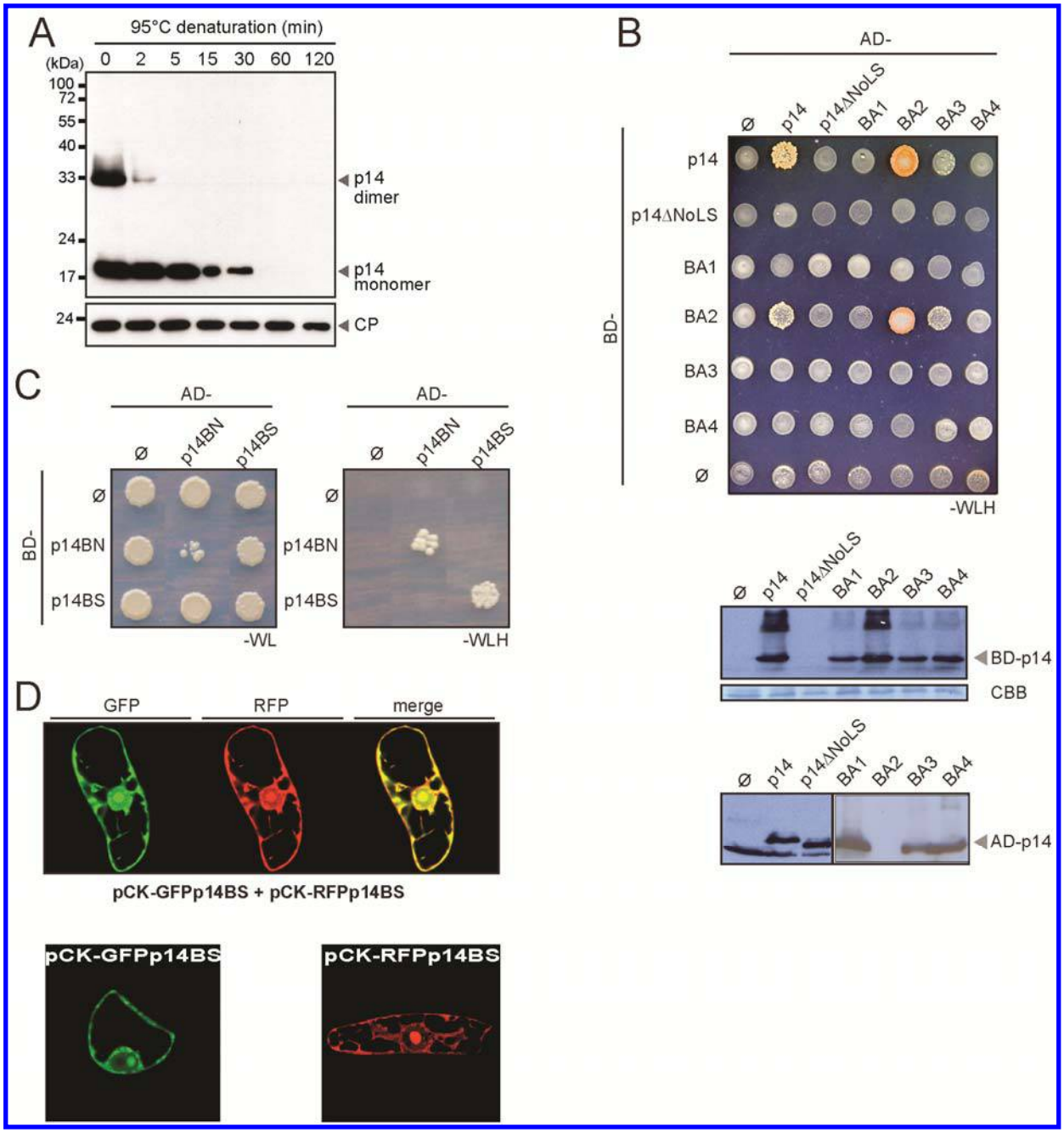

Fig. 5. Benyvirus cysteine-rich proteins (CRP) form dimers. **A,** Visualization of the thermosensitivity of p14 dimers by Western blotting of total protein extracts of *Chenopodium quinoa* local lesions infected by *Beet necrotic yellow vein virus* (BNYVV). Samples were heat treated at 95°C from 0 to 120 min. Bands corresponding to p14 and coat protein (CP) were detected by specific antisera. P14 monomer and dimer positions are indicated. Protein–protein interaction studies using **B,** yeast two-hybrid BNYVV p14 and nucleolar localization signal mutants or **C,** BNYVV and *Beet soil-borne mosaic virus* (BSBMV) p14 proteins were expressed in yeast as fusion proteins with the binding domain (BD) and activation domain (AD) of the GAL4 transcription factor. Selected recombinant yeast clones were grown on SD-WL media and then transferred onto selective SD minimal media (-WLH) to test for interaction. Expression of the fusion proteins in yeast cells was confirmed by Western analyses (B, lower panel). **D,** Subcellular localization of the BSBMV p14. Tobacco BY-2 cells were bombarded with pCK-EGFPp14BS, pCK-mRFPp14BS (lower panels), or both constructs (upper panel) to transiently express fusion proteins. FRET-FLIM interaction analyses were conducted on such co-transfected cells.

protein does not participate directly in the replication and cell-to-cell movement per se. Transcripts of RNA2 constructs carrying NoLS mutations (RNA2-BA1 to RNA2-BA4) were inoculated together with RNA1 to *N. benthamiana* plants and tested for systemic spread. The wt RNA1+2 induced a systemic infection on *N. benthamiana* 2 weeks after inoculation, as shown by viral RNA accumulation in the upper leaves (Figs. 1A and 6A). When the NoLS mutants were inoculated, only RNA2-BA1 and RNA2-BA3 were able to systemically spread in the upper leaves but the p14-BA1-expressing virus accumulated less efficiently than the wt and BA3 virus (Fig. 6A). Former tests in *C. quinoa* leaves showed that BA4 but not BA2 was unable to accumulate (Fig. 4B, left panel). WB analysis did not allow the detection of BA4 virus in the inoculated leaves of *N. benthamiana* (not shown). When a similar experiment was performed in *Beta macrocarpa* in the presence of RNA3 required for long-distance movement (Peltier et al. 2012), only the wt and BA3 mutant induced systemic symptoms (Fig. 6B) and accumulated in systemic leaves (data not shown). In this host, BA1 and BA2 were unable to move systemically. Sequencing of viral progeny in both hosts did not reveal any reversion of the introduced mutations. These results clearly illustrate the important role of BNYVV p14 in the long-distance movement of the virus.

DISCUSSION

BNYVV p14 VSR activity was demonstrated by distinct approaches (Andika et al. 2012; Dunoyer et al. 2002; Guilley et al. 2009; Kozlowska-Makulska et al. 2010; Zhang et al. 2005) and confirmed by this study. This protein acts on the silencing establishment and with a lower efficiency on established silencing in mesophyll tissues (Fig. 1A). This effect could be linked to benyvirus behavior during natural infection of sugar beet. Indeed, BNYVV infection occurs in the roots and rarely reaches the upper parts of the plant. Observation that p14 silencing-suppression activity is more efficient in roots than in leaves (Andika et al. 2005, 2012) and more efficient in vascular tissues than in mesophyll tissues is in agreement with such a trait. It is not yet known whether such tissue restriction is linked to a modulated mechanism of the p14 protein itself or to one of its as-yet-unknown partners. Because similar RNA-silencing-suppression patterns were obtained with BNYVV-p14 expressed in PVX vector (Zhang et al. 2005), the involvement of a cellular factor rather than a BNYVV product is suspected. CRP-mediated silencing-suppression activity of BNYVV was weaker than PCV p15 VSR in the viral context, which was further confirmed by the patch tests (Fig. 1) (Dunoyer et al. 2002). In an agroinfiltration test, p14 VSR activities reached a maximum at 4 days and then decreased along with p14 accumulation due to an as-yet-unknown phenomenon. Globally, the benyvirus p14 VSR were less efficient than the other VSR tested in this study. This trend was also found in the accumulation levels of 21- to 24-nucleotide siRNAs, the hallmark of a fully functional silencing pathway. In *N. benthamiana* 16C plants, benyvirus p14 proteins appear to be acting in an overall stabilization effect of GFP mRNA within 4 days without drastically affecting siRNA production when compared with the TuYV P0 protein, which targets ARGONAUTE 1 protein (Bortolamiol et al. 2007) and induces a strong reduction of the secondary siRNAs. Benyvirus p14 proteins were able to reduce the accumulation of P siRNAs and, consequently, reduced the amount of GF siRNA production. The intimate action mechanism is unknown but probably differs from the TCV VSR known to inhibit indirectly DCL4 mediated production of siRNAs (Azevedo et al. 2010; Deleris et al. 2006). Because secondary siRNAs were detected in reduced amounts, we concluded that p14 VSR act downstream of the dicer activity, probably by an interference with AGO-siRNA loading. Globally, these results are in agreement with a previous report (Zhang et al. 2005) that describes the mild effect of BNYVV p14 on the initiation of PTGS and its comparison with the nucleolar-targeted CMV 2b VSR (Diaz-Pendon and Ding 2008; Gonzalez et al. 2010). CMV 2b was recently shown to be implicated in both the inhibition of PTGS and RNA-directed DNA methylation by the sequestration of siRNAs and

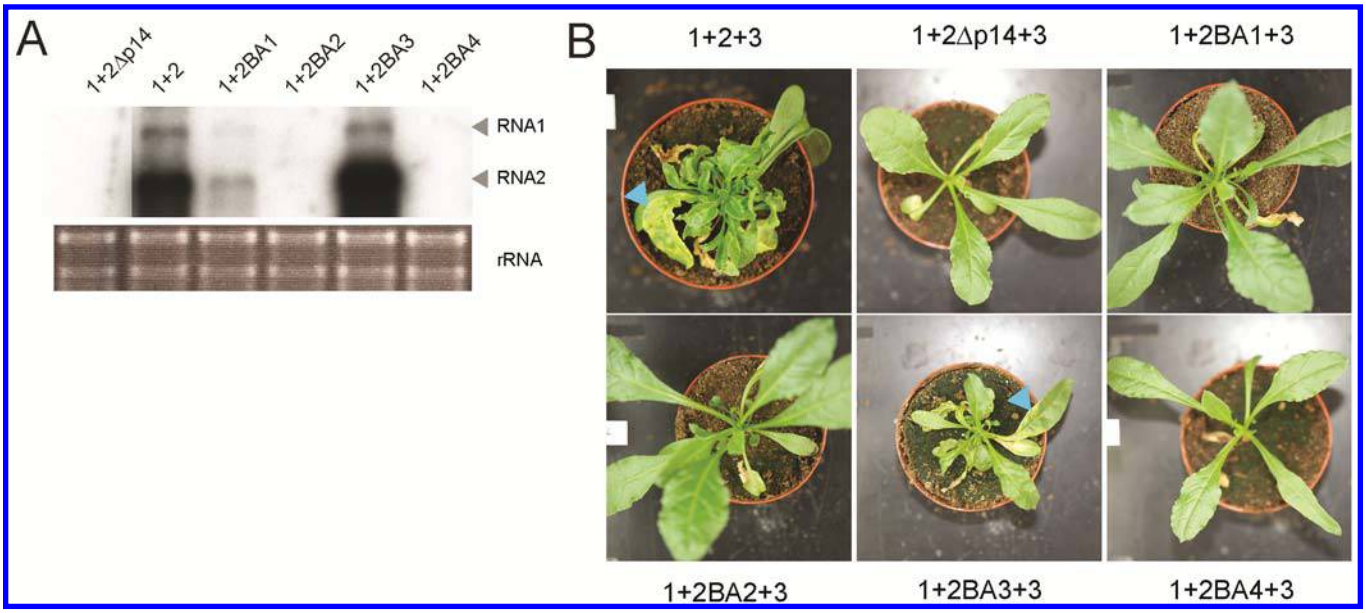

Fig. 6. Silencing-suppression deficiency affects long-distance movement of *Beet necrotic yellow vein virus* (BNYVV). **A,** Long-distance movement of BNYVV p14-nucleolar localization signal (NoLS) mutants in *Nicotiana benthamiana*. Plants were infected with in vitro transcripts of RNA1 supplemented with wild-type (wt) RNA2 or its p14-NoLS-mutated variants. Noninoculated upper leaves were harvested 15 days postinoculation (dpi) and analyzed by Northern blot using RNA1- and RNA2-specific riboprobes. Loading of RNA samples on the gel is visualized with ethidium bromide staining (rRNA) and the positions of viral RNA species are indicated on the right. **B,** Long-distance movement of BNYVV mutants in *Beta macrocarpa*. Seedlings were inoculated with the same RNA1+2 combinations as shown in A but supplemented with the RNA3 species essential for long-distance movement in this host. Plants were photographed 15 dpi. Yellowing symptoms (blue arrowheads) indicate systemic infection of wt and p14BA3 mutant viruses.

dsRNA precursors (Duan et al. 2012), an siRNA binding by CMV 2b also demonstrated by Gonzalez and associates (2012). Transgene transcriptional gene silencing inhibition by CMV 2b (Duan et al. 2012) is a property that was also proposed for BNYVV p14 protein (Zhang et al. 2005).

By focusing on the CRP and its expression in the viral context, we demonstrated the cytoplasmic and nucleolus localization of the benyvirus p14 proteins (Figs. 2B and D and 6D). Mutagenesis of basic residues confirmed the presence of the NoLS in BNYVV p14, as predicted by NoD (Scott et al. 2011), and precisely positioned it between amino acids 74 and 97. The existence of a nonpredicted NoLS within the BSBMV p14 protein was revealed by the subcellular distribution pattern, identical to BNYVV p14 localization (Fig. 5D). Furthermore, we were able to identify the four essential cystein residues involved in the Znf formation that are conserved among benyvirus CRP (Fig. 1) and other known CRP (Koonin et al. 1991).

Except for the p14EGFP construct, histidine-tagged, EGFP-p14 fusion protein and wt p14 expressed via the replicon vector were proven to be functional because they complemented efficiently an RNA2Δp14 mutant, leading to chlorotic local lesions and elevated virus multiplication levels similar to the wt. Therefore, because EG14 behaved like p14 and all the EGFP-p14BA variants behaved similarly to p14BA mutants, we conclude that the N-terminus-EGFP tag does not affect the intrinsic properties of p14 whereas the C-terminus fusion does.

Because all BNYVV p14 Znf and NoLS mutants replicated efficiently in protoplasts, the mutations introduced in the RNA2 p14 cistron did not interfere in *cis* with viral replication. Mutations affecting Znf folding decreased the stability of the corresponding EG14C^{68}A, EG14C^{71}A, EG14C^{105}A, and EG14C^{108}A proteins. Similarly, we found a severe destabilization of the p14BA4 protein that was not detected even in the presence of wt p14, indicating the essential role of residues 94 to 97 and, particularly, R^{97} for its stability. BA1 to BA3 mutations resulted in only a partial destabilization of the proteins, which still possessed a residual silencing-suppression activity. This lower VSR activity was not due to the reduced amount of protein accumulation, as shown by the use of p14His, which conserved VSR activity despite its lower accumulation. This allows us to uncouple VSR activity and protein accumulation level. However, the comparison between patch test VSR assays with infection tests highlighted a possible stabilization of p14 protein in the viral context. The nature of such synergistic effect will require further investigation. Thus, the lower RNA accumulation of the NoLS-mutated p14BA1, p14BA2, and, to a lesser extent, p14BA3 (Fig. 4B) is linked to a decreased VSR activity. When wt EG14 or p14 protein were used in complementation tests, the necrotic centers disappeared from the lesions, indicating a link between necrosis and the absence (RNA1+2Δp14) or disappearance of the VSR activity due to instability of the proteins at a later stage of infection (in the center of local lesions) and after 5 days post-agroinfiltration (data not shown).

Using biochemical and genetic tests, both benyvirus p14 proteins were shown to specifically form dimers (Fig. 5A and C). Only strongly self-interacting proteins were able to reach the nucleolus (p14 and p14-BA2), suggesting the requirement of a dimer formation for an efficient nucleolar targeting because nucleus- or nucleolus-distributing EG14-BA3 could interact with p14 and p14-BA2 but not with itself. EG14-BA1 and EG14-BA4 proteins merely diffused into the nucleus without reaching the nucleolus. The p14 nucleolus targeting also requires a functional Znf domain, suggesting the role of the Znf in both dimerization and protein stability. This highlights the outstanding importance of the ZnF-embedded NoLS stretch because some of the basic residues in NoLS are involved in the intrinsic properties of the p14 protein, as discussed above. Interestingly, the BdMoV p13 protein that efficiently complemented BNYVV-Δp14 virus (Guilley et al. 2009) accumulated in the nucleus but never reached the nucleolus of the infected cells (this study; data not shown) and no NoLS was predicted with NoD algorithm. Taken together, these results allow us to conclude that p14 silencing-suppression activity requires a functional Znf domain and NoLS basic rich residues but not the nucleolar localization of the protein.

The nucleolus gathers various functions from ribosome biogenesis in response against cellular stresses that are tightly regulated (Emmott and Hiscox 2009) and, therefore, are the target of viral pathogenesis factors (Hiscox 2007; Hiscox et al. 2010). Cytoplasmic replicative Nidovirales members such as *Arterivirus* and *Coronavirus* spp. require nucleolar targeting of their nucleocapsid protein (N) for efficient replication (Lee et al. 2006; Pei et al. 2008) that also involve interaction of the N proteins with fibrillarin (Yoo et al. 2003) or nucleolin (Chen et al. 2002), respectively. Some plant viruses are reported to produce proteins that similarly localize to the nucleolus and may influence viral outcome (e.g., *Alfalfa mosaic virus* CP whose NoLS motif appears important for both positive-strand accumulation and viral movement) (Herranz et al. 2012). In the case of *Ourmiavirus* spp., viral CP was also shown to localize in the nucleolus (Crivelli et al. 2011) but the incidence of such localization on the viral cycle has not been investigated. Another example is the *Beet black scorch virus* P7a movement protein that possesses basic residues crucial for nuclear and nucleolar localizations and virus infection (Wang et al. 2012).

As stated before, our experiments and previous studies rule out the direct involvement of p14 in the replication and cell-to-cell movement of BNYVV (Guilley et al. 2009). However, umbraviruses use fibrillarin and Cajal bodies for efficient long-distance spread within the plant host (Kim et al. 2007a and b). This leads to the important issue addressed in this report. We showed a relationship between VSR activity and BNYVV long-distance movement. By replacing p14 of RNA2 by the NoLS p14 mutants, we were able to show unambiguously that p14 protein is involved in long-distance movement. When inoculated to *N. benthamiana* plants, efficient viral systemic movement clearly occurred in the presence of RNA1 and RNA2-BA3. Although viral products were detected at a lower detection limit, BA1 was able to spread in the upper part of the plant at a decreased efficiency that could be linked to the weaker CP accumulation observed previously. When tested on *B. macrocarpa*, only p14-BA3 mutant was able to promote systemic movement of the virus, suggesting some cooperative effect of viral products on the p14 VSR that could not occur in agroinfiltration assays. In conclusion, p14 is essential for an efficient spread of BNYVV that appears unrelated to the nucleolar localization or to the dimer formation of p14. Thus, p14 CRP properties mark up a difference with the CMV 2b protein. It is not yet known which p14-nucleolar partners are recruited in the nucleolus during the viral cycle or for which purpose. Further proteomic studies, including pull-down analyses of the p14 protein partners obtained from nucleoli purified from BNYVV-infected cells, will provide some clues about the targets of the p14 protein.

MATERIALS AND METHODS

Silencing suppression in GFP-silenced *N. benthamiana* 16C by virus infection.

GFP-silenced plants were prepared by infiltration of *A. tumefaciens* C58 carrying pBin-GFP to expanded leaves of *N. benthamiana* 16C, as previously described (Voinnet et al. 1998). BNYVV was inoculated to a noninfiltrated leaf after 20 days.

Recovery of GFP expression was monitored by the observation of fluorescence under UV light and photographed 2 weeks after inoculation.

Agroinfiltration of *N. benthamiana*.

Agrobacteria were grown overnight at 28°C in Luria-Bertani medium supplemented with kanamycin (100 g/ml) and rifampicin (50 g/ml). Cells were centrifuged, washed, and resuspended to an optical density of 0.6 in 10 mM $MgCl_2$ and 200 M acetosyringone. Patch test and primary and secondary siRNA detection were performed as described (Bortolamiol et al. 2007; Kozlowska-Makulska et al. 2010). Briefly, leaves of *N. benthamiana* wt and 16C were agroinfiltrated with a mixture of *A. tumefaciens* cells (strain GV3101) containing pBin61 binary vectors carrying no VSR (–), TuYV P0 (Pfeffer et al. 2002), TCV p38 (Deleris et al. 2006), or BNYVV p14; or p14His, BSBMV p14, or HAp14 GFP and GF-FG hairpin sequences.

The binary vector pBin-GFP and pBin-GF-FG constructs were described elsewhere (Himber et al. 2003). BNYVV p14 and p14His were polymerase chain reaction (PCR) amplified with specific primers and cloned into pBin61 using the *Sma*I site. BSBMV p14 and p14HA amplicons were obtained from reverse-transcribed cDNA of the BSBMV MRM infectious isolate (Ratti et al. 2009) using specific primer sets. Fragments were digested with *Nco*I and *Bam*HI, filled in using Klenow (Promega France SARL, Charbonniéres-les-Bains, France), and introduced into the pBin61 *Sma*I site to produce pBin-p14BS and pBin-HA14BS. Restriction analyses and DNA sequencing validated all clones.

BNYVV infectious clones expressing wt and modified benyvirus CRP.

The full-length BNYVV clones for RNA1 and RNA2 (pB15 and pB2-14) were used as previously described (Hehn et al. 1995; Quillet et al. 1989). The p14 mutant RNA2 clones were constructed based on pB2-14 by the replacement of the region encompassing the p14 gene, between *Xba*I and *Stu*I sites. A stop codon was introduced in frame to obtain RNA2Δp14 (pB2-3722) (Hehn et al. 1995; Kozlowska-Makulska et al. 2010). All point mutations and some deletions were obtained by overlapping PCR-based mutagenesis. Sequence replacements for p14-BA mutants (pB2-BA1 to pB2-4) were conducted similarly. The RNA-3-based replicon vector, pRep0, was used as an expression vector for desired proteins (Schmidlin et al. 2005; Vetter et al. 2004). The genes coding for BNYVV p14 and its variants were inserted into pRep0 within the *Bam*HI site (pRep14, pRep14-BA1 to pRep14-4, and pRep14His). The pRepEGFP clone (Vetter et al. 2004) was used to produce BSBMV-p14-expressing replicons pRep14BS and pRepHA-14BS by replacing the EGFP *Nco*I and *Bam*HI fragment with p14BS- or HA-p14BS-digested amplicons. All pRepEGFP-p14 fusions were generated by the introduction of the p14 sequences within *Xma*I and *Bam*HI restriction sites (pRepEG14 and variants). The p14-EGFP construct was obtained by the replacement of the BNYVV p42 sequence within Rep42EGFP (Erhardt et al. 2000) by the p14 sequence using *Nco*I and *Xma*I restriction enzymes. All clones were validated by both restriction analyses and DNA sequencing.

In vitro transcription and infection procedures.

Full-length infectious clones of BNYVV and derivatives were transcribed in vitro as described previously (Hehn et al. 1995; Kozlowska-Makulska et al. 2010; Quillet et al. 1989; Schmidlin et al. 2005; Valentin et al. 2005) and served to infect *C. quinoa*, *N. benthamiana*, and *B. macrocarpa* leaves and BY-2 protoplasts using the usual procedures (Guilley et al. 2009; Klein et al. 2007; Rahim et al. 2007; Ratti et al. 2009; Valentin et al. 2005).

RNA analyses.

Agroinfiltrated patches or virus-infected tissues (pool of three local lesions or systemic leaf) were subjected to RNA extraction using TRIzol reagent following the manufacturer's recommendations. Protoplast RNA contents were purified using Polysomes buffer and phenol extraction followed by ethanol precipitation (Gilmer et al. 1992; Hehn et al. 1995). NB experiments were conducted as described previously using 12 g of total RNA for siRNA analyses, 5 g of total RNA for GFP mRNA detection, and the equivalent of one *C. quinoa* local lesion content (Bortolamiol et al. 2007; Guilley et al. 2009; Klein et al. 2007; Kozlowska-Makulska et al. 2010). When possible, RNA samples were adjusted at the same concentration and visualized on ethidium bromide–stained agarose gels. Specific ^{32}P-radiolabeled GFP or "GF" and "P" cDNA probes were used for hybridization of high and low molecular weight RNAs, respectively, as described (Himber et al. 2003). Detection of viral RNAs was performed as already described (Klein et al. 2007).

Protein analyses.

Agroinfiltrated leaves, systemic leaves, and pools of three local lesions were subjected to protein extraction using Laemmli buffer (Laemmli 1970), as described previously (Klein et al. 2007; Kozlowska-Makulska et al. 2010). Total protein extracts were heat denaturated and subjected to SDS-PAGE separation followed by the transfer onto Immobilon membranes. Immunodetections were performed as already described (Klein et al. 2007).

Yeast two-hybrid.

Experiments were performed as described previously (Klein et al. 2007) following the Clontech manufacturer's recommendations. Benyvirus CRP bait and prey sequences were cloned in frame with GAL4 BD (pGBKT7) and AD (pGADT7) using *Eco*RI or *Xma*I and *Sal*I or *Xho*I restriction sites, respectively.

BY-2 transient expression, CLSM, and FRET-FLIM analyses.

BSBMV p14 sequence was amplified with a specific set of primers. The amplicon was digested with *Xma*I/*Xba*I and inserted in the pCK-EGFP and pCK-mRFP vectors using the same restriction sites. EGFP and mRFP fusion proteins (GFPp14BS and RFPp14BS) were transiently expressed and visualized in BY-2 tobacco cells as described (Vetter et al. 2004). Lifetimes of EGFP-p14BS fluorescence were measured in the presence or absence of the mRFP-p14BS construct after 24 h by using the LIFA frequency domain fluorescence lifetime imaging system (Lambert Instruments, Roden, The Netherlands).

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LITERATURE CITED

Andika, I. B., Kondo, H., and Tamada, T. 2005. Evidence that RNA silencing-mediated resistance to beet necrotic yellow vein virus is less effective in roots than in leaves. Mol. Plant-Microbe Interact. 18:194-204.

Andika, I. B., Kondo, H., Nishiguchi, M., and Tamada, T. 2012. The cysteine-rich proteins of Beet necrotic yellow vein virus and Tobacco rattle virus contribute to efficient suppression of silencing in roots. J. Gen. Virol. 93:1841-1850.

Angell, S. M., and Baulcombe, D. C. 1997. Consistent gene silencing in transgenic plants expressing a replicating potato virus X RNA. EMBO (Eur. Mol. Biol. Organ.) J. 16:3675-3684.

Azevedo, J., Garcia, D., Pontier, D., Ohnesorge, S., Yu, A., Garcia, S., Braun, L., Bergdoll, M., Hakimi, M. A., Lagrange, T., and Voinnet, O. 2010. Argonaute quenching and global changes in dicer homeostasis caused by a pathogen-encoded GW repeat protein. Genes Dev. 24:904-915.

Baltimore, D. 1971. Expression of animal virus genomes. Bacteriol. Rev. 35:235-241.

Bortolamiol, D., Pazhouhandeh, M., Marrocco, K., Genschik, P., and Ziegler-Graff, V. 2007. The Polerovirus F box protein P0 targets ARGONAUTE1 to suppress RNA silencing. Curr. Biol. 17:1615-1621.

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

Brigneti, G., Martin-Hernandez, A. M., Jin, H., Chen, J., Baulcombe, D. C., Baker, B., and Jones, J. D. 2004. Virus-induced gene silencing in Solanum species. Plant J. 39:264-272.

Burgyan, J., and Havelda, Z. 2011. Viral suppressors of RNA silencing. Trends Plant Sci. 16:265-272.

Chen, H., Wurm, T., Britton, P., Brooks, G., and Hiscox, J.A. 2002. Interaction of the coronavirus nucleoprotein with nucleolar antigens and the host cell. J. Virol. 76:5233-5250.

Chiba, S., Kondo, H., Miyanishi, M., Andika, I. B., Han, C., and Tamada, T. 2011. The evolutionary history of *Beet necrotic yellow vein virus* deduced from genetic variation, geographical origin and spread, and the breaking of host resistance. Mol. Plant-Microbe Interact. 24:207-218.

Crivelli, G., Ciuffo, M., Genre, A., Masenga, V., and Turina, M. 2011. Reverse genetic analysis of Ourmiaviruses reveals the nucleolar localization of the coat protein in *Nicotiana benthamiana* and unusual requirements for virion formation. J. Virol. 85:5091-5104.

Deleris, A., Gallego-Bartolome, J., Bao, J., Kasschau, K. D., Carrington, J. C., and Voinnet, O. 2006. Hierarchical action and inhibition of plant dicer-like proteins in antiviral defense. Science 313:68-71.

Diaz-Pendon, J. A., and Ding, S. W. 2008. Direct and indirect roles of viral suppressors of RNA silencing in pathogenesis. Annu. Rev. Phytopathol. 46:303-326.

Ding, S. W. 2010. RNA-based antiviral immunity. Nat. Rev. Immunol. 10:632-644.

Ding, S. W., and Voinnet, O. 2007. Antiviral immunity directed by small RNAs. Cell 130:413-426.

Duan, C. G., Fang, Y. Y., Zhou, B. J., Zhao, J. H., Hou, W. N., Zhu, H., Ding, S. W., and Guo, H. S. 2012. Suppression of *Arabidopsis* ARGONAUTE1-mediated slicing, transgene-induced RNA silencing, and DNA methylation by distinct domains of the *Cucumber mosaic virus* 2b protein. Plant Cell 24:259-274.

Dunoyer, P., Pfeffer, S., Fritsch, C., Hemmer, O., Voinnet, O., and Richards, K. E. 2002. Identification, subcellular localization and some properties of a cysteine-rich suppressor of gene silencing encoded by peanut clump virus. Plant J. 29:555-567.

Dunoyer, P., Lecellier, C. H., Parizotto, E. A., Himber, C., and Voinnet, O. 2004. Probing the microRNA and small interfering RNA pathways with virus-encoded suppressors of RNA silencing. Plant Cell 16:1235-1250.

Emmott, E., and Hiscox, J. A. 2009. Nucleolar targeting: The hub of the matter. EMBO (EUR. MOL. BIOL. ORGAN.) Rep. 10:231-238.

Erhardt, M., Morant, M., Ritzenthaler, C., Stussi-Garaud, C., Guilley, H., Richards, K., Jonard, G., Bouzoubaa, S., and Gilmer, D. 2000. P42 movement protein of Beet necrotic yellow vein virus is targeted by the movement proteins P13 and P15 to punctate bodies associated with plasmodesmata. Mol. Plant-Microbe Interact. 13:520-528.

Gilmer, D., and Ratti, C. 2012. Benyvirus. Pages 1133-1138 in: Virus Taxonomy: Classification and Nomenclature of Viruses: Ninth Report of the International Committee on Taxonomy of Viruses. A. M. Q. King, M. J. Adams, E. B. Carstens, and E. J. Lefkowitz, eds. Elsevier, San Diego, CA, U.S.A.

Gilmer, D., Bouzoubaa, S., Hehn, A., Guilley, H., Richards, K., and Jonard, G. 1992. Efficient cell-to-cell movement of beet necrotic yellow vein virus requires 3′ proximal genes located on RNA 2. Virology 189:40-47.

Gonzalez, I., Martinez, L., Rakitina, D. V., Lewsey, M. G., Atencio, F.A., Llave, C., Kalinina, N. O., Carr, J. P., Palukaitis, P., and Canto, T. 2010. *Cucumber mosaic virus* 2b protein subcellular targets and interactions: Their significance to RNA silencing suppressor activity. Mol. Plant-Microbe Interact. 23:294-303.

Gonzalez, I., Rakitina, D., Semashko, M., Taliansky, M., Praveen, S., Palukaitis, P., Carr, J. P., Kalinina, N., and Canto, T. 2012. RNA binding is more critical to the suppression of silencing function of Cucumber mosaic virus 2b protein than nuclear localization. RNA 18:771-782.

Guilley, H., Bortolamiol, D., Jonard, G., Bouzoubaa, S., and Ziegler-Graff, V. 2009. Rapid screening of RNA silencing suppressors by using a recombinant virus derived from beet necrotic yellow vein virus. J. Gen. Virol. 90:2536-2541.

Hehn, A., Bouzoubaa, S., Bate, N., Twell, D., Marbach, J., Richards, K., Guilley, H., and Jonard, G. 1995. The small cysteine-rich protein P14 of beet necrotic yellow vein virus regulates accumulation of RNA 2 *in cis* and coat protein *in trans*. Virology 210:73-81.

Herranz, M. C., Pallas, V., and Aparicio, F. 2012. Multifunctional roles for the N-terminal basic motif of *Alfalfa mosaic virus* coat protein: Nucleolar/cytoplasmic shuttling, modulation of RNA-binding activity, and virion formation. Mol. Plant-Microbe Interact. 25:1093-1103.

Himber, C., Dunoyer, P., Moissiard, G., Ritzenthaler, C., and Voinnet, O. 2003. Transitivity-dependent and -independent cell-to-cell movement of RNA silencing. EMBO (Eur. Mol. Biol. Organ.) J. 22:4523-4533.

Hiscox, J. A. 2007. RNA viruses: Hijacking the dynamic nucleolus. Nat. Rev. Microbiol. 5:119-127.

Hiscox, J. A., Whitehouse, A., and Matthews, D. A. 2010. Nucleolar proteomics and viral infection. Proteomics 10:4077-4086.

Jupin, I., Richards, K., Jonard, G., Guilley, H., and Pleij, C. W. 1990. Mapping sequences required for productive replication of beet necrotic yellow vein virus RNA 3. Virology 178:273-280.

Katoh, K., and Toh, H. 2008. Recent developments in the MAFFT multiple sequence alignment program. Brief. Bioinf. 9:286-298.

Kim, S. H., Macfarlane, S., Kalinina, N. O., Rakitina, D. V., Ryabov, E. V., Gillespie, T., Haupt, S., Brown, J. W., and Taliansky, M. 2007a. Interaction of a plant virus-encoded protein with the major nucleolar protein fibrillarin is required for systemic virus infection. Proc. Natl. Acad. Sci. U.S.A. 104:11115-11120.

Kim, S. H., Ryabov, E. V., Kalinina, N. O., Rakitina, D. V., Gillespie, T., MacFarlane, S., Haupt, S., Brown, J. W., and Taliansky, M. 2007b. Cajal bodies and the nucleolus are required for a plant virus systemic infection. EMBO (Eur. Mol. Biol. Organ.) J. 26:2169-2179.

Klein, E., Link, D., Schirmer, A., Erhardt, M., and Gilmer, D. 2007. Sequence variation within *Beet necrotic yellow vein virus* p25 protein influences its oligomerization and isolate pathogenicity on *Tetragonia expansa*. Virus Res. 126:53-61.

Koonin, E. V., Boyko, V. P., and Dolja, V. V. 1991. Small cysteine-rich proteins of different groups of plant RNA viruses are related to different families of nucleic acid-binding proteins. Virology 181:395-398.

Kozlowska-Makulska, A., Guilley, H., Szyndel, M. S., Beuve, M., Lemaire, O., Herrbach, E., and Bouzoubaa, S. 2010. P0 proteins of European beet-infecting poleroviruses display variable RNA silencing suppression activity. J. Gen. Virol. 91:1082-1091.

Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680-685.

Lauber, E., Bleykasten-Grosshans, C., Erhardt, M., Bouzoubaa, S., Jonard, G., Richards, K.E., and Guilley, H. 1998. Cell-to-cell movement of beet necrotic yellow vein virus: I. Heterologous complementation experiments provide evidence for specific interactions among the triple gene block proteins. Mol. Plant-Microbe Interact. 11:618-625.

Lee, C., Hodgins, D., Calvert, J. G., Welch, S. K., Jolie, R., and Yoo, D. 2006. Mutations within the nuclear localization signal of the porcine reproductive and respiratory syndrome virus nucleocapsid protein attenuate virus replication. Virology 346:238-250.

Li, F., and Ding, S. W. 2006. Virus counterdefense: Diverse strategies for evading the RNA-silencing immunity. Annu. Rev. Microbiol. 60:503-531.

Lozano, I., and Morales, F. 2009. Molecular characterisation of *Rice stripe necrosis virus* as a new species of the genus *Benyvirus*. Eur. J. Plant Pathol. 124:673-680.

Moissiard, G., and Voinnet, O. 2006. RNA silencing of host transcripts by Cauliflower mosaic virus requires coordinated action of the four *Arabidopsis* dicer-like proteins. Proc. Natl. Acad. Sci. U.S.A. 103:19593-19598.

Moissiard, G., Parizotto, E. A., Himber, C., and Voinnet, O. 2007. Transitivity in *Arabidopsis* can be primed, requires the redundant action of the antiviral dicer-like 4 and dicer-like 2, and is compromised by viral-encoded suppressor proteins. RNA 13:1268-1278.

Niesbach-Klosgen, U., Guilley, H., Jonard, G., and Richards, K. 1990. Immunodetection in vivo of beet necrotic yellow vein virus-encoded proteins. Virology 178:52-61.

Pei, Y., Hodgins, D. C., Lee, C., Calvert, J. G., Welch, S. K., Jolie, R., Keith, M., and Yoo, D. 2008. Functional mapping of the porcine reproductive and respiratory syndrome virus capsid protein nuclear localization signal and its pathogenic association. Virus Res. 135:107-114.

Peltier, C., Hleibieh, K., Thiel, H., Klein, E., Bragard, C., and Gilmer, D. 2008. Molecular biology of the *Beet necrotic yellow vein virus*. Plant Viruses 2:14-24.

Peltier, C., Klein, E., Hleibieh, K., D'Alonzo, M., Hammann, P., Bouzoubaa, S., Ratti, C., and Gilmer, D. 2012. *Beet necrotic yellow vein virus* subgenomic RNA3 is a cleavage product leading to stable non-coding RNA required for long-distance movement. J. Gen. Virol. 93:1093-1102.

Pfeffer, S., Dunoyer, P., Heim, F., Richards, K. E., Jonard, G., and Ziegler-Graff, V. 2002. P0 of *Beet western yellows virus* is a suppressor of post-transcriptional gene silencing. J. Virol. 76:6815-6824.

Quillet, L., Guilley, H., Jonard, G., and Richards, K. 1989. In vitro synthesis of biologically active beet necrotic yellow vein virus RNA. Virology 172:293-301.

Rahim, M. D., Andika, I. B., Han, C., Kondo, H., and Tamada, T. 2007. RNA4-encoded p31 of *Beet necrotic yellow vein virus* is involved in efficient vector transmission, symptom severity and silencing suppression in roots. J. Gen. Virol. 88:1611-1619.

Ratcliff, F., Martin-Hernandez, A. M., and Baulcombe, D. C. 2001. Technical advance. *Tobacco rattle virus* as a vector for analysis of gene function by silencing. Plant J. 25:237-245.

Ratti, C., Hleibieh, K., Bianchi, L., Schirmer, A., Autonell, C. R., and Gilmer, D. 2009. Beet soil-borne mosaic virus RNA-3 is replicated and encapsidated in the presence of BNYVV RNA-1 and -2 and allows long distance movement in *Beta macrocarpa*. Virology 385:392-399.

Ruiz-Ferrer, V., and Voinnet, O. 2007. Viral suppression of RNA silencing: 2b wins the Golden Fleece by defeating Argonaute. Bioessays 29:319-323.

Schirmer, A., Link, D., Cognat, V., Moury, B., Beuve, M., Meunier, A., Bragard, C., Gilmer, D., and Lemaire, O. 2005. Phylogenetic analysis of isolates of *Beet necrotic yellow vein virus* collected worldwide. J. Gen. Virol. 86:2897-2911.

Schmidlin, L., Link, D., Mutterer, J., Guilley, H., and Gilmer, D. 2005. Use of a *Beet necrotic yellow vein virus* RNA-5-derived replicon as a new tool for gene expression. J. Gen. Virol. 86:463-467.

Scott, M. S., Troshin, P.V., and Barton, G. J. 2011. NoD: A nucleolar localization sequence detector for eukaryotic and viral proteins. BMC Bioinformatics 12:317.

Valentin, C., Dunoyer, P., Vetter, G., Schalk, C., Dietrich, A., and Bouzoubaa, S. 2005. Molecular basis for mitochondrial localization of viral particles during beet necrotic yellow vein virus infection. J. Virol. 79:9991-10002.

Vetter, G., Hily, J. M., Klein, E., Schmidlin, L., Haas, M., Merkle, T., and Gilmer, D. 2004. Nucleo-cytoplasmic shuttling of the *Beet necrotic yellow vein virus* RNA-3-encoded p25 protein. J. Gen. Virol. 85:2459-2469.

Voinnet, O. 2001. RNA silencing as a plant immune system against viruses. Trends Genet. 17:449-459.

Voinnet, O. 2005. Induction and suppression of RNA silencing: Insights from viral infections. Nat Rev Genet 6:206-220.

Voinnet, O. 2008. Post-transcriptional RNA silencing in plant-microbe interactions: A touch of robustness and versatility. Curr. Opin. Plant Biol. 11:464-470.

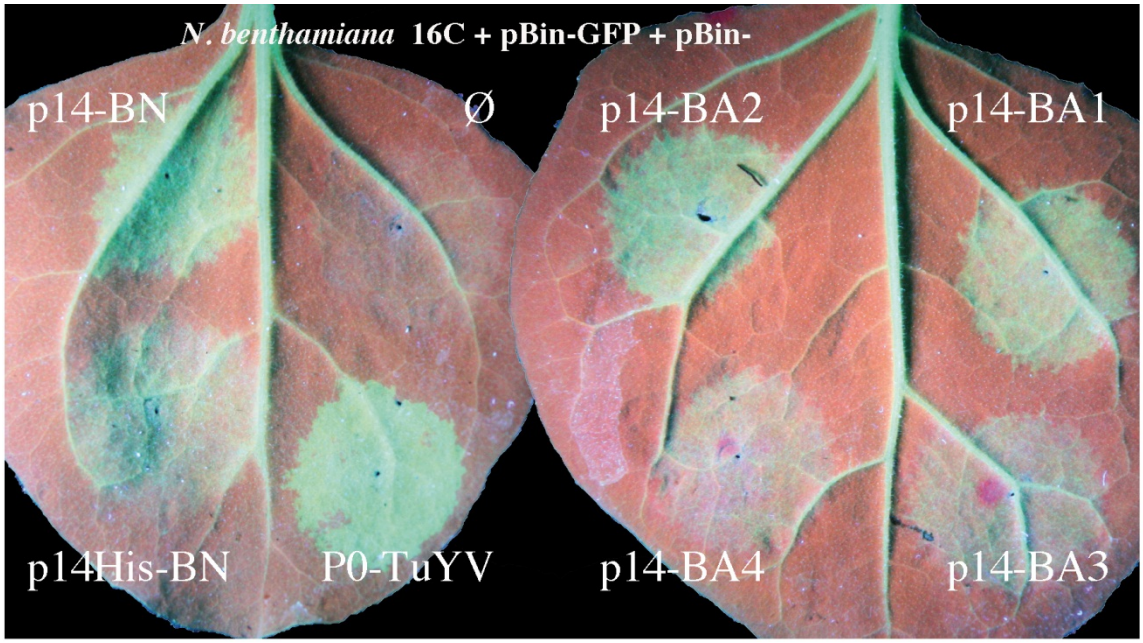
Voinnet, O., Vain, P., Angell, S., and Baulcombe, D. C. 1998. Systemic spread of sequence-specific transgene RNA degradation in plants is initiated by localized introduction of ectopic promoterless DNA. Cell 95:177-187.

Voinnet, O., Rivas, S., Mestre, P., and Baulcombe, D. 2003. An enhanced transient expression system in plants based on suppression of gene silencing by the p19 protein of *Tomato bushy stunt virus*. Plant J. 33:949-956.

Wang, X., Zhang, Y., Xu, J., Shi, L., Fan, H., Han, C., Li, D., and Yu, J. 2012. The R-rich motif of *Beet black scorch virus* P7a movement protein is important for the nuclear localization, nucleolar targeting and viral infectivity. Virus Res. 167:207-218

Yoo, D., Wootton, S. K., Li, G., Song, C., and Rowland, R. R. 2003. Colocalization and interaction of the porcine arterivirus nucleocapsid protein with the small nucleolar RNA-associated protein fibrillarin. J. Virol. 77:12173-12183.

Zhang, L., Wang, X., Li, D., Han, C., Zhai, Y., and Yu, J. 2005. Two virus-encoded RNA silencing suppressors, P14 of Beet necrotic yellow vein virus and S6 of *Rice black streak dwarf virus*. Chin. Sci. Bull. 50:305-310.



Supplementary Fig. S1. Viral suppressor of RNA silencing (VSR) activity of p14 NoLS mutants analyzed on *Nicotiana benthamiana* 16C. *N. benthamiana* 16C leaves were infiltrated with agrobacteria containing pBin-GFP silencing trigger together with empty binary vector (Ø) or binary vector expressing the VSR (p14-BN, p14His-BN, P0-TuYV and p14BAmutants). Leaves were photographed under UV light 4 days postinfiltration.

3. BSBMV p14 binds the "coremin" sequence

The yeast three-hybrid system is a method used to detect RNA-protein interactions *in vivo*. This technique consists in the expression in yeast cells of three hybrid molecules which assemble each other in order to activate a reporter gene (Bernstein *et al*., 2002). This system uses a transactivator protein consisting in a DNA binding domain and an activation domain that are functionally independent, meaning that they can be fused to other proteins (Fig. 3.2A).

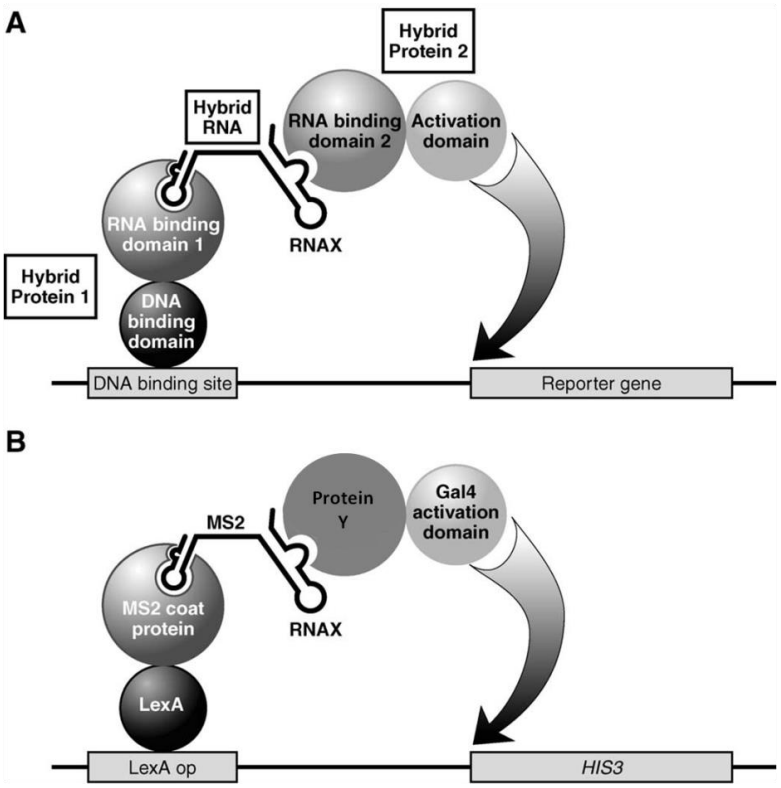


Fig. 3.2: Schematic illustration of yeast three hybrid test. (A) General strategy of the system. (B) Specific proteins and RNA components used (adapted from Bernstein *et al*., 2002).

The DNA binding domain (usually the LexA protein) is fused to the coat protein of the bacteriophage MS2 possessing sequence and structure specificity towards a small hairpin RNA structure. The second fusion protein contains the activation domain, typically Gal4AD fused to the the protein of interest (Protein Y). A chimeric RNA molecule is expressed in yeast and consists of the binding site for MS2 coat protein

and the RNA sequence of interest (RNA X, Fig. 3.2B). High specific binding of MS2-CP to its RNA target allows a binary complex to occur in the nucleus of the cells and tethered to LexAop sequences upstream of reporter gene. If the RNA X sequence of the chimeric RNA binds the fusion protein Y-AD, a ternary complex is formed at the vicinity of the reporter gene promoter (*HIS3)* that will be expressed (Fig. 3.2B). *HIS3* is the gene encoding imidazoleglycerol-phosphate dehydratase (His3p) and its expression confers the ability to grow on a medium lacking histidine. 3-amino-1,2,4-triazole (3-AT) is a competitive inhibitor of *HIS3* gene product and therefore cells on which His3p is high expressed can survive at higher concentrations of 3-AT in the medium. Thus, the level of 3-AT resistance of the yeast cells reflects the *HIS3* expression levels and consequently the strength of the RNA-protein interaction (Bernstein *et al*., 2002; Jaeger *et al*., 2004).

This system has been used to test the interaction between BNYVV p14 and the "coremin" sequence present in the RNA-3, which is required for viral long distance movement and the production and stabilization of ncRNA-3 (Peltier *et al*., 2012). Results obtained by Hleibieh (2010) showed that p14 binds specifically this stretch of 20 nucleotides until a 3-AT concentration of 10mM.

The possible interaction of BSBMV p14 with the "coremin" sequence has been investigated using the yeast strain YBZ1, whose genome encodes for the hybrid molecule LexA-MS2. The sequence of BSBMV p14 has been amplified from the available clone EUB22, digested *Eco*RI/*Sal*I and then inserted in the vector pGAD424 (Clontech), previously cut with the same enzymes. This vector expresses the protein of interest fused to the activation domain of the transcriptional factor, and also carries the nutritional marker *LEU2* that allows the cells to grow in a medium lacking leucine. The *wild type* "coremin" sequence and its mutated "K", "C" and "E" sequences were separately inserted in the plasmid pIIIMS2.1 which expresses the chimerical RNA molecule under a pol-III promoter and carries the *URA3* gene which ensures cells growing in a medium lacking uracile. Coremin "K" sequence is mutated into the loop ($C^{1239}G$, $G^{1240}A$, $A^{1241}C$), whereas "C" carries substitutions in both the stem and the loop ("K" mutations and $C^{1245}G$, $G^{1246}C$) and "E" is the complementary reverse sequence of the wild type "coremin" (Hleibieh, 2010; Peltier *et al.*, 2012) (Fig. 3.3A).

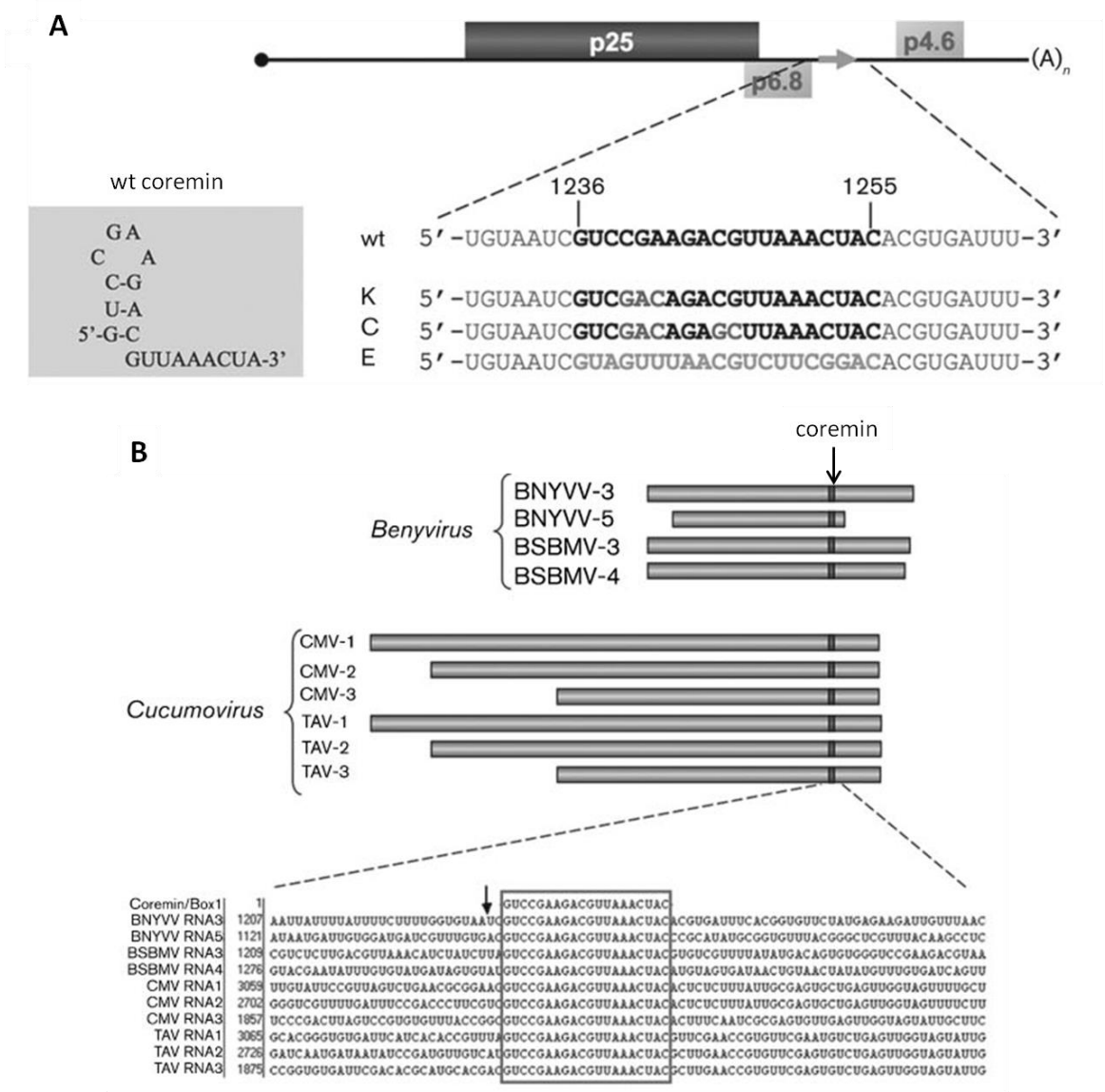


Fig. 3.3: (A) Mutagenesis of BNYVV RNA-3 coremin sequence was designed to replace 3 nts within the loop (K), supplemented by a stem destabilization (C) or to reverse the orientation of the sequence (E). (B) The coremin sequence is present in Benyviruses RNAs and is known as Box1 sequence in cucumoviruses. The arrow specifies the 5' extremity of the ncRNA-3 (adapted from Peltier *et al*., 2012).

In our experiments we used as positive control the well characterized interaction between the human histone HBP, expressed by the vector pACT, with the *wild type* hairpin of the 3' UTR replication-dependent histone mRNA, encoded by pIIIMS2.1WT (Jaeger *et al*., 2004).

The empty vector pGADø and the clones pGAD_p14BSBMV and pACT_HBP have been separately transformed in the yeast cells with pIIIMS2.1ø, pIIIMS2.1_WT, pIIIMS2.1_coremin, pIIIMS2.1_K, pIIIMS2.1_C or pIIIMS2.1_E clones. Diploids cells were selected on synthetic defined (SD) medium lacking both leucine and uracile (SD-UL). Interactions were then tested on plates of SD-UL medium lacking histidine (SD-ULH) with different concentration of 3-AT. Yeasts cotransformed with pGAD_p14BSBMV and either pIIIMS2.1_coremin or pIIIMS2.1_K grew until a concentration of 5mM 3-AT, whereas no growth was observed in yeasts carrying pGAD_p14BSBMV and either pIIIMS2.1ø, pIIIMS2.1_WT, pIIIMS2.1_C or pIIIMS2.1_E.

Our results demonstrated that BSBMV p14, as BNYVV p14, is able to bind the "coremin" sequence, but only in presence of low concentration of 3-AT (Fig. 3.4). Such results indicated that p14s possess RNA binding activities with different affinities towards the coremin sequence since BNYVV p14 binds coremin up to 10 mM 3-AT (Hleibieh, 2010).

Expression of BSBMV p14 fused to the activation domain has been confirmed through western blot using a specific Anti-AD antibody (Sigma) (Fig. 3.5).

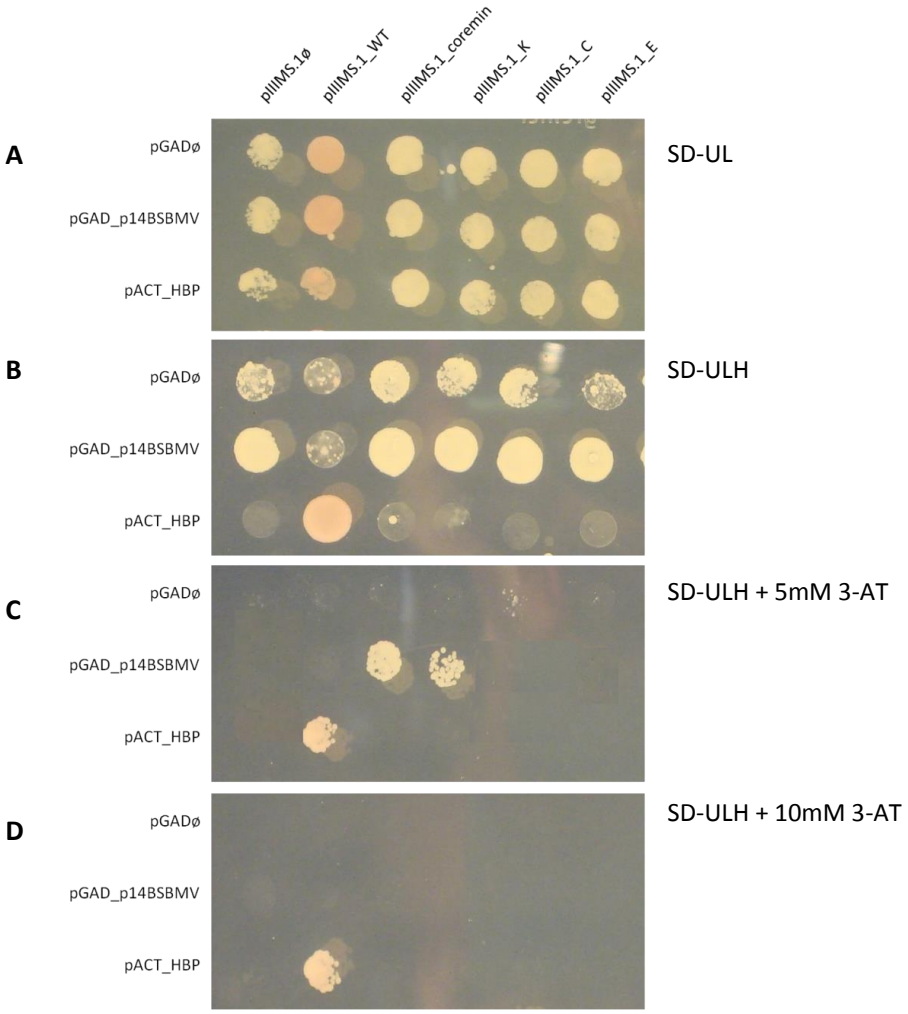


Fig. 3.4. Yeast three-hybrid test performed in YBZ1 strain. (A) Diploid yeasts are able to grow on a medium lacking both uracile and leucine. Selected diploids yeasts are subsequently plated on a medium lacking uracile, leucine and histidine (B) and with different concentrations of 3-AT (C, D) in order to test interaction beetween the protein of interest and the chimerical RNA molecules. BSBMV p14 binds the *wild type* coremin and K sequences up to 5mM of 3-AT (C). The positive control is represented by the well characterized interaction between the HBP protein and the WT hairpin (Jaeger *et al*., 2004).

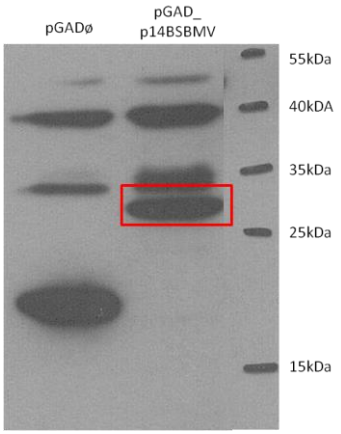


Fig. 3.5. Red box evidences a band of 27kDa (BSBMV p14 fused to the activation domain of about 13kDa). The fusion protein is detected by an anti-AD antibody (Sigma). Bands of 35, 40 and 50 kDa correspond to cellular proteins recognized by the primary or secondary antibody.

4. Discussion

BNYVV RNA-3 is involved in the viral pathogenicity and its "core" region (1033-1257 nts) is required for viral long distance movement (Lauber *et al*., 1998). Within this region, a stretch of 20 nucleotides, named "coremin" has been identified. The "coremin" sequence is present as well in BNYVV RNA-5, BSBMV RNA-3 and -4 and in viral RNAs of cucumoviruses *Cucumber mosaic virus* (CMV) and *Tomato aspermy virus* (TAV) (Ratti *et al*., 2009) (Fig. 3.3B).

In the CMV, the "coremin" sequence is involved in the accumulation of the non-coding RNA-5 which carries this sequence at its 5' end (de Wispelare and Rao, 2009). Structure analysis showed that it forms a hairpin and that its mutations can influence the viral pathogenicity (Thompson *et al*., 2008).

In BNYVV, the "coremin" sequence is required for the production and stabilization on the ncRNA-3, which is a cleavage product, involved in viral long distance movement. Mutations in the "coremin" motif lead to the disappearance of the ncRNA-3 and the absence of viral systemic spread on *B. macrocarpa*. These data highlight the importance of the "coremin" sequence for the long-distance movement of the virus, however it is not clear if this is due to the presence of full-lentgh RNA-3, ncRNA-3 or both (Peltier *et al*., 2012).

BSBMV p14 showed to specifically interact with the "coremin" sequence *in vivo.* This interaction persists until a concentration of 5mM of 3-AT, in contrast with the known BNYVV p14/coremin interaction which last up to 10mM 3-AT (Hleibieh, 2010). However, it has to be considered that in the natural host plant, such as *B. macrocarpa*, the strength of this interaction could be likely altered by other factors.

This work demonstrated the VSR function of Benyviruses p14s and the requirement of BNYVV p14 for long distance movement in *N. benthamiana* and *Beta* species. Moreover, yeast three-hybrid experiments provided evidence for an interaction between VSR and the coremin sequence, both needed for the viral long distance movement. Such binding has been confirmed with crosslinking experiments for BNYVV p14. (Hleibieh, 2010). Interestingly, only homodimers of BNYVV p14 bind the "coremin" sequence. The role of the ncRNA-3 is not clear yet. This ncRNA is stabilized

by the “coremin” sequence and is required for systemic spread of the virus. Recent data also indicate that “coremin” or ncRNA3 likely play a key role in the suppression of RNA silencing. Hleibieh (2010) demonstrated that *C. quinoa* leaves inoculated with viral transcripts of BNYVV RNA-1 + -2Δp14 show small necrotic lesions due to the absence of VSR. The typical chlorotic lesions appear if BNYVV RNA-3 is added to the previous combination but not if a mutated coremin version of RNA-3 is used. This restoration of local lesion phenotype is somehow linked to the action of a VSR. The only difference between wild type and mutated RNA-3 resides in the “coremin” sequence. Thus, it could be concluded that the RNA-3 is directly involved in this suppression of PTGS supporting and particularly the ncRNA-3. One elegant hypothesis that needs to be verified is that the overproduction of ncRNA-3 could saturate the silencing machinery of the cell, as proposed for the Adenovirus VA-RNA (Peltier *et al*., 2012; Andersson *et al*., 2005; Lu and Cullen, 2004) or saturate the exonuclease XRN1 as described for flaviviruses (Schnettler *et al*., 2012; Moon *et al.*, 2012).When BNYVV RNA-3 is expressed in yeast, ncRNA-3 is produced but not in a mutant deficient in the production of the XRN1 exonuclease (David Gilmer, unpublished, personal communication). Both plant and animal viruses seem to share common mechanism of silencing suppression.

References

Andersson M.G., Joost Haasnoot P.C., Xu N., Berenjian S., Berkhout B., Akusjarvi G. (2005). Suppression of RNA interference by Adenovirus virus-associated RNA. Journal of Virology, Vol. 79, No.15, p.9556-9565.

Baumberger N., Tsai C.H., Lie M., Havecker E., Baulcombe D.C. (2007). The polerovirus silencing suppressor P0 targets Argonaute proteins for degradation. Curr. Biol., 17, 1609-1614.

Bayne e.H., Rakitina D.V., Morozov S.Y., Baulcombe D.C. (2005). Cell-to-cell movement of potato potexvirus X is dependent on suppression of RNA silencing. Plant J., 44, 471-482.

Baumberger, N., and Baulcombe, D.C. (2005). Arabidopsis ARGONAUTE1 is an RNA Slicer that selectively recruits
microRNAs and short interfering RNAs. Proc Natl Acad Sci U S A 102, 11928-11933.

Bortolamiol D., Pazhouhandeh M., Marrocco K., Genschik P., Ziegler-Graff V. (2007). The polerovirus F box protein P0 targets Argonaute1 to suppress RNA silencing. Curr. Biol., 17, 1615-1621.

Bernstein E., Caudy A.A., Hammond S.M., Hannon G.J. (2001). Role for a bidentate ribonuclease in the initiation step of RNA interference. Nature, 409, 363-366.

Bernstein D.S., Buter N., Stumpf C., Wickens M. (2002). Analyzing mRNA-protein complexes using a yeast three-hybrid system. Methods, 26, 123-141.

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

Brodersen, P., Sakvarelidze-Achard, L., Bruun-Rasmussen, M., Dunoyer, P., Yamamoto, Y.Y., Sieburth, L., Voinnet, O. (2008). Widespread translational inhibition by plant miRNAs and siRNAs. Science 320, 1185-1190.

Burgyan J., Havelda Z. (2011). Viral suppressors of RNA silencing. Trends. Plant Sci., 16, 265-272.

Carrington J.C., Kasschau K.D., Johansen L.K. (2001). Activation and suppression of RNA silencing by plant viruses. Virology, 281, 1-5.

Chiu M.H., Chen I.H., Baulcombe D.C., Tsai C.H. (2010). The silencing suppressor P25 of Potato virus X interacts with Argonaute1 and mediates its degradation through the proteasome pathway. Mol. Plant Pathol., 11, 641-9.

Csorba T., Bovi A., Dalmay T., Burgyan J. (2007). The p122 subunit of tobacco mosaic viurs replicase is a potent silencing suppressor and compromises both small interfering RNA and microRNA-mediated pathways. J. Virol., 81, 11768-11780.

Csorba T., Lozsa R., Hutvagner G., Burgyan J. (2010). Polerovirus protein P0 prevents the assembly of small RMA-containing RISC complexed and leads to degradation of ARGONAUTE1. The Plant Journal, 62, 463-472.

de Wispelaere, M., Rao A.L. (2009). Production of cucumber mosaic virus RNA5 and its role in recombination. Virology, 384, 179-191.

Deleris A., Gallego-Bartolome J., Bao J., Kasschau K.D., Carrington J.C. et al. (2006). Hierarchical action and inhibition of plant Dicer-like proteins in antiviral defense. Science, 313, 68-71.

Derrien B., Baumberger N., et al. (2012). Degradation of the antiviral component ARGONAUTE1 by the autophagy pathway. Proc. Natl. Acad. Sci. USA, 109(39), 15942-15946.

Diaz-Pendon J.A., Ding S.W. (2008). Direct and indirect roles of viral suppressors of RNA silencing in pathogenesis. Annu. Rev. Phytopathol., 46, 303-326.

Ding S.W., Voinnet O. (2007). Antiviral immunity directed by small RNAs. Cell, 130, 413-426.

Dunoyer P., Pfeffer S., Fritsch C., Hemmer O., Voinnet O., Richards K.E. (2002). Identification, subcellular localization and some properties of a cycteine-rich suppressor of gene silencing encoded by peanut clump virus. Plant. J., 29, 555-567.

Elbashir S.M., Lendeckel W., Tuschl T. (2001). RNA interference is mediated by 21- and 22- nucleotide RNAs. Gene Dev., 15, 188-200.

Ekwall, K. (2004). The RITS complex-A direct link between small RNA and heterochromatin. Mol Cell 13, 304-305.

Fire A., Xu S., Montgomery M.K., Kostas S.A., Driver S.E., Mello C.C. (1998). Potent and specific genetic interference by double-stranded RNA in Caenorhabditis elegans. Nature, 391, 806-811.

Gilmer D., Bouzoubaa S., Hehn A., Guilley H., Richards K., Jonard G. (1992). Efficient cell-to-cell movement of beet necrotic yellow vein virus requires 3' proximal gene located on RNA-2. Virology, 189, 40-47.

Hamera S., Son X., Su L., Chen X., Fang R. (2012). Cucmber mosaic virus suppressor 2b binds to AGO4-related small RNAs and impairs AGO4 activities. The Plant Journal, 69, 104-115.

Hamilton A.J., Baulcombe D.C. (1999). A species of small antisense RNA in post-transcriptional gene silencing in plants. Science, 286, 950-952.

Hehn A., Bouzoubaa S., Bate N., Twell D., Marbach J., Richards K., Guilley H., Jonard G. (1995). The small cysteine-rich protein p14 of Beet necrotic yellow vein virus regulates accumulation of RNA-2 in cis and coat protein in trans. Virology, 210, 73-81.

Himer C., Dunoyer P., Moissiard G., Ritzenthaler C., Voinnet O. (2003). Transitivitiy-dependent and – independent cell-to-cell movement of RNA silencing. EMBO J., 22, 4523-4533.

Hleibieh K. (2010). Étude des propriétés biologiques de la protéine p25, de l'ARN3 et de la protéine p14 du Beet necrotic yellow vein virus : vers de nouvelles stratégies de luttes antivirales. Thesis Univerisity of Strasbourg.

Jaeger S., Eriani G., Martin F. (2004). Results and prospects of the yeast three-hybrid system. FEBS Letters, 556, 7-12.

Kozlowska-Makulska A., Guilley H., et al. (2010). P0 proteins of European beet-infecting poleroviruses display variable RNA silencing suppression activity. J. Gen. Virol., 91(Pt 4), 1082-1091.

Lakatos L., Szittya G., Silhavy D., Burgyan J. (2004). Molecular mechanism of RNA silencing suppression mediated by p19 protein of tombusviruses. EMBO J., 23, 876-884.

Lakatos L., Csorba T., Pantaleo V., Chapman E.J., Carrington J.C. et al. (2006). Small RNA binding is a common strategy to suppress RNA silencing by several viral suppressors. EMBO J., 25, 2768-2780.

Lauber E., Guilley H., Tamada T., Richards K.E., Jonard G. (1998). Vascular movement of beet necrotic yellow vein virus in Beta macrocarpa is probably dependent on an RNA3 sequence domain rather than a gene product. Journal of General Virology, 79, 385-393.

Li, F., and Ding, S.W. (2006). Virus counterdefense: diverse strategies for evading the RNA-silencing immunity. Annu. Rev. Microbiol. 60, 503-531.

Lindbo J.A., Silva-Rosales L., Proebsting W.M., Dougherty W.G. (1993). Induction of a highly specific antiviral state in transgenic plants: implications for regulation of gene expression and virus resitance. Plant Cell, 5, 1749-1759.

Lu S., Cullen B.R. (2004). Adenovirus VA1 noncoding RNA can inhibit small interfering RNA and microRNA biogenesis. Journal of Virology, Vol. 78, No.23, p.12868-12876.

Merai Z., Kerenyi Z., Kertesz S., Magna M., Lakatos L. et al (2006). Double-stranded RNA binding may be a general plant RNA viral strategy to suppress RNA silencing. J. Virol., 80, 5747-5756.

Moon S. L., Anderson J.R, et al. (2012). A noncoding RNA produced by arthropod-borne flaviviruses inhibits the cellular exoribonuclease XRN1 and alters host mRNA stability. RNA, 18(11), 2029-2040.

Napoli C., Lemieux C., Jorgensen R. (1990). Introduction of a chimeric chalcone synthase gene into petunia results in reversible co-suppression of homologous genes in trans. The plant cell, 2, 279-289.

Niesbach-Klosgen U., Guilley H., et al. (1990). Immunodetection in vivo of beet necrotic yellow vein virus-encoded proteins. Virology, 178(1), 52-61.

Peltier C., Klein E., Hleibieh K, D'Alonzo M., Hammann P., Bouzoubaa S., Ratti C., Gilmer D. (2012). Beet necrotic yellow vein virus subgenomic RNA3 is a cleavage product leading to stable non-coding RNA required for long-distance movement. J. Gen. Virol., 93 (Pt 5), 1093-102.

Qu F., Ren T., Morris T.J. (2003). The coat protein of Turnip crinkle virus suppresses port-transcriptional gene silencing at an early initiation step. J. Virol., 77, 511-522.

Ratti C., Hleibieh K., Bianchi L., Schirmer A., Autonell C.R., Gilmer D. (2009). Beet soil-borne mosaic virus RNA-3 is replicated and encapsidated in the presence of BNYVV RNA-1 and -2 and allows long distance movement in Beta macrocarpa. Virology, 385, 392-399.

Romano M., Macino G. (1998). Quelling: transient inactivation of gene expression in Neurospora crassa by transformation with homologous sequences. Mol. Microbiol., 6, 3343-3353.

Schnettler E., Sterken M.G., et al. (2012). Noncoding flavivirus RNA displays RNA interference suppressor activity in insect and Mammalian cells. J. Virol., 86(24), 13486-13500.

Shiboleth Y.M., Haronsky E., Leibman D., Arazi T., Wassenegger M. et al. (2007). The conserved FRNK box in HC-Pro, a plant viral suppressor of gene silencing, is required for small RNA binding and mediates symptom development. J. Virol., 81, 13135-13148.

Thompson J.R., Buratti E., de Wispelaere M., Tepfer M. (2008). Structural and functional characterization of the 5' region of subgenomic RNA5 of cucumber mosaic virus. J. Gen. Virol., 89, 1729-1738.

Verchot J, Angell SM and Baulcombe DC (1998). In vivo translation of the triple gene block of potato virus X requires two subgenomic mRNAs. J. Virol., 72, 8316-20.

Voinnet O., Pinto Y.M., Baulcombe D.C. (1999). Suppression of gene silencing: a strategy used by diverse DNA and RNA viruses. Proc. Natl. Acad. Sci USA, 96, 14147-14152.

Voinnet O. (2001). RNA silencing as a plant immune system against viruses. Trends Genet., 17, 449-459.

Voinnet O. (2005). Non-cell autonomous RNA silencing. FEBS Lett., 579, 5858-5871.

Waterhouse P.M., Graham H.W., Wang M.B. (1998). Virus resistance and gene silencing in plants can be induced by simoultaneous expression of sense and anti-sense RNA. Proc. Natl. Acad. Sci. USA, 95, 13959-13964.

Zhang X., Yuan Y.R., Pei Y., Lin S.S., Tuschl T. et al. (2006). Cucumber mosaic virus-encoded 2b suppressor inhibits Arabidopsis Argonaute1 cleavege activity to counter plant defense. Genes Dev., 20, 3255-3268.

General Discussion and Conclusions

The aim of my study was to investigate both the molecular interactions between the *Beet necrotic yellow vein virus* and the *Beet soil-borne mosaic virus* and the mechanisms involved in the pathogenesis of these viruses that belong to the same genus and share similar host range, vector, morphology and genome organization (Lee *et al*., 2001).

BNYVV helper strain is able to replicate and encapsidate BSBMV RNA-3 and -4 allowing long distance movement and the transmission through the vector (Ratti *et al*., 2009; D'Alonzo *et al*., 2012). Previous studies about BNYVV/BSBMV interactions showed results that vary greatly depending on the approach used (Rush, 2003). In the United States these viruses have been found in the same plant but no chimeric forms have been described in nature so far. Thus, one of the aims of my PhD project consisted to investigate the possible synergistic or antagonistic effect between these two *Benyvirus* and to verify the possibility that BNYVV/BSBMV chimeras may be generated.

To address the effect of one virus on the other, a possibility resides on the mechanical inoculation of leaves with known amounts of each virus. Since viruses are constituted with four different particles, it is rather difficult to be sure about the amount of each RNA species in the inoculum. Therefore, I needed to develop a method that permit the quantification of the amount of each RNA species provided per cell. *In vitro* transcription allows such quantitative control but represent an expensive approach. I decided to construct full-length infectious clones under the control of the 35S promoter and to perform agroinfiltration to bypass the problems of viral ratios and costs. Different strategies have been exploited to overcome cloning problems and finally BNYVV and BSBMV agroinfectious clones have been produced, with the exception of BSBMV RNA-4 agroclone.

As described in our paper published in *Molecular Plant Pathology*, BNYVV agroclones are fully functional since those are able to reproduce a complete viral cycle, from replication to the transmission. Such constructs are suitable for the infection of *N. benthamiana*, *B. vulgaris* and *B. macrocarpa.* This method represents an important achievement for studying benyviruses and opens new possibilities for research studies.

In the context of my study, agroclones represent a suitable tool for the verification of synergistic or antagonistic effects, as well as cross-protection, between BNYVV and other viruses affecting sugar beet, such as BSBMV. Direct agroinfection of *B. vulgaris* roots allows a quick and economic assay to test for Rhizomania resistance since a large number of sugar beet plants could easily be infected and the viral title detected can be considered a measure of BNYVV resistance of the cultivar.

Preliminary experiments performed with BSBMV agroclones revealed that even these clones were able to induce typical symptoms on *N. benthamiana* and *B. macrocarpa* agroinfected leaves, but the virus didn't move systemically in these plants. On this basis, we discovered that BSBMV RNA-2 clone doesn't allow the viral encapsidation. *In vitro* and *in vivo* (EUB22 and AgroBS-2 clones) derived sequences carry two point mutations in the Read-Through domain when compared to the published sequence (Lee *et al*., 2001): a guanine mutated in adenine ($G^{1567}A$) and a thymine mutated in adenine ($T^{2000}A$). Interestingly, CP-RT domain of BNYVV is known to initiate particle formation (Schmitt *et al*., 1992; Tamada *et al*., 1991) and these two mutations will be corrected in order to obtain fully functional infectious clones of BSBMV RNA-2. However, the implication of other RNA-2 encoded proteins in the RNA encapsidation cannot be ruled out. Thus, exploiting BNYVV/BSBMV protein similarity, complementation tests will be performed through mechanical inoculation of *C. quinoa* leaves using BSBMV RNA-1 and -2 *in vitro* transcripts added to viral replicons expressing different BNYVV RNA-2 proteins.

Once this aspect will be solved, the availability of all BNYVV and BSBMV agroclones will give us the tools necessary to test antagonistic or synergistic effects between these Benyviruses in mixed infections.

During my work, I demonstrated that BNYVV/BSBMV chimeras are viable. The biological properties of chimeras mixing house keeping genes have been investigated both with the use of *in vitro* transcripts and agroclones. BoStras12 and StrasBo12 chimeras have been tested on *C. quinoa* protoplasts and leaves demonstrating their ability to replicate and move from cell to cell. As StrasBo12 chimera carries mutations that do not allow long distance movement, the interpretation of symptoms outcome is

controversial. However, when compared with *wild type* strains, the BoStras12 chimera induced large necrotic lesions with chlorotic border and was able to move long distance in *N. benthamiana* and to induce similar symptoms in upper leaves. BNYVV VSR was functional in infected tissues. Such severe symptoms were linked to the absence of expression of the BSBMV p14 suppressor of post-transcriptional gene silencing. Indeed, the necrosis disappeared when BSBMV p14 was added to the inoculum *via* a replicon vector. This hypersensitive-like response of the host suggests a higher aggressiveness of the chimera that could explain why Benyviruses recombinants don't arise in nature. Of course, such experiments need to be performed on the natural host of the viruses. For this purpose, the same experiments will be conducted on *B. macrocarpa* and *B. vulgaris* plants. We will also analyze the behavior of *Benyvirus* chimeras in natural transmission conditions and test the hypothesis of a hypersensitive defense response by quantifying the expression of pathogenesis related proteins (such as PR1), which increase during HR using *wild type* strains as controls.

The data obtained with chimeras highlighted the role of the p14 VSRs. BSBMV p14 is able to fully complement BNYVV p14 functions but, conversely, BNYVV p14 is unable to complement BSBMV p14 in the presence of BSBMV RNA-1 since necrosis arose. This result suggests the existence of a link between the BSBMV p14 protein and its cognate RNA-1 that will be the object of further investigations through immunoprecipitation, yeast two- or three-hybrid test. Interestingly, a link exists between genomic RNAs of BNYVV isolates since few recombinants are detected in fields contaminated with multiple isolates. The *in silico* study performed on concatenated CP and p25 sequences (Schirmer *et al*., 2005) demonstrated that RNA-2, RNA-3 and RNA-5 when present are linked one another. The existence links between genome segments is also described for multipartite viruses such as influenza that implies RNA-RNA interactions (Gavazzi *et al.*, 2013). Therefore, protein-RNA interactions and RNA-RNA interactions will be studied to explain such particular link between p14 VSR (or RNA-2) and RNA1.

During this work, I demonstrated that BSBMV is able to replicate BNYVV RNA-5 and its derived replicon vector Rep5. The capability to use Rep5 together with BSBMV RNAs open new possibilities to express proteins in this viral context. Therefore we will be

able to investigate the effect of BNYVV RNA-3 encoded protein (p25) expression in BSBMV context in *B. vulgaris*. BNYVV p25 has been associated to rhizomania symptom expression (Tamada *et al*., 1999) and root proliferation (Peltier *et al*., 2010). If rhizomania syndrome occurs even in a BSBMV context, this will represent the final demonstration of the direct implication of the p25 protein in root proliferation. Experiments will be performed either with the use of a natural isolate or *in vitro* transcripts when BSBMV RNA-2 clone will be corrected for efficient encapsidation.

Within the study of molecular mechanisms involved in the pathogenesis of beet soil-borne viruses, the role of BNYVV and BSBMV p14s has been investigated. BNYVV p14 has already been identified as a suppressor of post-transcriptional gene silencing (Dunoyer *et al*., 2002), and BSBMV p14 has been suspected to possess a similar function on the basis of sequence similarity with BNYVV p14.

My work using patch test in *N. benthamiana* 16C demonstrated that both Benyviruses p14s are viral suppressors of RNA silencing (VSR) acting downstream of the production of primary siRNA generated by Dicer proteins, without interfering with the transitivity.

These cysteine-rich proteins with a zinc-finger domain are able to bind nucleic acids and a putative nucleolar localization signal (NoLS) has been predicted in BNYVV p14. We demonstrated that both p14s localize in the cytoplasm and in the nucleolus and form homodimers. However, experiments performed using several BNYVV p14 mutants showed that the silencing suppression activity requires a functional ZnF domain and NoLS basic rich residues but not the nucleolar localization of the protein.

The most important result of this study is the evidence that BNYVV p14 is required for long distance movement in *N. benthamiana* and *B. macrocarpa*. Moreover, yeast three-hybrid test showed that p14s bind the “coremin” sequence *in vivo*. This stretch of 20 nucleotides is required for the production and stabilization of the ncRNA-3 produced by the RNA-3 maturation and involved in viral systemic movement. Recent data also indicate that the “coremin” sequence or ncRNA-3 may play a role in the suppression of RNA silencing. One hypothesis that needs to be verified is that the overproduction of ncRNA-3 could saturate the silencing machinery of the cell, as already proposed for some human viruses, and particularly Flaviviruses. Thus, both

BNYVV p14 and the “coremin” sequence seem to be involved in both the long distance movement and the suppression of post-transcriptional gene silencing.

In conclusion, this study contributed to improve knowledge about BNYVV and BSBMV biology, behavior and mechanisms involved in the viral pathogenicity. We constructed new and powerful tools to carry on experiments about Benyviruses that open new possibility of research. Future works will be addressed to further investigate the suppression of post-transcriptional gene silencing in *Benyvirus* genus, the role of the “coremin” sequence and ncRNA-3 in BNYVV, and mechanisms that prevent the origin of BNYVV/BSBMV chimeras in natural mixed infections.

References

D'Alonzo M., Delbianco A., Lanzoni C., Autonell C.R., Gilmer D., Ratti C. (2012). Beet soil-borne mosaic virus RNA-4 encodes a 32 kDa protein involved in symptom expression and in virus transmission through Polymyxa betae. Virology; 423:187-94.

Dunoyer P., Pfeffer S., Fritsch C., Hemmer O., Voinnet O., Richards K.E. (2002). Identification, subcellular localization and some properties of a cysteine-rich suppressor of gene silencing encoded by peanut clump virus. Plant Journal, 29, 555-567.

Gavazzi C., Isel C., et al. (2013). An in vitro network of intermolecular interactions between viral RNA segments of an avian H5N2 influenza A virus: comparison with a human H3N2 virus. Nucleic Acids Res., 41(2), 1241-1254.

Lee L., Telford E.B., Batten J.S., Scholthof K.B., Rush C.M. (2001). Complete nucleotide sequence and genome organization of beet soil borne mosaic viru, a proposed member of the genus Benyvirus. Archives of Virology, 146, 2243-2453.

Peltier C., Schmidlin L., Klein E., Taconnat L., Prinsen E., Erhardt M., Heintz D., Weyens G., Lefebvre M., Renou J.P., Gilmer D. (2010). Expression of beet necrotic yellow vein virus p25 protein induces hormonal changes and a root branching phenotype in Arabidopsis thaliana. Trangenic Res., 20, 443-466.

Ratti C., Hleibieh K., Bianchi L., Schirmer A., Rubies Autonell C., Gilmer D. (2009). Beet soil borne mosaic virus RNA-3 is replicated and encapsidated in the presence of BNYVV RNA-1 and -2 and allows long distance movement in Beta macrocarpa. Virology, 385, 392-399.

Rush C.M. (2003). Ecology and epidemiology of benyviruses and plasmodiophorid vectors. Annual Review Phythopathology, 41, 567-592.

Schirmer A., Link D., Cognat V., Moury B., Beuve M., Meunier A., Bragard C., Gilmer D., Lemaire O. (2005). Phylogenetic analysis of isolates of beet necrotic yellow vein virus collected worldwide. Journal of General Virology, 86, 2897-2911.

Schmitt C., Balmori E., Jonard G., Richards K.E., Guilley H. (1992). In vitro mutagenesis of biologically active transcripts of beet necrotic yellow vein virus RNA-2: evidence that a domain of 75 kDa read-through protein is important for efficient virus assembly. Proceedings of the National Academy of Sciences USA, 89, 5715-5719.

Tamada T., Kususme T. (1991). Evidence that the 75K readthrough protein of beet necrotic yellow vein virus RNA-2 is essential for transmission by the fungus Polymyxa betae. Journal of General Virology, 72, 1497-1504.

Tamada T., Uchino H., Kusume T., Saito M. (1999). RNA3 deletion mutants of beet necrotic yellow vein virus do not cause rhizomania disease in sugar beet. The American Phytopathological Society, vol.89, no.11, 1000-1006.

Appendix A

Material and Methods

A.1 Organisms

Test plants

Host plants generally used for BNYVV and BSBMV research studies are:

- ***Chenopodium quinoa***: it belongs to *Chenopodiaceae* family and it is a local host of BNYVV and BSBMV. Depending on the inoculum composition, viral infections result in small chlorotic, yellow or necrotic lesions in leaves.
- ***Nicotiana benthamiana***: it is a systemic host of BNYVV and BSBMV and belongs to the *Solanaceae* family.
- ***Beta macrocarpa*** and ***B. vulgaris***: both belong to the *Chenopodiaceae* family and represent the natural host of BNYVV and BSBMV.

Bacteria

Bacteria generally used are:

- ***Escherichia coli*** strain **MC1022:** it is used for cloning and amplification. This strain allows blue/white colony screening in presence of IPTG and Xgal.
- ***Agrobacterium tumefaciens*** strain **GV3101**: this bacteria is used for agroinfiltration and carries rifampicin selectable marker.

Yeasts

Two strains of *Saccharomyces cerevisiae* have been used:

- **AH109**: for two-hybrid test to detect protein-protein interactions.
- **YBZ1**: for three-hybrid test to detect RNA-protein interactions.

N. tabacum BY-2 cells

These cells are used to investigate protein subcellular localization *in vivo*, they can be quickly transfected and are easily observable by conventional microscopy approaches.

A.2 RNA and DNA processing

Trizol©total RNA extraction (Life technologies)

Trizol© total RNA extraction was performed according to manufacturer's protocol. Fresh or frozen leaves and roots (100-200 mg) were crushed in a sterile 1.5 ml eppendorf with 1 ml of Trizol buffer. After 5 min at room temperature, 200 µl of chloroform are added and tubes are vigorously shaken by hand and incubate again at room temperature for 2 min. After centrifugation for 15 min at 12,000 g and 4°C, the aqueous phase is transferred in a fresh tube and 0.5 ml of isopropanol is added. RNA precipitation requires incubation for 20 min at room temperature then the RNA is pelleted, through centrifugation at 12,000g for 30 min at 4°C and washed with 1 ml of 70% ethanol. RNA pellet is dried 5 min and then resuspended in sterile water. RNA quality and quantity are analyzed on agarose gel and by spectrophotometer.

RNA-DNA amplification and visualization

Polymerase chain reaction (PCR) is a powerful method that permits to generate millions of DNA copies starting from a limited amount of nucleic acid. While DNA is immediately suitable for such amplification, RNA must be reverse transcribed.

- **Reverse transcription**: The reverse transcription aims to synthesize the complementary DNA strand (cDNA) of each RNA molecules. The cDNA is then amplified by PCR. Moloney murine leukemia reverse transcriptase (M-MLV RT) (Promega, Madison, CA) was used for the common production of short fragments (up to 1-2 kb). RNA samples, mixed with 1 µl reverse primer (25 µM) and nuclease-free water up to 5 µl final volume, is first heated 10 min at 65°C in a T3000 Thermal Cycler (Biometra) to disrupt secondary structures. The elongation step is performed at 37°C in 1 h after the addition of 4 µl of 5X buffer (250 mM Tris-HCl, 375 mM KCl, 15 mM MgCl2 and 50mM DTT), 2 µl dNTPs (10 mM), 0.25 µl M-MLV RT (200 U/µl) and 3.75 µl steril and nuclease-free water. High quantities of long cDNA fragments (1.5 – 6.0 kb) were

synthesized using ImProm-II Reverse Transcriptase (Promega, Madison, CA). The denaturation step is identical to that described for M-MLV RT, and then 4 µl ImProm-II 5x-reaction buffer, 1.2 µl MgCl2 (25 mM), 1 µl dNTPs (10 mM), 0.5 µl RNasin Ribonuclease Inhibitor (40 U/µl), 1 µl Improm-II RT and 7.3 µl nuclease-free water were added. Elongation step is performed in 60 min at 42°C followed by RT inactivation at 70°C for 15 min.

- **Polymerase chain reaction (PCR)**: Two types of thermostable DNA polymerases synthesizing dsDNA were used in the presented experiments. Go Taq© Flexi DNA polymerase (Promega, Madison, CA) was used to amplify 1-2 kb fragments that didn't require high accuracy of copying, e.g. viruses detection or clones screening. Five µl of cDNA from RT step is mixed with 5 µl Go Taq® Flexi 5X buffer, 2.5 µl MgCl2 (25 mM), 0.75 µl dNTPs (10 mM), 1 µl of each primers (10 µM), 0.12 µl of Go Taq® DNA polymerase (5 U/µl) and sterile water up to a final volume of 25 µl. Pfu Ultra II Fusion Hotstart Polymerase (Agilent Technologies, Santa Clara, California, USA) was preferred when DNA fragments need to be subsequent cloned or sequenced. The reaction mix includes 2.5 µl PfuUltra II 10X reaction buffer (containing MgCl2), 1 µl of dNTP mix (25mM each dNTP), 1 µl of each forward and reverse primer (10 µM), 0.5 µl PfuUltra II Fusion HS DNA Polymerase (5 U/µl) and 5 µl cDNA from reverse transcription, nuclease-free water was added to a final volume of 25 µl.

Separation of DNA fragments on agarose gel by electrophoresis

DNA fragments may be separated according to their size. For such purpose, gels are prepared by melting Multi Purpose agarose (Roche, Mannheim, Germany) in 1x-concentrated Tris-Borate-EDTA buffer (TBE, 89 mM Tris-borate, 8.9 mM boric acid and EDTA 2 mM). The agarose concentration can vary between 0.7 and 2.0%, depending on the fragments size. Using Biorad Power Pac 300 or Modell

1000/500 power supply (Biorad, Hercules, CA), DNA fragments are subsequently forced to migrate through the gel in TBE buffer 1x-concentrated towards the anode, as being negatively charged. Then the gel is stained on 200 ml of Ethidium bromide solution (0.1 mg/ml) that allow double strand DNA visualization under UV light. Using 1 kb or 100 bp DNA ladders (Promega, Madison, CA), the approximate size of the observed fragments can be determined.

Purifications of nucleic acids

DNA and RNA molecules may be purified for further manipulations.

When a single-type/length of DNA had to be selected among molecules of different sizes, all fragments were separated on agarose gel and the fragment of interest was selected and excised from the gel. DNA was subsequently extracted by using affinity columns of Wizard SV gel and PCR clean-up system (Promega, Madison, CA) and eluted with nuclease-free sterile water.

In order to purify DNA molecules the phenol-chloroform method is generally used. Equal volumes of hydrophobic phenol:chlorofom:isoamyl alcohol (25:24:1, pH 4.5) solution and DNA are mixed and centrifuged 15 min at 14,000 g at 4°C. The upper aqueous phase containing DNAs molecules is then precipitated with two volumes of 100% ethanol, 16 µl NaCl (5 M) and 1 µl glycogen (10 mg/ml) at -20°C during 20 min. After 20 min centrifugation at 14,000 g at 4°C, the pellet is washed with 70% ethanol, dried and resuspended in nuclease-free sterile water.

The same protocol is also used for RNA purification, but in the precipitation step, glycogen and NaCl are replaced by Sodium Acetate (final concentration 150mM).

A.3 Cloning

Vectors

- **pCK-eGFP** and **pCK-RFP**: these plasmids are used to produce GFP or RFP fused proteins. They harbor an ampicillin resistance gene for the selection of transformed bacteria. The plasmids contain the 35S promoter sequence to guide the expression of fluorescent fusion proteins in

eukaryotic cell. pCK plasmids were used for biolistic transformation of BY-2 cells.

- **pBin61**: this plasmid of 12.9 kb is derived from the pBin19 plasmid and was used in agroinoculation experiments. pBin61 harbors a kanamycin resistance gene and contains a T-DNA between the left and right border sequences. Inside this T-DNA a 35S promoter sequence is followed by a multiple cloning site and a35S terminator sequence.
- **pGADT7**: pGADT7 is an 8 kb plasmid that contains the GAL4 activation domain (AD) sequence placed upstream of a HA epitope tag sequence and a MCS. Genes ligated into the MCS are thus expressed as a GAL4AD-HA fusion protein. This fusion protein is expressed under the control of the constitutive ADH1 promoter. An ampicillin resistance gene allows selection of transformed bacteria and the LEU2 nutritional marker that allows auxotroph yeast carrying pGADT7 to grow on a synthetic medium lacking Leucine.
- **pGBT9**: this 5.5 kb vector allows expression of GAL4BD fusion proteins under the control of the ADH1 promoter. It harbors an ampicillin resistance gene and the TRP1 nutritional marker.
- **pJL89**: this binary vector has been used to produce BNYVV and BSBMV agroclones. It carries a rifampicin selectable marker.
- **pIII/MS2**: this vector is used in three-hybrid test and harbour the URA3 selectable marker. The cDNA of the RNA sequence of interest in cloned in this vector under the control of the pol III.

Enzyme digestion

In order to obtain the desired final construct with the sequence of interest inserted in a specific vector, DNA has to be treated with restriction enzymes. Restriction enzymes recognize specific DNA sequences and cleave the double-strand to produce cohesive or protrusive extremities. One µg of template DNA is

incubated with 0.4 µl of restriction enzyme (10 U/µl) and the appropriate restriction buffer at 37°C for 3 hours.

Dephosphorylation

In order to avoid self-ligation of the restricted plasmid and favor the insertion of the fragment of interest, phosphate groups of 5'-extremities of the linearized vector should be removed. Thus, the fragment to be inserted brings the only phosphate groups available and insertion is the only way to get circularization. One µg of linearized vector is mixed with 1 µl of alkaline phosphatase from calf intestine (20 U/µl) (Roche), 2 µl dephosphorylation buffer 10x-concentrated (0.5 M Tris-HCl, 1 mM EDTA pH 8.5) and water to 20 µl final volume. The reaction is performed at 37°C for 1 hour.

Ligation

Ligation reaction was performed using the Rapid DNA ligation kit (Fermentas) according to the manufacturer's protocol. Usually, 1 µl of vector (50 ng/µl), 3 µl of insert (50 ng/µl), 3 µl Rapid DNA ligation buffer5X, 1 µl DNA ligase (1 U/µl) and nuclease-free sterile water up to 15 µl were used for each reaction, following incubation for at least 1 hour at room temperature. After phenol:chloroform purification and precipitation with ethanol, the ligation products are resuspended in 3 µl of nuclease-free sterile water and then used for electroporation.

Transformation of bacteria through electroporation

One and half µL of plasmid DNA was added to 25/40 µL of electro-competent bacteria cells and the mixture was transferred to a special cuvette with two electrodes on its sides. Electroporation was carried out in a cell-electroporator (BioRad®, Hercules, CA) using the following settings: 125 µF capacitance, 200Ω (for *E. coli*) or 400Ω (for *A. tumefaciens*) resistance and 2.5V voltage. After electroporation, 500µl of LB medium was added and cells were left 30 min at

37°C (or 28°C for *A. tumefaciens*) for recovery. The transformed cells were spread onto solid LB medium containing the appropriate antibiotic.

Plasmid extraction

Transformed *E. coli* cells were grown overnight in 5 mL LB medium and plasmids were extracted by the alkaline lysis method. Cell culture was briefly centrifuged, supernatant was discarded and pellet was resuspended in 100 µL resuspension solution (50 mM glucose, 25 mM Tris and 10 mM EDTA). 200 µL lysis solution (2N NaOH and 1% SDS) was added and homogenized by inverting tubes. Cell lysis was incubated no longer than 5 min, then 150 µL of neutralization solution (3M CH3COOK, 23 mL CH3COOH and H2O to 200 mL) was added and mixed by inverting. Tubes were centrifuged for 5 min at 15000 rpm, subsequently supernatant was transferred to a new tube. One volume of phenol/chloroform was added, tubes were vortexed vigorously and centrifuged 10 min at 15000 rpm. The plasmid DNA containing aqueous phase was precipitated with 2 volumes of 100% ethanol and 200 mM NaCl for 30 min at -20°C. Tubes were then centrifuged for 30 min at 15000 rpm in a cooled centrifuge. The pellet was washed in 400 µL 70% ethanol to dissolve salt then dried. Finally, pellet was finally resuspended in 100 µL H2O.

A.4 Viral infection

***In vitro* transcription**

This technique is used to produce viral single-stranded RNAs from DNA clones. Two conditions are required to allow efficient transcription:

a) a transcription promoter should be upstream the DNA sequence that has to be transcribed,

b) DNA should be linearized where transcription has to stop.

The RiboMAXTM Large Scale RNA Production System – T7 kit (Promega, Madison, CA) was used to synthesize RNAs of interest, following manufacturer's protocol. DNA template (1 µg) was mixed with 4 µl Transcription Buffer 5x, 1.5 µl

of each rATPs (100 mM), rUTPs (100 mM), rCTPs (100mM), 0.06 µl of rGTPs (100 mM), 0.5 µl of RNAseOUT (40 U/µl) (Promega, Madison, CA), 1 µl of m7G5'ppp5' CAP analog (Promega, Madison, CA), 2 µl of T7 enzyme mix and sterile water up to a final volume of 20 µl. After incubation at 37°C for 30 minutes, 1.5 µl of rGTPs (100 mM) was added to the reaction mix that was then left for 3 h 30 min at 37°C to complete transcription.

Mechanical inoculation onto test plant

Leaves of host plants were mechanical rub-inoculated with viral RNA and/or transcribed RNAs. Each leaf was dusted with Celite to promote mechanical lesions and facilitate penetration of transcribed RNAs into plant cells, and then gently rubbed with the inoculum solution composed by 10 µg of each RNAs transcripts, 10 µl of potassium phosphate buffer (0,5 M KH2PO4 pH 7.5), 8 µl of macaloid 0.5% and sterile water up to 100 µl. After 7 days lesions from inoculated leaves were recovered and used for total RNAs or proteins extraction.

Agroinfiltration

Agroinfiltration is an efficient methods for transient expression of gene in plants. Transformed *A. tumefaciens* cells were grown O/N at 28°C in 5 ml of liquid LB medium supplemented with rifampicin (50 µg/ml) and kanamycin (100 µg/ml). Bacteria were centrifuged for 10 min at 5.000g and pellet was resuspended in MA buffer (10 mM MgCl2, 200 µM acetosyringone), adjusting the OD600nm to 0.6. Bacteria were then incubated at room temperature for 3 hours. Leaves of *Nicotiana benthamiana* plants were lightly incised with a scalpel and then infiltrated with the bacterial suspension using a syringe without the needle. Between 2 to 5 days post-infiltration leaves were harvested for analysis.

A.5 Western Blot

Protein extraction from plant material

Inoculated tissues were directly homogenized in 2x Laemmli buffer (125 mM Tris-HCl pH 6.8, 20% glycerol, 5% SDS, 0.01% bromophenol blue, 5% β-mercapatoethanol), boiled 3 min at 95°C to denature proteins and then centrifuged for 5 min at 13,000g to pellet cellular debris.

Separation of proteins under denaturing conditions (SDS-PAGE)

Proteins were separated according to their molecular weight on SDS-polyacrylamide gels. However, these were composed of two gels with distinct functions. The proteins separation occurs in the lower resolving gel, whereas the upper stacking gel was prepared at lower polyacrylamide concentration and is necessary for protein concentration. The stacking gel consists of 4% acryl-bisacryl (37,5:1), 125 mM Tris-HCl pH 6.8, SDS 0.1%. The resolving gel 10% acryl-bisacryl (37,5:1), 275 mM Tris-HCl pH 8.8, 0.1% SDS). The final addition of 0.05% TEMED and 0.1% ammonium persulfate catalyses the polymerization of acrylamide with bisacrylamide. The electrophoresis was performed at 80/100V in appropriated running buffer (25 mM Tris-Base pH 8.3, 192 mM glycine, 0.1% SDS).

Immunodetection of proteins by Western Blot

Proteins were transferred from the gel to a Hybond-PTM (GE Healthcare, Little Chalfont, Buckinghamshire, UK) polyvinylidene fluoride membrane in an electroblot apparatus (BioRad®, Hercules, CA). Prior to transfer, membranes were washed in 100% ethanol, rinsed in deionized water and incubated in transfer buffer (25 mM Tris-HCl; 0.2 mM glycine; pH 8.6) for a minimum of 15 min. Transfer was carried out at 4°C under 500mA and 80V current for 1-2 hours, depending on the protein size. After transfer the membrane was saturated in a 2.5% milk powder - 1x PBS (phosphate buffered saline) solution containing 0.5% Tween20R, for 1 hour at room-temperature or overnight at 4°C. The membrane was then incubated in milk-PBS-Tween containing the primary antibody at an

adequate dilution for 3-4 hours at room-temperature. Consequently, the membrane was washed 3-4 times in PBS-Tween buffer and incubated in milk-PBS-Tween containing the secondary antibody at an adequate dilution. This antibody is conjugated with horseradish peroxidase (HRP) for subsequent immunostaining. Three washing steps in PBS-Tween were performed, then proteins were detected by chemiluminescence using a Lumi-LightPlus Western Blotting Kit (Roche), which contains the substrate, and subsequent exposure to an autoradiography film.

10x PBS buffer composition: KH2PO4 (2.04 g/L) Na2HPO4 (14.24 g/L), KCl (2.01 g/L) and NaCl (87.66 g/L), pH adjusted to 7.4.

A.6 Northern blot

Radioactive probe preparation

The Promega® Prime-a-gene® labeling system was used to label PCR products with radioactive 32P. The reaction was performed as indicated by manufacturer: 2 µL of linear DNA is added to 31 µL H2O and heated at 95°C for 2 min. Ten µL of 5x reaction buffer (containing random synthetic hexadeoxynucleotide primers), 0.66 µL dATP (100 mM), 0.66 µL dTTP (100 mM), 0.66 µL dGTP (100 mM), 2 µL BSA (2 mg/mL), 2.5 µL α-32P labeled dCTP (corresponding to 25 µCi) and 0.5 µL DNA polymerase (Klenow fragment) were added and mixture was incubated at 37°C for 1 hour. Unincorporated dNTPs were eliminated on a Sephadex G-25 column (GE Healthcare, Little Chalfont, Buckinghamshire, UK) by spinning at 3000 rpm for 2 min. The probe was denatured by heating at 95°C for 5 min prior to hybridization.

Low molecular weight RNA analysis

For analyses of low molecular weight RNA, 15 µg of total RNA extract were denatured in 20 µL of 50% deionized formamide by heating at 65°C for 5 min, then rapidly cooled down on ice. Two µL of blue loading solution (50% glycerol, bromophenol blue, xylene cyanol) were added to samples prior to loading.

Polyacrylamide-urea gels were prepared (17.5% polyacrylamide: acrylamide/bisacrylamide 19:1; 8M urea; 0.5x TBE; 75 µL of 25% APS and 15 µL TEMED were added to polymerize 15 mL) and submitted to a 30 min pre-electrophoresis at 400V in 0.5x TBE migration buffer. Samples were loaded and migration was performed at 80-200V. Prior to transfer, gels were stained in ethidium bromide and photographs were taken under short wavelength UV light to get a loading control. Transfer of sRNAs to Hybond-NXTM (GE Healthcare, Little Chalfont, Buckinghamshire, UK) membranes, previously washed in TBE buffer, was carried out at 400 mA and 80V for 1 hour in 0.5x TBE buffer. Membrane was rinsed in 4x SSC and RNAs were UV-cross-linked to the membrane in a cross-linker. Membranes were pre-hybridized at 42°C for at least one hour in Perfect Hyb PlusTM hybridization buffer (Sigma-Aldrich, St. Louis, MO). Probe was added to buffer and hybridization was carried out overnight at 42°C. Washing steps were performed at 50°C in 2xSSC/2%SDS buffer, then twice in 1xSSC/1%SDS buffer, for 15 min each. Radioactive labeled sRNAs were detected by autoradiography.

20x SSC buffer: 3M NaCl, 300 mM trisodium citrate.

High molecular weight RNA analysis

For analyses of high molecular weight RNA, 2-5 µg of total RNA extract were denatured in 18 µL RNA loading buffer by heating at 65°C for 5 min then chilled on ice. Samples were loaded on a denaturing agarose gel (1% agarose, 1x HEPES, 6% formaldehyde) and migration was performed at 50-80V in 1x HEPES buffer. Gels were photographed under UV light prior to transfer. Transfer to Hybond-NXTM (GE Healthcare, Little Chalfont, Buckinghamshire, UK) membranes, previously rinsed in water and in 20x SSC for 30 min, was carried out overnight by capillarity in 20x SSC. The membrane was then rinsed in 4x SSC and RNAs were UV-cross-linked to the membrane. Membranes were pre-hybridized at 60°C for at least one hour in Perfect Hyb PlusTM hybridization buffer (Sigma-Aldrich, St. Louis, MO). Probe is added to buffer and hybridization is carried out overnight

at 55°C. Washing steps were performed at 65°C two times in 2xSSC/0.1%SDS buffer, then in 0.2xSSC/0.1%SDS buffer, for 30 min each. Radioactive labeled RNAs were detected by autoradiography.

A.7 Yeast transformation using the LiAc method

A yeast preculture was grown overnight in 50 mL YPD medium. This preculture was used to inoculated a 300 mL YPD culture aiming at preparing competent yeast cells for transformation. The initial OD600 was adjusted to 0.1-0.2. The culture was incubated at 30°C for about 3 hours under constant shaking at 250 rpm. When the OD600 reached 0.4-0.6, yeast cells were collected by centrifugation at 1000g for 5 min, then resuspended in 25 mL H2O. Cells were centrifuged again at 1000g for 5 min, supernatant was discarded and cells were resuspended in 1 mL of 1x TE/LiAc solution (10x: 1M lithium acetate (LiAc); 100 mM Tris and 10 mM EDTA, pH 7.5). For transformation, 10 µL carrier DNA (salmon sperm DNA, 10 mg/mL), previously denatured by boiling for 10 min then incubated on ice, were added to 300-400 ng of plasmid DNA (of each vector for co-transformation), then 100 µL of yeast cells were added and mixed by inverting tubes. Six hundred µL PEG/LiAc/TE (for 10 mL: 8 mL 50% PEG, 1 mL 1x LiAc and 1 mL 1x TE) was added to each transformation, mixture was gently vortexed, incubated at 30°C for 45 min and then 42°C for 20 min before cooling them rapidly on ice for 5 min. Cells were then sedimented by spinning, supernatant was discarded and cells were resuspended in 500 mL TE buffer and plated on selective medium plates. Plates were incubated at 30°C for 3 days.

Protein extraction from yeast cells

For protein extractions, 10 mL of yeast cultures grown in SD-LW medium for 2-3 days at 30°C, was centrifuged at 13000 rpm for 5 min. Pellet was washed in H2O and resuspended in 150µL extraction buffer (1.85M NaOH; 7.5% β-mercaptoethanol). One hudred and fifty µL 55% TCA were added and incubated on ice for 10 min. After centrifugation at 15000 rpm for 15 min at 4°C, as much

supernatant as possible was discarded and 50 µL PBS and 50 µL 2x Laemmli buffer were added. Proteins were denatured at 65°C for 5 min, several µL of 1N NaOH were added to restore the neutral pH of the solution, displayed by a blue color, and the proteins were loaded on polyacrylamide gels for electrophoresis and subsequent western blot analysis.

A.8 Biolistic transformation of BY-2 cells

This technique was used to analyze the subcellular localization of RFP or GFP fusion proteins.

Microcarrier preparation

Sixty mg of tungsten particles were washed in 1 mL 100% ethanol by vigorous vortexing for 3 min. Particles were sedimented by spinning for 1 min and supernatant was discarded, then particles were washed 3 times in 1 mL of water by vortexing for 1 min. Finally, the particles were resuspended in 500 µL of sterile 50% glycerol solution. In this solution, particles can be used immediately or stored at 4°C for up to 3 months. For DNA coating, 30 µL of particles solution was mixed with 5-10 µg of plasmid (at least 1µg/µL concentration is required) and vortexed for up to 10 min. Successively, 25 µL of 2.5M CaCl2, 10 µL of 0.1M spermidin and 1 mL of 100% ethanol were added and vortexed for 3 min after each step. Particles were briefly spinned-down and the supernatant was removed. A washing step in 70% ethanol was performed, then particles were resuspended in 40-50 µL 100% ethanol for subsequent bombardment.

Cell preparation and bombardment

A 3-4 days old BY-2 cell culture, grown at 25°C under 150 rpm shaking, was used for biolistic bombardment experiments. 10-12 mL of cells were spread on filter paper and put on small BY-2 medium plates (10 cm of diameter). Plates with cells were bombarded with a 5-6 µL aliquot of microcarrier suspension in a Biolistic PDS-1000/HeTM particle delivery system. Bombarded cells were incubated at

25°C for one night in the dark before confocal laser scanning microscopy observations.

A.9 Protoplast preparation

Small 2-weeks old *C. quinoa* plants were used for protoplast preparation. Well-expanded leaves were chosen and washed 3 times in sterile water to remove soil. 0.5–1 mm leaf strips from the middle part of a leaf were cut using a fresh sharp razor blade without tissue crushing at the cutting site. Strips from 10-20 leaves were directly put into a 0.4M mannitol solution, then leaf strips were transferred into 12 mL prepared enzyme solution (20 mM MES pH 5.4; 1.5% cellulase R10; 0.4% macerozyme R10; 0.4M mannitol and 20 mM KCl) by dipping both sides of the strips (completely submerged). The digestion is carried out, without shaking, in the dark at least for 3 h at 25°C. The enzyme solution should turn green after a gentle swirling motion, which indicates the release of protoplasts. Protoplasts were released by gentle shaking at 50 rpm for 15 min, then the protoplast suspension was filtered, after wetting the 100 µm filter with W5 solution (0.1M MES; 4M NaCl; 1M CaCl2; 0.5M KCl; 0.8M mannitol and 10% glucose). The flow-through was centrifuged at 100g to pellet the protoplasts in a 30-ml round-bottomed tube for 1–2 min. As much supernatant as possible was removed and the protoplast pellet was resuspended in 9 mL of W5 solution by gentle swirling and this suspension was used for subsequent treatment with fixative.

A.10 Confocal microscopy

Confocal laser scanning microscopy was performed using a Zeiss® LSM510 laser scanning confocal microscope. Excitation/emission wavelengths were 488 nm/505 to 545 nm for eGFP and 543 nm/585 to 615 nm for RFP. Images were acquired using LSM510 version 2.8 software (Carl Zeiss Imaging Solutions GmbH, Gottingen, Germany).

Appendix B

Primer sequences

Primer	**Sequence (5' – 3')**
BNYVV1AgroF	AAATTCGATTCTTCCCATTCG
BNYVV1AgroR	TTTTTTTTTTTTTTTATATCAATATACTG
BNYVV1R202-221	GGTGAATCGGTTCAGTTGTT
BNYVV2AgroF1	AAATTCTAACTATTATCTCCATTGAATAG
BNYVV2AgroR1	TTTTTTTTTTTTTTTCAATATACTGAAAAC
BNYVV3AgroF	AAATTCAAAATTTACCATTAC
BNYVV3AgroR	TTTTTTTTTTTTTTTGTCAATATACTGAC
BNYVV4AgroF	AAATTCAAATCTCAAAATATATTTG
BNYVV4AgroR	TTTTTTTTTTTTTTTGTCAATATACTGACAG
BNYVV5AgroF	AAATTCAAAGTACTTTCATATTG
BNYVV5AgroR	TTTTTTTTTTTTTTTGTCAATACACTGAC
BSBMV1AgroF	AAATTCGATCTTTCCCACCCAC
BSBMV1AgroR	TTTTTTTTTTTTTTTATATCAATACACTG
BSBMV1R13*Nco*I	CCCATGGTGATACAATACCTC
BSBMV1F12*Stu*I	CACAGGCCTCCTATCTTCGG
BSBMV2AgroF	AAATTCTAATTATTATCTCCATTG
BSBMV2AgroR	TTTTTTTTTTTTTTTCAATAAACTGAAAATAAACC
BSBMV2R*Mlu*I	TACGCGTGCCCATCGGTCG
BSBMV2F*Stu*I	CAGGCCTCCCATTGGGTTGTTCC
BSBMV3AgroF	AAATTTAAATCTATCACCACATTAGG
BSBMV3AgroR	TTTTTTTTTTTTTTTCTTCAATATACTGAAGG
BSBMV4AgroF	AAATTCAAAACTCAAAAATATAATTTTG
BSBMV4AgroR	TTTTTTTTTTTTTTTCAATAAACTGAAAATAC
BSBMVp14*Nco*IF	AAACCATGGAGAAAAGTAACAGCATAG
BSBMVp14*Bam*HIR	AAAGGATCCTTAGACAACATTGTTGTCCAACTC
BSBMVp14Ha *Bam*HIR	AAAGGATCCTCATGCATAATCAGGAACATCATAAGGATAGACA ACATTGTTGTCCAACTC
BSBMVp14*Sma*IF	AAACCCGGGGAGAAAAGTAATAGCATAG
BSBMVp14*Sal*IR	AAAGTCGACTTAGACAACATTGTTGTCCAACTC
BSBMVp14*Eco*RIF	AAAGAATTCATGGAGAAAAGTAATAGCATAG
BSBMVp14*Xba*IR	AAATCTAGATTAGACAACATTGTTGTCCAACTC

Résumé francais

“Mécanismes moléculaires à l’origine de la pathogenicité de phytovirus de betterave sucrière transmis par un vecteur tellurique”

Le virus des nervures jaunes et nécrotiques de la betterave (*Beet necrotic yellow vein virus*, BNYVV) est l’agent infectieux responsable de la rhizomanie de la betterave sucrière, une maladie caractérisée par une prolifération anarchique du chevelu racinaire. Le *Beet soil-borne mosaic virus* (BSBMV) appartient également au genre Benyvirus mais n’est retrouvé qu’en Amérique du Nord. Ce virus, identifié pour la première fois au Texas, est morphologiquement et génétiquement semblable au BNYVV mais sérologiquement éloigné. Compte tenu des différences moléculaires existant, le BSBMV et BNYVV correspondent à deux espèces virales distinctes.
Mon projet de thèse a consisté à étudier les interactions moléculaires entre le BNYVV et le BSBMV et rechercher les mécanismes impliqués dans la pathogénicité de ces deux virus.
Des clones complets cDNA infectieux du BNYVV étaient disponibles, tout comme ceux de BSBMV. Compte tenu de l’aspect versatile de l’obtention de transcrits infectieux de ces différents clones, j’ai entrepris de produire des clones cDNA de chacun des ARN viraux sous contrôle d’un promoteur constitutive végétal pour initier l’infection par agroinfiltration.
Les plantes hôtes *Chenopodium quinoa* et *Nicotiana benthamiana* ont été inoculées par des transcrits et agroinfiltrées pour initier l’infection virale et étudier l’interaction entre les ARN génomiques 1 et 2 des deux virus et étudier les propriétés de constructions chimères. En parallèle à ce travail, j’ai réalisé la caractérisation du suppresseur de RNA silencing du BSBMV en le comparant à celui du BNYVV.

Keywords: Benyvirus, agroinfection, post-transcriptional gene silencing, p14, chimeras.

Résumés anglais

“Molecular mechanisms involved in the pathogenesis of beet soil-borne viruses”

The genus *Benyvirus* includes the most important and widespread sugar beet viruses transmitted through the soil by the plasmodiophorid *Polymyxa betae*. In particular *Beet necrotic yellow vein virus* (BNYVV), the leading infectious agent that affects sugar beet, causes an abnormal rootlet proliferation known as rhizomania. *Beet soil-borne mosaic virus* (BSBMV) is widely distributed in the United States and, up to date has not been reported in others countries.
My PhD project aims to investigate molecular interactions between BNYVV and BSBMV and the mechanisms involved in the pathogenesis of these viruses.
BNYVV full-length infectious cDNA clones were available as well as full-length cDNA clones of BSBMV RNA-1, -2, -3 and -4. Handling of these cDNA clones in order to produce *in vitro* infectious transcripts need sensitive and expensive steps, so I developed agroclones of BNYVV and BSBMV RNAs, as well as viral replicons allowing the expression of different proteins.
Chenopodium quinoa and *Nicotiana benthamiana* plants have been infected with *in vitro* transcripts and agroclones to investigate the interaction between BNYVV and BSBMV RNA-1 and -2 and the behavior of artificial viral chimeras. Simultaneously I characterized BSBMV p14 and demonstrated that it is a suppressor of post-transcriptional gene silencing sharing common features with BNYVV p14.

Keywords: Benyvirus, agroinfection, post-transcriptional gene silencing, p14, chimeras