MEMOIRE

Présenté devant l'Université Victor Ségalen Bordeaux2

Pour obtenir

L'HABILITATION A DIRIGER DES RECHERCHES (HDR)

par

Frédéric Revers

Chargé de Recherche 1ère classe INRA

Unité Mixte de Recherche 1090 Génomique Diversité et Pouvoir Pathogène

IFR 103 Biologie Végétale Intégrative

« Du virus à la plante : bases génétiques et moléculaires des interactions Plante/Potyvirus »

Soutenue le 22 octobre 2009 devant le jury composé de :

Rapporteurs :

Dominique Roby, Directrice de Recherche CNRS, Toulouse

Carole Caranta, Directrice de Recherche INRA, Avignon

Stéphane Blanc, Directeur de Recherche INRA, Montpellier

Examinateurs :

Christophe Robaglia, Professeur, Université Aix-Marseille, Marseille

Michel Hernoud, Professeur, Université Victor Segalen Bordeaux2, Bordeaux

SOMMAIRE

CURRICULUM VITAE

Nom : REVERS
Prénom : Frédéric
Date de naissance : 13 avril 1968
Lieu de naissance : Bordeaux
Situation familiale : marié, deux enfants
Service militaire: accompli du 1/10/92 au 30/9/93

ENSEIGNEMENTS SUIVIS ET DIPLOMES ACQUIS

1993-1997 : **Thèse de doctorat** de l'Université Victor Ségalen Bordeaux 2 (mention "Sciences Biologiques et Médicales", Option " Biologie-Santé") : Bases moléculaires des propriétés biologiques du virus de la mosaïque de la laitue (LMV): variabilité et organisation génomique. Soutenue le 14 mai 1997.

1988-1992 : Elève de l'Ecole Normale Supérieure (ENS) de Cachan.

1988-1989 : Obtention de la **Licence de Biochimie** à l'Université Paris VII.

1989-1990 : Obtention de la **Maîtrise de Biochimie** à l'Université de Paris VII.

1990-1991 : Préparation et obtention de **l'Agrégation de Biochimie**.

1991-1992 : **Diplôme d'Etudes Approfondies** (D.E.A.) de biologie-santé, option "Pathologies microbiennes et virales" de l'Université de Bordeaux II. Mention "assez bien". Stage de DEA effectué à la Station de Pathologie Végétale du centre INRA de Bordeaux. Titre du rapport: "Développement de techniques de détection du virus des taches foliaires chlorotiques du pommier (ACLSV) par amplification de séquences (PCR)".

1985-1988 : Classes préparatoires au lycée M. Montaigne (Bordeaux). Concours d'entrée aux grandes écoles. Reçu au concours d'entrée à l'ENS de Cachan.

1987-1988 : Baccalauréat C

POSITION ACTUELLE

Depuis le 1er janvier 2003 : **Chargé de Recherche 1ème classe** dans l'Unité Mixte de Recherche, Génomique Diversité et Pouvoir Pathogène (ex Génomique du Développement et du Pouvoir Pathogène) (UMR GDPP 1090) à l'INRA de Bordeaux.

POSITIONS ANTERIEURES

1er septembre 1997 - 31 décembre 2002 : CR2 dans l'équipe de Virologie Végétale de l'Unité de Recherche en Santé Végétale (URSV) du Centre INRA de Bordeaux qui depuis janvier 2000 est intégrée à l'Institut de Biologie Végétale Moléculaire (IBVM) au sein de l'Unité Mixte de Recherche, Génomique du Développement et du Pouvoir Pathogène (UMR GDPP 1090) à l'INRA de Bordeaux.

15 septembre 1997 - 30 juin 1999 : Séjour dans le groupe de recherche d'Andy Maule, du département de Virologie, au Centre John Innes, Norwich, Angleterre.

Octobre 1996 - Août 1997 : **Attaché Temporaire à l'Enseignement et à la Recherche** (ATER) à l'Université Victor Ségalen Bordeaux 2. Enseignement en microbiologie (DEUG) et en biologie moléculaire (Licence de biochimie). Quatrième année de thèse à la Station de Pathologie Végétale du Centre INRA de Bordeaux.

Octobre 1993 - septembre 1996 : **Allocataire Moniteur Normalien** (AMN) à la Station de Pathologie Végétale du Centre INRA de Bordeaux. Enseignement en microbiologie (DEUG) et en biologie moléculaire (Licence de biochimie) à l'Université de Bordeaux II.

Octobre 1992 - septembre 1993 : **Scientifique du Contingent** (Service National) dans le Laboratoire de Biophysique Moléculaire, INSERM U386, Université de Bordeaux II dirigé par J.J. Toulmé. Sujet de recherche: "détection par photoaffinité de protéines cellulaires fixant les hybrides ADN/ARN " (encadré par C. Cazenave).

Septembre 1988 - septembre 1992 : **Elève-professeur** fonctionnaire stagiaire de l'ENS de Cachan.

ENCADREMENT D'ETUDIANTS / POSTDOCS

- **Depuis 1997**, **encadrement direct** de 2 postdocs (Juillet 2003 à juin 2004, décembre 2004 à juin 2006), d'une doctorante (2004-2007), de 3 DEA/DESS/M2 (2000-2001, 2004 et 2007-2008), de 2 M1 (2009), de deux BTS (2001, 2009) et d'un étudiant de maitrise (2001).
- **Participation au comité de thèse** de Chloé Marchive (UMR PBV, IBVM INRA Bordeaux ; directeur de thèse : Said Hamdi, soutenue fin 2006).
- **Tutorat de doctorant :** Sébastien Pons, école doctorale Sciences de la vie et de la santé (SVS), Université Bordeaux2 (2006-2009).

ACTIVITES D'ENSEIGNEMENT

- **Enseignement** depuis l'année universitaire 2003-2004 au niveau du **Master Recherche 2ième année** (Sciences de la vie, mention Biologie Santé, spécialité « Biologie et Biotechnologie des plantes », UE interaction plante-pathogène et épidémiologie) de l'Université Bordeaux2 : 2 heures de TD/an (3h à compter de la rentrée 2007) sur les gènes de résistance R.

- Participation régulière depuis 2004 au **jury du Master Recherche 1ère et 2ième année** (Sciences de la vie, mention Biologie Santé, spécialité Microbiologie (de 2004 à 2007) et spécialité Biologie et Biotechnologie des plantes (depuis 2007)) de l'Université Bordeaux2 pour l'épreuve de soutenance du mémoire bibliographique (1er semestre) et du stage de recherche (2ième semestre).
- Participation en tant que « professionnel » aux **jurys d'examen du BTS** « Bioanalyses et contrôles » pour des épreuves de TP de 2006 à 2008.
- Participation en 1999 à **l'organisation d'un module d'enseignement de Biologie Moléculaire** organisée par la formation permanente du centre INRA de Bordeaux (environ 50 heures): préparation et encadrement de travaux pratiques et intervention de 2 heures dans un module théorique.

PRODUCTION SCIENTIFIQUE

Depuis 1994 : 16 publications dans revues à comité de lecture , 457 citations, h = 10 (au 15 août 2009)

- Revues à comité de lecture : 16 articles
- Revues sans comité de lecture : 8 articles
- Revues de vulgarisation : 0 articles
- Chapitres d'ouvrage : 7
- Communications invitées : 1
- Communication à des congrès (au 15 août 2009): 72 (35 communications orales et 38 posters) dont 21 par F. Revers (12 posters, 9 communications orales)

EVALUATION ET ANIMATION DE LA RECHERCHE

RESPONSABILITES AU SEIN DU LABORATOIRE

- **Agent chargé de la prévention** (ACP) de janvier 2000 à décembre 2006 de l'équipe de Virologie. Participation à un groupe de travail sur les risques chimiques sur le centre INRA de Bordeaux (production d'une plaquette en septembre 2002)
- **Animateur** d'un groupe de travail sur la **prévention du risque incendie** à l'Institut de Biologie Végétale Moléculaire (dont fait partie l'UMR GDPP) de 2000 à 2007.
- **Responsable des commandes** "équipement" de l'équipe de Virologie et **gestion des réparations** d'équipements.
- **Pilote du processus** « Gestion des ressources humaines » dans le cas de la mise en œuvre de la démarche qualité pour la gestion de l'utilisation et du fonctionnement de la serre S3 du centre INRA de Bordeaux gérée par l'équipe de virologie.
- **Membre élu au conseil de service** (corps des chercheurs) de l'UMR GDPP depuis janvier 2007.
- **Animation d'un groupe (1 MC, 1 IE, 2 TR) et Encadrement direct** d'un TR (Luc Sofer depuis mai 2006) et d'un IE (Patrick Cosson depuis février 2006) et co-encadrement d'un TR (Mélodie Bousquet depuis septembre 2008).

COORDINATION DE PROJET

- **Co-animateur du projet** « Modifications de la régulation du génome consécutives à l'infection par un Potyvirus : Etude comparative entre la tomate et *Arabidopsis thaliana* » soutenu par **l'Action Structurante Tomate INRA** en 2003.
- **Co-animateur du projet** « Déterminants génétiques et moléculaires de l'interaction entre Potyvirus et *Arabidopsis thaliana* » financé par le **département SPE** (2005-2007).
- **Coordinateur du projet ANR-Génoplante** « ViroMouv » (projet ANR-08-GENM-016): Identification de facteurs de l'hôte impliqués dans le mouvement à longue distance des virus de plante (2009-2011, avec 2 autres partenaires).

EVALUATION DE LA RECHERCHE

- **Membre de jurys de concours** INRA : IE (2005), TR (2000, Président de jury).
- **Membre de Commissions d'évaluation collective pilotées par l'AERES :** UMR Santé de la Vigne et du Vin de Colmar (Février 2008).
- **Membre de la Commission Scientifique Spécialisée** « Biologie des Interactions Hôtes-Agresseurs, Symbiotes et Commensaux » (BIHASC, CSS 7) de l'INRA (Evaluation individuelle des chercheurs) (2007-2010).
- **Evaluation de projets de recherche pour les appels à projet du département SPE :** 11 projets en 2008, 8 projets en 2009 (en tant que membre du conseil scientifique SPE).
- **Expert auprès de l'Office allemand d'échanges universitaires** (DAAD) : 1 dossier expertisé en 2009.

PARTICIPATION AU CONSEIL SCIENTIFIQUE DE DIVERSES STRUCTURES

- **Membre élu du Conseil Scientifique du Département** « Santé des Plantes et Environnement » de l'INRA **depuis 2006.**

ACTIVITES D'ANIMATION

- **Animateur d'un club « doctorant »** de 1994 à 1997 au sein du centre INRA Bordeaux-Aquitaine
- **Secrétaire Général de la Société Française de Phytopathologie** : 1er mandat de 2005 à 2007, 2ième mandat depuis 2008 (Organisation et animation des conseils d'administration (1/an), gestion des adhésions et du site web http://www.sfp-asso.org/index.php).
- **Membre du comité d'organisation et du comité scientifique des Rencontres de Virologie Végétales** Edition 2009. Construction du site web des RVV (https://colloque2.inra.fr/rencontres_virologie_vegetale) en collaboration avec le service communication de l'INRA de Bordeaux (Je suis **Directeur de publication et co-gestionnaire du site**). En prévision : **organisateur principal pour les éditions 2011 et 2013**.
- **Membre du comité scientifique du 7ème Colloque National de la Société Française de Phytopathologie** tenu à Lyon du 8 au 11 Juin 2009**.**

RESPONSABILITES EDITORIALES

- **Revue** régulière **d'articles scientifiques** (en moyenne 4-5 articles par an pour MPMI, J. Virol, J. Virol. Methods, EJPP).

ACTIVITES
DE RECHERCHE

Du virus à la plante : bases génétique et moléculaire des interactions Plante/Potyvirus

ACTIVITES DE RECHERCHE

Classiquement lors de la soutenance d'une HDR, nous retraçons l'ensemble de nos activités de recherche généralement décomposé en 2 périodes, une allant de la thèse au recrutement en tant que chercheur, la deuxième depuis le recrutement. Je ne dérogerai pas à cette présentation classique, même si les deux périodes d'activité que je vais développées vont se rapporter surtout à l'un et à l'autre des deux acteurs majeurs de mes recherches, c'est-à-dire le virus d'un coté, la plante de l'autre, et que ces deux périodes d'activité partagent le même axe de recherche, l'étude des interactions plante/virus. Cela s'explique notamment par le fait que j'ai effectué quasiment toute mon activité de recherche au sein de la même équipe, celle de virologie végétale de l'INRA de Bordeaux animée par Messieurs Candresse et Le Gall.

En effet, lors de mon DEA (1991-1992) et de ma thèse (octobre 1993- mai 1997), mon activité s'est porté sur les virus, en particulier le virus des tâches foliaires chlorotiques du pommier (ACLSV) en DEA avec pour objectif de développer une technique de détection par PCR, et le virus de la mosaïque de la laitue (LMV) en thèse. Cette thèse puis celles qui ont suivi au sein du laboratoire ont permis de développer des outils et des connaissances de virologie moléculaire qui ont été à la base du projet actuel du laboratoire. Cette période est donc celle « DU VIRUS… ».

Suite à mon recrutement au sein de l'équipe de virologie végétale de Bordeaux réalisé juste après ma thèse (septembre 97), j'ai alors démarré des recherches sur la plante hôte et cela constitue la deuxième période d'activité. Elle a démarré par un séjour postdoctoral de 21 mois (septembre 97-juillet 99) dans l'équipe d'Andy Maule au Centre John Innes à Norwich pour travailler sur le pathosystème Pois/virus de la mosaïque du pois transmis par la graine (PSbMV, genre Potyvirus) et s'est poursuivi à Bordeaux sur le pathosystème *Arabidopsis thaliana*/LMV. Cette période est donc celle « …A LA PLANTE »

DU VIRUS…

1.1. Recherches pré-doctorales

J'ai réalisé mon DEA (biologie-santé, option "Pathologies microbiennes et virales", Université Bordeaux2) de septembre 1991 à juin 1992 dans la Station de Pathologie Végétale du centre INRA de Bordeaux à laquelle mon laboratoire de Virologie Végétale actuel appartenait. Le titre de ce premier stage de recherche fut "Développement de techniques de détection du virus des taches foliaires chlorotiques du pommier (ACLSV) par amplification de séquences (PCR)". Ce stage fut ma première expérience dans le domaine de la virologie végétale et m'a permis de m'initier à divers techniques de biologie moléculaire. Parmi les résultats obtenus, je citerai la mise au point d'un test de détection de l'ACLSV par Immunocapture-RT-PCR qui a fait l'objet de 2 publications dans Acta Horticulturae (**revue sans comité de lecture, Publications [19] et [23]**).

Suite à ce DEA, j'ai réalisé non pas immédiatement ma thèse mais un séjour d'un an (octobre 1992-Septembre 1993) dans le laboratoire de Biophysique Moléculaire, (INSERM U386, Université de Bordeaux II) dirigé par J.J. Toulmé comme scientifique du Contingent dans le cadre de mon service national. Mon sujet de recherche fut: "détection par photoaffinité de protéines cellulaires fixant les hybrides ADN/ARN " (encadré par C. Cazenave). Cette expérience fut pour moi riche puisqu'elle me permettait de parfaire ma formation en recherche avant d'aborder ma thèse et de séjourner dans un nouveau laboratoire dépendant d'un organisme de recherche différent de l'INRA. De plus, cela m'a permis de produire deux nouvelles publications dans des revues à comité de lecture (**Publications [1] et [7]**).

1.2. Thèse de doctorat

En octobre 1993, désireux de poursuivre mon activité de recherche dans le domaine de la phytopathologie, j'ai démarré ma thèse de doctorat (mention "Sciences Biologiques et Médicales", Option " Biologie-Santé", Université Bordeaux2) grâce à un financement d'allocation moniteur normalien (AMN), ayant été jusqu'à la fin de mon DEA élève de l'Ecole Normale Supérieure (ENS) de Cachan. J'ai effectué cette thèse dans mon équipe actuelle mais à ce moment là toujours intégrée à la station de pathologie végétale à l'INRA de Bordeaux, sous l'encadrement de Thierry Candresse et Olivier Le Gall.

Cette thèse intitulée «Bases moléculaires des propriétés biologiques du virus de la mosaïque de la laitue (LMV): variabilité et organisation génomique » fut la première étude menée au laboratoire sur les interactions moléculaires entre plante et virus. A cette époque, les données sur l'identification de déterminants viraux impliqués dans les interactions plante/virus étaient encore peu nombreuses et le couple laitue (*Lactuca sativa*) / LMV présentait plusieurs particularités qui en faisait un modèle de choix pour ce type d'étude. Le LMV appartient au groupe des potyvirus, groupe de virus phytopathogènes le plus important tant par le nombre de virus identifiés que par les dégâts causés aux cultures dans le monde entier. Au moins deux gènes de résistance au LMV ont été décrits chez la laitue, $mo1^1$ et $mo1^2$, gènes récessifs, les plus efficaces et les plus utilisés en lutte génétique contre le LMV chez les laitues cultivées. Le LMV se caractérise par une large variabilité biologique qui s'exprime notamment dans la sévérité des symptômes induits, dans sa capacité à être transmis par les semences de laitue et dans sa capacité à contourner les gènes de résistance *mo1* de la laitue. L'apparition récente d'isolats de LMV capables de contourner l'ensemble des gènes de

résistance a suscité de l'intérêt pour une meilleure compréhension des interactions moléculaires entre la laitue et ce virus.

Un projet collaboratif a alors été initié en 1993 entre notre équipe et celle d'Hervé Lot, DR à la station de pathologie végétale à l'INRA d'Avignon. L'objectif principal du projet était de réaliser une caractérisation moléculaire du LMV afin d'identifier coté virus les déterminants moléculaires impliqués dans plusieurs processus biologiques tels que contournement des gènes de résistance *mo1*, sévérité des symptômes ou transmission par la graine. La stratégie retenue fut de produire des clones ADNc infectieux à partir de deux souches de LMV différant par leurs propriétés biologiques à partir desquels des clones chimères pourraient être construits et analysés pour leurs propriétés biologiques.

Etant donné le peu de données moléculaires disponibles sur le LMV au début de ma thèse, mon travail a consisté à :

1. étudier la variabilité moléculaire de ce virus afin d'identifier deux isolats proches sur le plan moléculaire mais relativement distinct de par leurs propriétés biologiques

2. séquencer le génome complet d'un des deux isolats de LMV

3. tenter de construire par longue PCR un clone ADNc infectieux de cet isolat.

Pour étudier la variabilité moléculaire du LMV, nous avons opté pour le séquençage de 3 régions du génome viral, une région à l'extrémité 5' du génome viral correspondant à la protéine P1 et deux régions à l'extrémité 3' correspondant à la jonction protéine NIb / protéine de capside (CP) et à l'extrémité C-terminale de la CP, de 10 isolats de LMV.

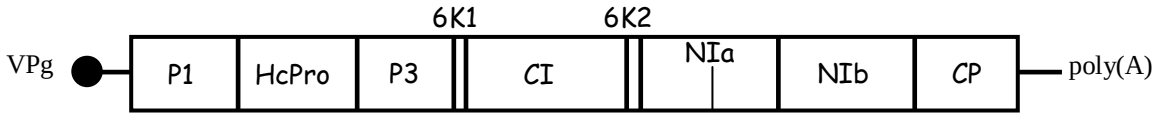

Figure 1 : Organisation du génome des potyvirus. La polyprotéine est représentée par le rectangle. Les sites de clivage sont représentés par des traits séparant chacune des régions des 10 protéines matures.

Le fait de choisir 3 régions plutôt qu'une a été motivé par un travail préalable que j'ai effectué en début de thèse où nous avons révélé des fréquences de recombinaisons élevés entre isolats d'un même potyvirus. Sélectionner nos deux isolats de LMV sur la base de séquence d'une seule région engendrait en effet le risque de sélectionner des isolats à priori proches sur cette région mais relativement distant sur le reste du génome lié à de possibles événements de recombinaison. Les résultats sur la fréquence élevée de recombinaisons chez les potyvirus d'une part, et sur la variabilité moléculaire du LMV d'autre part sont présentés dans les deux publications suivantes (**Publications [2] et [3]*)***.

Journal of General Virology (1996), 77, 1953–1965. Printed in Great Britain

Frequent occurrence of recombinant potyvirus isolates

F. Revers,[1] O. Le Gall,[1] T. Candresse,[1] M. Le Romancer[2] and J. Dunez[1]

[1] Station de Pathologie Végétale, INRA, BP 81, 33883 Villenave d'Ornon Cedex, France

[2] Station de Pathologie Végétale, INRA, BP 29, 35650 Le Rheu, France

We have performed a systematic search for recombination in the region encoding coat protein and the 3′ non-translated region in natural isolates of potyviruses, the largest group of plant RNA viruses. The presence of recombination, and the localization of the cross-over points, were confirmed statistically, by three different methods. Recombination was detected or suspected in 18 out of 109 potyvirus isolates tested, belonging to four out of eight virus species, and was most prevalent in potato virus Y, clear in bean common mosaic virus, and possible in bean yellow mosaic and zucchini yellow mosaic viruses. Recombination was not detected in the four other potyvirus species tested, including plum pox virus, despite the availability of numerous sequences for this last species. Though it was not specifically researched, no evidence for inter-specific recombination was found. For several reasons, including the fact that only a minor portion of the genome was analysed, the above figures certainly represent an underestimate of the extent of recombination among isolates of potyviruses, which might thus be a common phenomenon.

Introduction

There is growing evidence that RNA recombination is a major evolutionary factor in plant RNA viruses (Koonin, 1991; Simon & Bujarski, 1994). Instances of recombination between genomic viral RNAs have been described for natural isolates of tobraviruses (Robinson *et al.*, 1987; Goulden *et al.*, 1991), bromoviruses (Allison *et al.*, 1989), viroids (Rezaian, 1990), nepoviruses (Rott *et al.*, 1991; Le Gall *et al.*, 1995 *a*), potyviruses (Cervera *et al.*, 1993) and luteoviruses (Rathjen *et al.*, 1994; Gibbs & Cooper, 1995). The phenomenon has also been observed in laboratory isolates of cucumoviruses (Fernández-Cuartero *et al.*, 1994), nepoviruses (Le Gall *et al.*, 1995 *b*), hordeiviruses (Edwards *et al.*, 1992), carmoviruses (Cascone *et al.*, 1990) and bromoviruses (Bujarski & Kaesberg, 1986; Rao & Hall, 1990), and was particularly well studied in the last two cases (Carpenter & Simon, 1994; Nagy & Bujarski, 1995). Recombination between viral and cellular RNAs has also been described, both in natural (Mayo & Jolly, 1991; Sano *et al.*, 1992) and experimental (Greene & Allison, 1994) systems.

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The sequence of the CP region of PVY-LB has been deposited in GenBank, accession number X92078.

To date, however, no efforts have been directed at a systematic search of viral genomes for recombination. The potyvirus group is particularly well suited for such a search for several reasons. Firstly, it is the largest genus of plant viruses (Ward & Shukla, 1991), with many members partially or fully sequenced. Secondly, RNA recombination is known to be possible in this genus: it has been described between field isolates of plum pox virus (Cervera *et al.*, 1993) and may also have occurred between an isolate of turnip mosaic virus and a cellular sequence (Sano *et al.*, 1992). We have attempted to detect recombinant isolates in several potyviruses. Our approach, inspired by that used by Chenault & Melcher (1994) on cauliflower mosaic virus, is based on the search for virus isolates showing different clustering properties in trees constructed using different short regions of their genomes. The results obtained may then be confirmed by various statistical means.

Using this approach, we demonstrate the occurrence of recombination in the region encoding the capsid protein (CP) and in the 3′ non-translated region (3′NTR) of several isolates of potato virus Y (PVY) and bean common mosaic virus (BCMV). Potential recombinants were also observed for bean yellow mosaic virus (BYMV) and zucchini yellow mosaic virus (ZYMV), but apparently not for watermelon virus II (WMV2), turnip mosaic virus (TuMV), plum pox virus (PPV) and papaya ringspot virus (PRSV).

0001-3870

Methods

■ **Viral sequences.** Nucleotide sequences with accession numbers as follows were retrieved from the GenBank database (the designations used in this paper for the different isolates are indicated in parentheses when applicable).

BCMV: L11890 (Mex), L12470 (US1), L15332 (NL1), L19472 (NL2), L19473 (US5), L19474 (US7), L19539 (CH2), L21766 (NL4), S66251/S66275 (NL1b), S66252/S66279 (NY15), S66253/S66280 (BlCMV), U05771 (PStV-Bl2), X63559 (PStV-Bl1), Z15057 (B), Z21700 (PStV-Ib).

BYMV: D00490 (GDD), D00604 (CS), D28819 (MB4), S77515 (S), X53684 (DG), X81124 (MI).

PPV: D13751, M21847, M92280, U27652, S57404, S57405, X16415, X56258, X57975, X57976, X81073, X81074, X81075, X81076, X81077, X81078, X81079, X81080, X81081, X81082, X80183, X81084. A corrected version of sequence X16415 was used (M. Ravelonandro, personal communication).

PRSV: D00594, D00595, D50591, I06996, S89893, U14736, U14737, U14738, U14739, U14740, U14741, U14742, U14743, U14744, X67672, X78557.

PVY: D00441 (Fr), D12539 (O4), D12570 (T), M81435 (US), M95491 (H), U06789 (VN), U09508 (N US), U09509 (O7), U10378 (H VN), U25672 (Ch2), S74810 (36), S74813 (T13), X14136 (O1), X54058 (Ch1), X54611 (Hu), X54636 (N Rus), X68221 (Chil), X68222 (Pot US), X68223 (Eur H), X68224 (NsNr), X68225 (MsNr), X68226 (O'), X79305 (NL2), X92078 (LB). In addition, the sequences of the following isolates, which are not in the databases, were included: PeMV (Dougherty *et al.*, 1985), Is (Rosner & Raccah, 1988), Go16 (Wefels *et al.*, 1989), NL1 (van der Vlugt *et al.*, 1993), Th (Hataya *et al.*, 1990), O2 (Lawson *et al.*, 1990) and M1, partially sequenced by one of us (Le Romancer, 1993).

TuMV: D10601, D10927, L12396, X52804, X65978, X81140, X81141, X83968.

WMV2: D00535, I06999, I06186, L22907.

ZYMV: D00593 (Flo), D00692 (Con), I07016 (Pat15), L29569 (Reun), L31350 (Cal), M35095 (Is), X62662 (Sin).

The three sub-fragments of these nucleotide sequences used in this study were called N-ter, C-ter and 3'NTR. The N-ter region is defined as that encoding the hyper-variable amino-terminal extension of the CP gene (up to, but excluding, the amino acid homologous to the first lysine in the motif KDKDVNAG of PVY-N; see Rybicki & Shukla, 1992). The C-ter region is that encoding the carboxy terminus of the CP [from, and including, the motif RYGLIRN in PVY-N (see Rybicki & Shukla, 1992) and including the termination codon]. The 3'NTR, when available, is the 3' non-translated region, excluding the termination codon.

■ **Computer analysis of the sequences.** Multiple alignments of the selected regions were obtained using the program ClustalV (Higgins *et al.*, 1992). Distance matrices were calculated from these alignments based on the Dayhoff PAM matrix (Dayhoff *et al.*, 1983), using the program DNADIST in version 3.5 of the PHYLIP package (Felsenstein, 1989). Clustering was done from these matrices using the program NEIGHBOR in PHYLIP, implementing the UPGMA (average linkage clustering) method. Unrooted trees could finally be printed using the utility program DRAWTREE in PHYLIP. The statistical significance of the branching order was estimated by performing 100 replications of bootstrap resampling of the original alignment using SEQBOOT (Felsenstein, 1985), and synthesizing the resulting set of trees using CONSENSE.

In addition, the statistical test for recombination developed by Sawyer (1989) was used, as implemented in the program VTDIST (kindly provided by Dr S. Sawyer), to statistically validate the recombination events suspected from the clustering tree topologies. Dr Sawyer also provided us with an unreleased version of this program, VTDIST2, that allows pair-wise comparisons of the input sequences. In this test, the totally conserved positions are omitted ('condensation') from the alignment, and for each sequence pair a set of fragments is defined between successive sites where these two sequences differ. Two working parameters are defined, MCF (maximal size of the condensed fragments), and SSCF (sum of the squares for condensed fragments). Two additional parameters (MUF and SSUF) are defined similarly but omitting the condensation step. Statistical comparison of the parameter values obtained with random permutations of the sequences or with the actual data provides an estimation of the significance of the size distribution of the fragments, and hence of the likelihood that genetic rearrangements have occurred. We found that the results of the VTDIST analysis became very difficult to interpret when sequences that were identical or differed at only one position were present in the dataset. Therefore, in some cases we inserted only one sequence of such pairs into the dataset analysed by VTDIST.

The recombination breakpoints were tentatively localized using the maximum chi-squared approach described by Maynard-Smith (1992), as implemented in the program RecSite (O. Le Gall, unpublished). This statistical method searches along a recombinant sequence for the limit between the blocks, and estimates the probability for the null hypothesis (no recombinant structure). In addition, RecSite determines the percentage identity between the sequences in each block, which helps to understand the structure of the recombinant sequence.

Results

PVY

A databank and literature search allowed identification of the region encoding the CP of 29 PVY isolates, 12 of which also included the 3'NTR. The pepper mottle virus isolate sequenced by Dougherty *et al.* (1985) was included in our analysis since it is now considered to be a strain of PVY (Robaglia *et al.*, 1989). In addition, we sequenced the CP–3'NTR of two more isolates, LB and M1, except for a region in M1 internal to the CP coding region (from positions 8954–9097, numbered according to the complete sequence of isolate Fr, as in Robaglia *et al.*, 1989). Three regions in the PVY sequences were selected and analysed as described above. The N-ter region extends from positions 8573–8659, the C-ter region from positions 9119–9376 and the 3'NTR region from positions 9377–9704 of isolate Fr.

The general topologies of the trees constructed using the N-ter, C-ter and 3'NTR regions are similar (Fig. 1), with two major sub-groups of isolates being delineated beside a less homogeneous set of isolates, as already described by van der Vlugt *et al.* (1993). Sub-groups I and II roughly correspond to the N and O pathogenicity and serology sub-groups of PVY respectively. The rest of the PVY isolates cluster in a third sub-group in the N-ter tree, though with a lower bootstrap value (47%), and are even more widely dispersed in the C-ter tree (Fig. 1*b*). This set of isolates corresponds to the third sub-

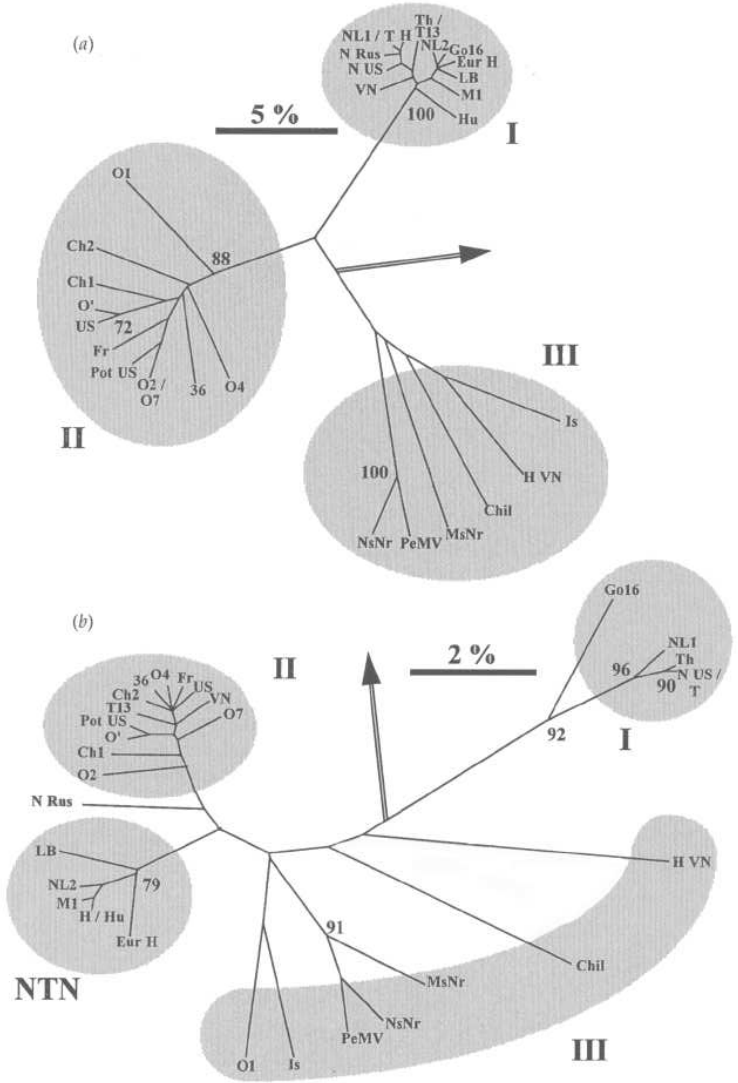


Fig. 1. Clustering trees derived from the N-ter (*a*), C-ter (*b*) and 3′NTR (*c*) regions of PVY, as defined in the text. The different sub-groups discussed in the text are shaded. The branching of the corresponding sequences of PStV-Bl2, used as an outgroup, is indicated by a double arrow. The percentage of bootstrap replicates in which each node was recovered is given when above 70%, with the omission of the 100% value for the outgroup branch. The scale bar represents, for the branch lengths, 5% (*a*) and 2% (*b*, *c*) substitutions per site.

Table 1. VTDIST analysis of the potyviruses studied

Virus	Parameter†	Parameter value†	*P*-value‡ (%)	SD above sim. mean§	SD of sims‖
BCMV	SSCF	272142	0·09	4·42	11889·66
	MCF	126	39·57	0·12	26·95
	SSUF	5925582	0·31	3·70	277659·95
	MUF	583	32·88	0·30	122·54
BYMV	SSCF	17736	0·01	5·99	777·48
	MCF	56	0·20	4·74	5·94
	SSUF	605209	< 0·01	8·26	26720·09
	MUF	362	0·04	5·80	33·91
PPV*	SSCF	1621475	3·60	2·05	91193·46
	MCF	195	32·49	0·47	27·06
	SSUF	25187052	42·66	0·11	2369358·76
	MUF	674	62·77	−0·34	82·58
PRSV*	SSCF	171854	10·73	1·26	9584·78
	MCF	168	5·86	1·79	27·17
	SSUF	3845941	17·57	0·88	198327·01
	MUF	703	9·23	1·53	118·65
PVY*	SSCF	1225061	< 0·01	13·51	28537·01
	MCF	230	0·75	2·48	29·76
	SSUF	21199878	< 0·01	12·82	621145·16
	MUF	767	1·16	2·11	93·02
TuMV*	SSCF	6896	21·08	0·71	596·99
	MCF	25	55·58	−0·20	5·79
	SSUF	724803	41·21	0·03	86153·17
	MUF	251	73·70	−0·67	71·27
WMV2	SSCF	4689	57·83	−0·40	1170·72
	MCF	53	57·51	−0·31	13·52
	SSUF	982188	33·42	0·30	185768·77
	MUF	792	39·50	0·37	169·87
ZYMV	SSCF	35126	< 0·01	6·18	2713·347
	MCF	137	0·68	3·03	19·867
	SSUF	1408284	1·35	2·92	131904·01
	MUF	673	13·34	1·17	137·92

* Only the CP coding region was considered in the analysis reproduced here.
† See text for parameter definitions.
‡ Proportion of permuted datasets, out of 10000, yielding higher scores than the actual data.
§ Number of standard deviations above simulation mean.
‖ Standard deviation of the simulations.

group described by van der Vlugt *et al.* (1993), and will thus be named sub-group III in this paper. In the C-ter tree, isolate Eur H and all of the isolates (H, Hu, NL2, LB and M1) belonging to the NTN biotype (necrotic-tuber necrosis: Le Romancer & Kerlan, 1992; Le Romancer *et al.*, 1994; van den Heuvel *et al.*, 1994) are clustered together close to sub-group II.

Several PVY isolates show markedly different affinities depending on the genomic region considered. Such is clearly the case for isolates T13 and VN, which shift from sub-group I in the N-ter tree to sub-group II in the C-ter tree and in the 3′NTR tree for VN (no sequence information is available for T13 in this region). Isolates N Rus and NTN (including Eur H) also have different positions in the different trees, being found in sub-group I of the N-ter tree, and shifting to a position slightly removed from the main sub-group II cluster in the C-ter tree (as already described above for the NTN and Eur H isolates), and well within sub-group II in the 3′NTR tree (3′NTR sequences are not available for some NTN isolates and for Eur H). The bootstrap value of sub-group II is low in the C-ter tree, but the high value of sub-group I in this tree allows us to draw the conclusion that these isolates significantly 'leave' this sub-group in this region, and for the isolates for which the

3′NTR was analysed this is confirmed with a 100% bootstrap value for sub-group II. Isolate Fr clusters in sub-group II in both the N-ter and the C-ter trees, but in sub-group I in the 3′NTR tree, and H VN similarly shifts from sub-group III to sub-group II in the 3′NTR tree. Isolate O1 clusters in sub-group II in the trees derived from N-ter (with a bootstrap value of 88%) and 3′NTR (bootstrap value of 100%) but in sub-group III in the C-ter tree, though with a low bootstrap value (58% for the branching between O1 and Is; data not shown). These 12 isolates are thus good candidates for being the results of RNA recombination. In the case of isolate O1, two successive recombination breakpoints could lead to the double shift observed.

In the second step of our analysis, we proceeded to evaluate the statistical significance of these potential recombination events. Sawyer (1989) devised a statistical test for genetic rearrangement based on the imbalance in size distribution of regions in which pairs of sequences within a dataset are identical. We used a dataset containing the entire CP sequences, rather than the terminal regions used previously, to run the VTDIST program implementing this test. The parameters considered differed significantly from a randomized dataset, with a probability lower than 1·2% of the null hypothesis that no recombination occurred (Table 1). A similar result was obtained when another dataset was used, in which the entire CP-3′NTR sequences were included, but in which the number of isolates was restricted to those for which this information was available (not shown). This analysis provides strong statistical evidence for RNA recombination in PVY, and confirms the previous analysis made on the basis of the topology of the trees; however, it does not tell us which sequences are recombinant.

In order to answer this question statistically, we performed two further types of analysis using VTDIST, either by comparing the sequences pair-wise or by comparing each of them with consensus sequences representative of each sub-group, as defined in Fig. 1. First of all, we selected sequences clustering unequivocally in the same manner in all the trees to represent their sub-groups. Sub-group I thus contained isolates Go16, NL1, N US, T and Th, sub-group II contained isolates O2, O4, O7, O′, US, Ch1, Ch2, 36 and Pot US, and sub-group III contained isolates Is, PeMV, Chil, NsNr and MsNr. Consensus sequences were then inferred for each sub-group from a multiple alignment made using ClustalV. In sub-group III, the 3′NTR sequence has been determined only for isolates Is and PeMV, so that a consensual sequence was not possible to determine, and the PeMV sequence was arbitrarily used as the 'consensus' in this region.

All of the PVY sequences were then analysed using VTDIST in a reduced dataset consisting of the three consensus sequences and the isolate to be analysed (Table 2). Depending on their availability for each isolate, either the CP–3′NTR or only the CP coding region was used for these analyses. The 3′NTR of PeMV was excluded from the analysis because of the previous arbitrary choice of this sequence as the consensus for sub-group III. Analysis of isolate M1 was restricted to the region for which its sequence is known. For Fr and H VN, comparisons were made only with isolates for which the sequence of the entire CP–3′NTR is known. The SSCF-associated *P*-values are shown for all the PVY isolates in the first column of Table 2. All of the 12 isolates detected above as possible recombinants, on the basis of their clustering, were confirmed to be so at the 3% level, except for Hu and O1. We also compared all the pairs of PVY sequences using VTDIST2, and for each sequence calculated the mean of the *P*-values obtained when this sequence was compared with isolates representing each subgroup. The results of this pair-wise analysis are given in columns two to four of Table 2. This approach allows us to determine not only which isolates are recombinant, but also between which sub-groups they shift. Again, the results described above were confirmed at the 3% level, except for isolate O1. This time, isolate Hu was confirmed to be a putative recombinant between sub-groups I and II. One of the parents of H VN was predicted to belong to sub-group II, but no prediction could be made as to its second parent using this approach.

Finally, we looked for the location of the recombination breakpoints in the candidate PVY recombinant isolates. For this analysis, we used the approach described by Maynard-Smith (1992), as described above. This approach first determines the structure of a recombinant sequence, and then attributes a significance level to this structure. Here again, each of the PVY sequences was compared with the consensus representing each sub-group. The results of this last analysis are given in columns five and six of Table 2. Using this approach, all the putative recombinants determined above were confirmed at the 3% level, except O1 (but this approach is not optimal for detecting multiple recombinants; Maynard-Smith, 1992). No recombinant structure could be detected that connected with the low *P*-values found for sub-group II isolates O4, O′, O2 and Ch2. Visual inspection of the alignment confirmed that in these four isolates, the significantly high chi-squared value maps to a position close to either end of the alignment, where random mutations in the query sequence weigh more heavily on the statistics than elsewhere (data not shown).

In all cases the structures, as displayed in column five of Table 2, confirm the polarity of recombination predicted from the cluster analysis, except again for isolate O1. At least six different recombination breakpoints were detected in the eleven recombinant isolates (because the recombinant nature of O1 could not be confirmed, this isolate is not included here), four of them in the coding region and two of them in the 3′NTR. As an example, Fig. 2 shows the sequence alignments around the five recombination breakpoints found between sub-groups I and II. In all of these isolates except Fr, the sequences 5′ to the recombination point are of sub-type I and those 3′ to this point are of sub-type II. The location of the recombination

Table 2. Statistical determination of the PVY recombinant isolates and prediction of their recombination breakpoints

PVY isolate	VTDIST*				Maynard-Smith	
	%cons†	%I‡	%II‡	%III‡	Structure/site§	P‖
Go16	7·58	32·60	44·20	21·02	Not applicable	6·1
NL1	48·37	67·46	56·87	45·06	Not applicable	99·8
N US	46·17	58·55	51·10	40·77	Not applicable	9·3
T	22·38	59·92	54·74	45·27	Not applicable	7·2
Th	8·12	64·12	43·07	62·65	Not applicable	64·9
VN	< 0·01	0·03	0·63	47·61	I/8714-8715/II	< 0·1
N Rus	< 0·01	0·21	2·04	59·78	I/8747-8748/II	< 0·1
T13	< 0·01	0·26	0·33	60·14	I/9137-9144/II	< 0·1
LB	< 0·01	0·15	1·24	36·46	I/9170-9183/II	< 0·1
H	< 0·01	0·16	1·46	41·49	I/9170-9183/II	< 0·1
Hu	13·10	2·09	2·92	62·48	I/9170-9183/II	0·3
NL2	2·06	1·83	0·51	58·15	I/9170-9183/II	< 0·1
Eur H	2·75	0·70	0·54	67·54	I/9170-9183/II	< 0·1
M1	< 0·01	NT	NT	NT	I/9170-9183/II	< 0·1
Fr	< 0·01	< 0·01	< 0·01	34·37	II/9455-9466/I	0·1
O4	27·78	68·79	19·79	65·40	None found	0·3
O7	20·75	60·94	22·01	43·95	Not applicable	27·7
US	54·28	75·98	28·95	68·58	Not applicable	20·8
O′	7·87	22·23	15·58	76·35	None found	1·0
O2	77·74	32·76	35·44	67·00	None found	1·1
Ch1	78·62	67·23	73·29	34·58	Not applicable	48·5
Ch2	94·26	66·53	14·74	73·99	None found	3·1
36	11·69	33·81	22·91	81·19	Not applicable	13·4
Pot US	73·31	21·70	34·29	70·17	Not applicable	40·4
O1	78·81	54·97	47·05	43·05	Not applicable	23·0
H VN	< 0·01	22·45	< 0·01	24·92	III/9378-9379/II	2·1
Is	17·00	33·30	73·10	8·11	Not applicable	53·1
PeMV	18·79	37·39	70·35	32·12	Not applicable	98·5
Chil	70·87	25·39	22·48	36·03	Not applicable	26·1
NsNr	13·18	48·16	73·73	40·34	Not applicable	96·8
MsNr	31·96	70·52	83·24	38·13	Not applicable	59·7

* SSCF-associated *P*-values (as percentages) determined using VTDIST. NT, Not tested.
† Comparison of the PVY sequences with the consensus representing the sub-groups defined in Fig. 1.
‡ Mean *P*-value of the null hypothesis, as determined after pair-wise comparisons of each sequence with those of sub-groups I, II or III.
§ Recombination breakpoint as predicted by RecSite; the sequences 5′ to the site indicated are of the type indicated on the left; those 3′ to it are of the type indicated on the right.
‖ Percentage probability of the null hypothesis against this structure, as determined after testing 1000 randomly mutated sequences.

breakpoint within the C-ter region in isolates LB, H, Hu, Eur H, NL2 and M1 explains their clustering slightly outside the rest of sub-group II in the C-ter tree (Fig. 1*b*).

It is striking to note that all the isolates (H, Hu, NL2, LB and M1) belonging to the biological type NTN are recombinants between sub-groups I and II, with recombination breakpoints within the same short region. Isolate Eur H, whose biological type is not as clearly defined (Sudarsono *et al.*, 1993), is characterized by the same recombination breakpoint. Whether or not there is a link between this recombination event and the NTN phenotype cannot be ascertained at the moment. These isolates could either have a common (recombinant) ancestor that had the NTN phenotype independently of its recombinant status, or the NTN phenotype could be a direct result of the recombination event, which could then have happened several times independently. The first hypothesis is strengthened by the presence, at several positions within the CP–3′NTR, of nucleotide variations unique to these isolates (not shown).

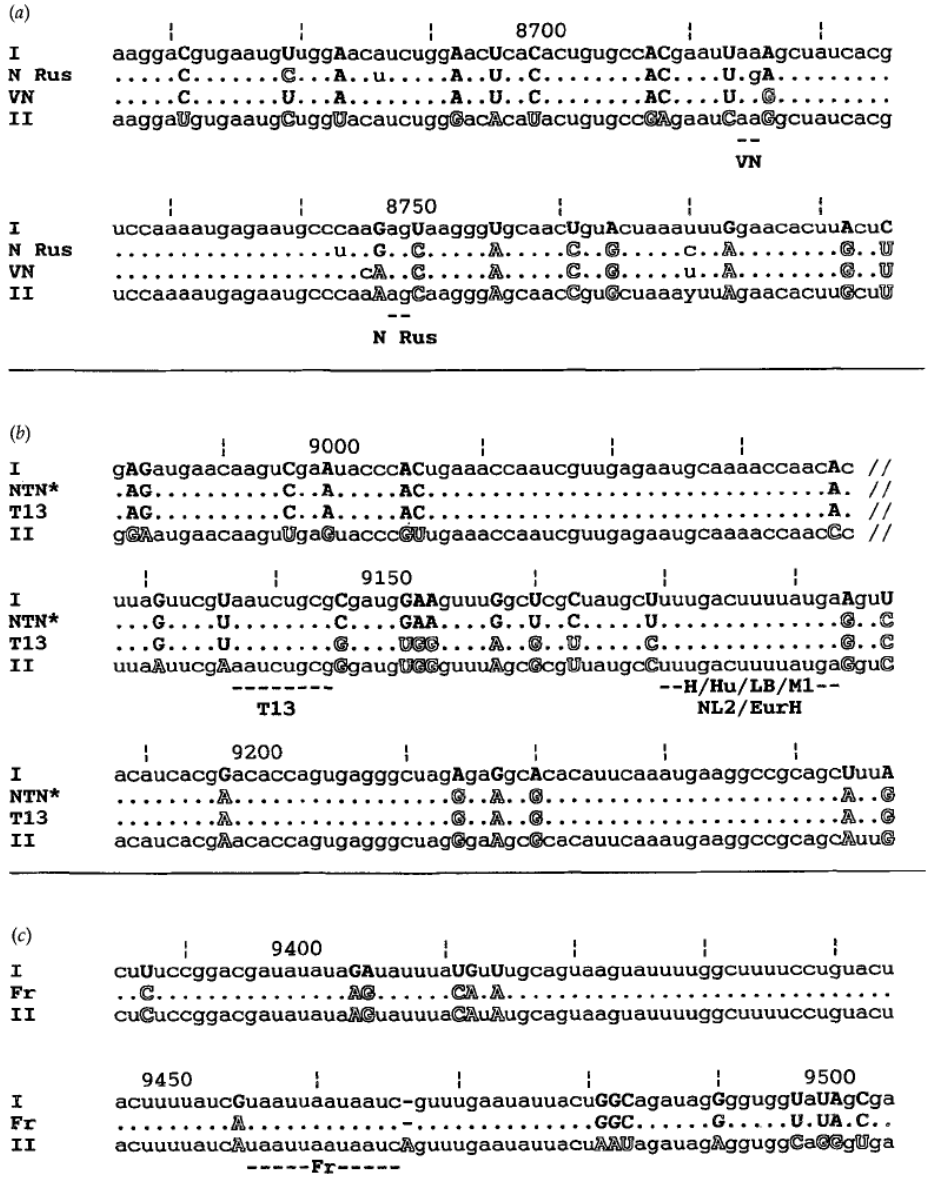
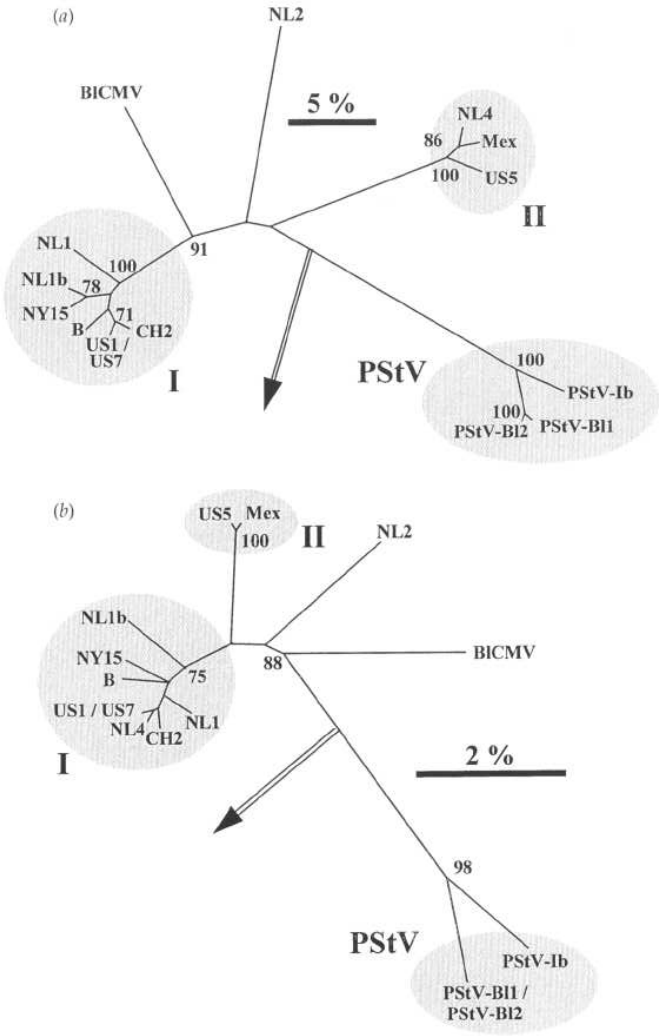


Fig. 2. Alignments of the putative recombinant PVY sequences shown with the consensus sequence of sub-types I (line I) and II (line II). Only the regions around the recombination breakpoints are shown [(*a*) recombination breakpoints in isolates VN and N Rus, (*b*) in isolates T13, LB, H, Hu, NL2, Eur H and M1, (*c*) in isolate Fr]. At the polymorphic sites, the nucleotides are shown in capitals, in bold for those of sub-type I, and in outline for those of sub-type II. The locations of the putative recombination breakpoints are indicated by dashes below the alignments. NTN* denotes the consensus sequence for isolates H, Hu, NL2, LB and M1, all of them of the NTN biotype, and Eur H, of uncharacterized biotype. The numbering is that of isolate Fr (Robaglia *et al.*, 1989), the last digit of each figure indicating the corresponding position. Y denotes C/U when these nucleotides were equally represented at a given position in a sub-group. Dots indicate nucleotides identical to the consensus; // indicates a deletion, in the part of the alignment displayed in (*b*), of a region with no polymorphic sites between sub-types I and II (positions 9039–9127).

BCMV

The nucleotide sequences of 15 BCMV isolates are available in GenBank, and all contain the 3′NTR. The sequences of isolates of peanut stripe (PStV) and black-eye cowpea mosaic (BlCMV) viruses were also included in this analysis since they have been shown to represent isolates of BCMV (McKern *et*



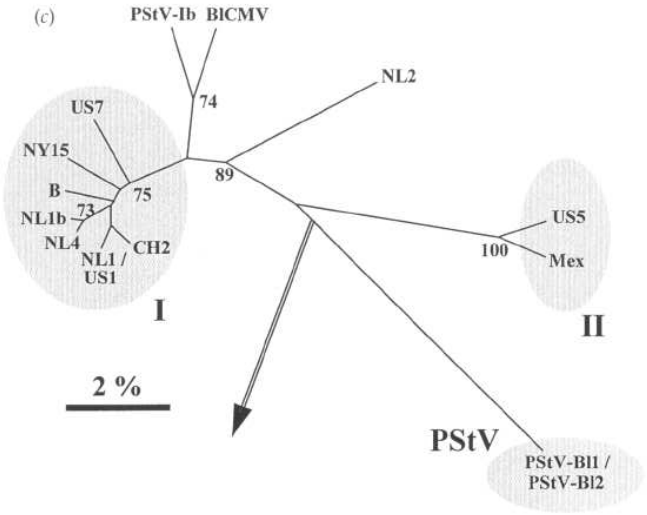


Fig. 3. Clustering trees derived from the N-ter (*a*), C-ter (*b*) and 3'NTR (*c*) regions of BCMV, as defined in the text. The sub-groups discussed in the text are shaded. The branching of the corresponding sequences of PVY-Fr, used as an outgroup, is indicated by a double arrow. The percentage of bootstrap replicates in which each node was recovered is given when above 70%, with the omission of the 100% value of the outgroup branch. The scale bar represents, for the branch lengths, 5% (*a*) and 2% (*b*, *c*) substitutions per site.

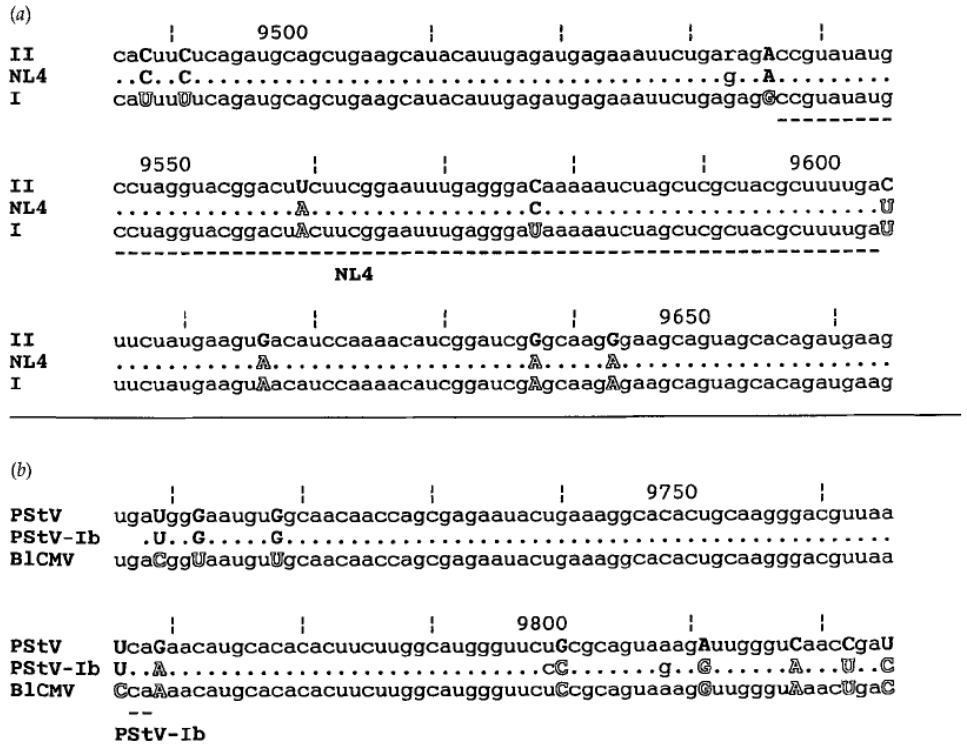


Fig. 4. Alignments of the putative recombinant BCMV sequences with the consensus sequence of various sub-types (I, II, PStV or BlCMV). Only the regions around the recombination breakpoints are shown [(*a*) recombination breakpoint between sub-types I and II in isolate NL4, (*b*) between PStV and BlCMV in isolate PStV-Ib). At the polymorphic sites, the nucleotides are shown in capitals, in bold for those of sub-type II or PStV, and in outline for those of sub-type I or BlCMV. The locations of the putative recombination breakpoints are indicated by dashes below the alignments. The numbering is that of PStV-Bl2 (Gunashinge *et al.*, 1994), the last digit of each figure indicating the corresponding position. R denotes G/A when these nucleotides were equally represented at a given position in a sub-group. Dots indicate nucleotides identical to the consensus.

al., 1992; Vetten *et al.*, 1992; Khan *et al.*, 1993; Saiz *et al.*, 1994). On the other hand, an additional sequence referenced as BCMV, that of strain NL3 (accession number U20818), was not included in our analysis since Khan *et al.* (1993) showed that this isolate should be considered as the member of a distinct virus species. As in the PVY analysis, three regions were selected and analysed. With nucleotide numbering according to the complete sequence of isolate PStV-Bl2 (Gunashinge *et al.*, 1994), the region N-ter extends from positions 8945–9118, C-ter from 9548–9808 and 3′NTR from 9809–10061.

Fig. 3 shows the trees constructed using the N-ter, C-ter and 3′NTR regions. There are three main groups in each tree. A large sub-group (sub-group I) contains most of the isolates, and two smaller sub-groups (sub-groups II and PStV) contain two sequences each, Mex/US5 in sub-group II and PStV-Bl1/PStV-Bl2 in sub-group PStV. In addition, the sequences of BlCMV and BCMV-NL2 diverge in two other directions and could thus be the only currently sequenced representatives of two additional sub-groups. However, two isolates, NL4 and PStV-Ib, cluster differently in the trees displayed in Fig. 3. NL4 belongs to sub-group II in the N-ter tree, but to sub-group I in the C-ter and 3′NTR trees, and PStV-Ib belongs to sub-group PStV in the N-ter and in the C-ter trees, but is closer to BlCMV in the 3′NTR tree. These observations, supported by high bootstrap values, indicate that these two isolates are possible recombinants.

When the statistical relevance of this possible recombination was estimated using VTDIST, a high probability for recombination within the 15 BCMV sequences was found according to the SS parameters, but not to the M parameters (Table 1). Such a difference in the prediction made with the two types of parameter has been described in some cases by Sawyer (1989). Again, a 'consensus' sequence was determined for the five sub-groups shown in Fig. 3. Mex was arbitrarily chosen to represent sub-group II, and similarly PStV-Bl2 was chosen for the PStV subgroups. The consensus sequence for sub-group I was determined and found to be identical with that of isolate US1. BlCMV and NL2 were considered to be the only representatives of their respective sub-groups. When each of the BCMV sequences was compared, using VTDIST, to these five consensus sequences, only isolates NL4 and PStV-Ib

gave SSCF-associated *P*-values lower than 3% (data not shown). Isolates US7 and NL1 also gave relatively low *P*-values (4·93% and 8·90% respectively), and none of the other isolates had a *P*-value lower than 32% (data not shown). This result confirms the hypothesis that isolates NL4 and PStV-Ib are recombinants. The pair-wise comparisons, performed as for PVY in Table 1, again confirmed this result at the 1% level, and indicated that NL4 was probably a recombinant between sub-groups I and II, and that PStV-Ib was probably a recombinant between the PStV and the BlCMV sub-groups (data not shown), as predicted above from the cluster analyses.

The Maynard-Smith approach was then used to determine the fine structure of these two recombinant isolates (Fig. 4). The recombination breakpoint was mapped between positions 9537 and 9603 in isolate NL4, with sub-type II sequences 5′ to this region and sub-type I sequences 3′ to it. In PStV-Ib, the breakpoint was located between positions 9767 and 9768, with PStV-type sequences upstream and BlCMV-type sequences downstream.

BYMV

The nucleotide sequences of six BYMV isolates, all of them including their 3′NTR, were available in GenBank. As above, three regions were selected and clustering trees derived thereof. No major differences were observed in the topology of these trees, except that the isolates MB4 and MI converged to DG in the 3′NTR and that GDD diverged from DG in this same region (data not shown). The program VTDIST found evidence at the 0·01% level for genetic rearrangement in BYMV when the SS parameters were considered, and at the 0·2% level with the M parameters (Table 1), and VTDIST2 confirmed, at the 2% level, the implication of MB4 and MI in this process (data not shown). The closer relationship of MB4 with MI and DG, as well as the divergence of GDD, in the 3′NTR were confirmed by visual inspection of the entire alignment (data not shown) but, in the rest of the CP–3′NTR region, no other available sequence could be related to these isolates. The Maynard-Smith approach did not detect any significant mosaic structure in isolates MB4, MI or GDD (not shown). In conclusion, it cannot be excluded that recombination has occurred in BYMV, between isolates belonging to a sub-group represented by DG, and another one not represented in the available dataset. Thus, the case of BYMV, no firm conclusion about the presence or absence of recombination is possible, essentially because of the relatively restricted dataset.

ZYMV

The nucleotide sequences of seven ZYMV isolates, all including their 3′NTR, were available in GenBank. Regions were defined and clustering trees derived as above. In all the trees, two sub-groups could be defined, one containing Sin and Reun, and another one containing four other sequences (data not shown). Isolate Is lies between these two sub-groups. Within the major sub-group, isolates Con and Cal cluster together and Pat15 is slightly aside. Isolate Flo clusters with Pat15 except in the C-ter tree where it is closer to Cal and Con (data not shown), and could thus be a double recombinant, detected despite the overall close relationships between the isolates of this sub-group. In the dataset provided (the isolate Reun, very divergent in the 3′NTR, was excluded), VTDIST detected possible recombination at significant levels except with parameter MUF (Table 1). The Maynard-Smith approach showed that, at the 1% confidence level, the segment of Flo extending to position 9169 was of the Pat15 type, then of the Con/Cal type up to position 9407, and then shifted back to Pat15 (the numbering refers to Cal, as in Wisler *et al.*, 1995) (data not shown). Flo could thus be a double recombinant between related ZYMV isolates; however, these observations rely on only 13 polymorphic sites in the intermediate region.

PPV, PRSV, TuMV and WMV2

Multiple alignments of the regions designated as described in Methods were obtained for PPV, PRSV, TuMV and WMV2, and used to infer clustering trees. For each of these viruses, the topologies of the trees derived from the different regions were not significantly different (data not shown). Thus, we have not detected any evidence for recombination between the sequenced isolates of these viruses in the CP–3′NTR region. As above, we then analysed these sequence sets using VTDIST (Table 1). As described in Methods, PRSV accessions I06996 and U14740, and TuMV accessions D10927 and X81141 were not considered in this VTDIST analysis because they are identical to, or differ at only one position from another sequence. The VTDIST analysis failed to detect significant probability of recombination either when all the available sequences for a given virus were considered in only their CP coding region (PPV, PRSV and TuMV; see Table 1) or when only those for which the 3′NTR information is available were considered in their CP–3′NTR region (PPV, PRSV, TuMV, data not shown; WMV2, see Table 1). The lowest percentage found for the null hypothesis was 3·6% for the PPV SSCF parameter, but this was not confirmed by any other PPV parameter, and close examination of the data showed that this low value was due to a pattern of conserved and very divergent regions in the El-Amar sequence (X56258), so that, even for PPV, recombination was ruled out.

Discussion

So far, only two possible or probable instances of recombination in the genus *Potyvirus* have been described (Sano *et al.*, 1992; Cervera *et al.*, 1993). In this work we have systematically searched for the presence of RNA recombination in potyvirus sequences present in the databases, or published. We focused on the coat protein region because it is the one that is most often targeted in sequencing projects, leading to a larger available dataset than for any other region of the genome. We first focused on PVY, since this virus is of

considerable economic importance which has prompted more studies than on other potyviruses, resulting in a particularly large (and indeed continuously increasing) dataset. The search was then extended to other potyviruses for which the CP gene sequences of at least four different isolates were available in the databases.

We first used a simple approach for rapid identification of putative recombinant isolates, similar to the one that allowed Chenault & Melcher (1994) to demonstrate the occurrence of recombination in a plant DNA virus, cauliflower mosaic virus. The two terminal domains of the region encoding the CP were analysed separately, and the trees obtained were compared. In the absence of recombination, the general topologies of the trees should be similar, whereas if recombination had occurred in some isolates, the corresponding branches would be expected to differ in their location in the two trees, being more closely related to a different parental cluster in each tree. An advantage of this approach is that the parents themselves do not have to be present in the dataset, as long as other members of the cluster to which they belong are present. A statistical analysis was then performed on the entire CP-encoding region, so as to confirm the occurrence of recombination suspected from the tree topology. Using this approach, we identified a number of recombinant isolates in the case of PVY and of BCMV. Recombination was also proposed in BYMV and ZYMV, but no recombination was detected in PPV, PRSV, TuMV and WMV2. In total, recombination in the CP–3′NTR region of the genome was demonstrated in 13 isolates (with at least nine independent recombination breakpoints), and suspected in five more, out of a total of 109 isolates tested. The recombinant isolates, found in 50% (4/8) of the virus species tested, thus represent between 8% (9/109) and 17% (18/109) of the total population examined.

The reasons for the difference in prevalence of recombination between different virus species are not clear. For some viruses (WMV2, TuMV), the size of the sample (four sequences for WMV2, eight for TuMV) may simply have been too small. Such an explanation cannot, however, apply to PPV and PRSV, for which large datasets were available. In the case of PPV, a possible explanation could be the different geographical distributions of the two major groups of isolates (Candresse *et al.*, 1995), which would clearly preclude mixed infections, a prerequisite for recombination to take place. Indeed, the only PPV recombinant isolate detected so far (Cervera *et al.*, 1993) was isolated in former Yugoslavia, one of the few countries where both types of PPV isolates occur together. Another explanation, which cannot be ruled out, is that individual potyviruses could differ significantly in their recombination rates due to differences in the intrinsic properties of their replication machineries.

The approach that we have used has, however, several limitations that actually lead to underestimating the extent of recombination. First of all, only viable recombination products can be detected, so that in this work 'recombinant' means 'viable recombinant'. Secondly, it is very important to have a dataset large enough to establish a clear clustering of the different isolates (the uncertainty about recombination in BYMV is an illustration of this point), and also to increase the chance that it contains a recombinant isolate. Thirdly, we would have failed to identify recombination between closely related isolates since no visible shift in the clustering of such a recombinant isolate would have resulted. This is illustrated by ZYMV-Flo, a possible recombinant between two closely related isolates, which shows a barely detectable shift between the different trees. Fourthly, multiple recombination events might escape detection since these types of isolate will cluster similarly in regions situated on the two sides of the two recombination breakpoints. Finally, the non-detection of recombination in the CP–3′NTR region does not preclude the possibility of recombination in other parts of the genome. The region we have chosen is, in potyviruses, about one-tenth the entire size of the genome; thus, if recombination breakpoints are evenly distributed along the entire genome, our approach would only have detected about one-tenth of the recombination events between the isolates represented in the dataset. Indeed, in the case of PPV, one of the viruses for which we did not detect any recombination event in this study, RNA recombination with a cross-over point outside the region analysed in this work, in the putative viral polymerase coding domain, has been described (Cervera *et al.*, 1993). This last limitation can be removed only when more complete virus sequences are known.

Although we made no specific efforts to detect interspecific recombination, such events would have been characterized by the presence of highly divergent isolates in some of the trees (comparable to that of the outgroup sequences used in Figs 1 and 3), something we never observed in our dataset. In addition, interspecific recombination would also have been detected by the VTDIST program. We therefore conclude that, if it exists, interspecific recombination has to have a frequency much lower than intraspecific recombination in the genus *Potyvirus* (less than 1 isolate in 109). There may be several reasons for the paucity of interspecific recombination. First of all, the products of such an event could be less likely to be viable than those of intraspecific rearrangement. Alternatively, recombination might not occur at all because lower levels of sequence identity may not allow the template switch by the viral polymerase. Finally, recombination between different virus species might be hindered by spatial separation of the virus replication sites (different host species or sub-cellular, cellular, tissue, or geographical localization, etc.).

The genetic shuffling caused by recombination may lead to the appearance of new virus isolates in the field, some of which may thus acquire new biological properties (as may have happened with the NTN biotype of PVY, as discussed above). Recombination could also happen, as described for a bromovirus (Greene & Allison, 1994), between a replicating virus and the product of a transgene introduced into the genome of a

crop plant for protection purposes. In this respect, it is worthwhile to note that we did not detect interspecific recombination. However, spatial separation, suggested above to be a possible inhibitor of interspecific recombination, might differ in the cases of transgenic versus infected plants.

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Biological and Molecular Variability of Lettuce Mosaic Virus Isolates

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ABSTRACT

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Lettuce mosaic potyvirus (LMV) causes severe disease of commercial lettuce crops. LMV isolates show wide biological variability, particularly in their ability to overcome the resistance genes described in *Lactuca sativa*. For a better understanding of the molecular interaction between lettuce and LMV, biological and molecular characterization of a collection of 10 LMV isolates known to differ in virulence or aggressiveness was performed. The ability of these isolates to overcome the resistance genes was reevaluated under standardized conditions. To study the molecular variability of LMV, an immunocapture-reverse transcription-polymerase chain reaction technique, coupled with direct sequencing, was used to obtain nucleotide sequence data from three short regions of the LMV genome. Clustering analysis was performed and compared to the biological properties of the 10 isolates. Three groups of LMV isolates were discriminated based on the molecular data. These groups appear to correlate with the geographic origin of the isolates rather than with their pathogenicity. Sequence comparison with California isolates clearly showed that the California isolates are related to the western European isolates, raising the possibility of past exchanges of LMV between western Europe and California.

Lettuce mosaic virus (LMV), a member of the genus *Potyvirus*, causes severe disease of commercial lettuce crops. LMV is a seed- and aphid-transmitted virus with worldwide distribution. Symptoms observed in infected plants include dwarfing, poor heading, mottling, and vein clearing (7).

The complete nucleotide sequences of the genomes of two LMV isolates (LMV-0 and LMV-E) have been determined ([27]; EMBL accession X97704 for LMV-0 and X97705 for LMV-E). The genome of LMV is a single, positive-sense 10,080-nt RNA molecule polyadenylated at its 3′ end. It contains a unique large open reading frame encoding a 3,255-amino acid polyprotein.

LMV isolates show a large array of biological variability, with variations in the severity of symptoms induced, seed transmissibility, and ability to overcome the three resistance genes described in lettuce (*Lactuca sativa*) cultivars (24,25). Two of these genes, $mo1^1$ and $mo1^2$, are recessive and are believed to be either closely linked or allelic ([7,20,25]; B. Maisonneuve and H. Lot, *unpublished data*). These two genes have been introgressed into lettuce cultivars for LMV management. Depending on the particular genetic background and LMV isolate considered, the presence of $mo1^1$ and $mo1^2$ results in either the absence of virus multiplication or reduced virus multiplication and subsequent absence of symptoms (25). A third gene, *Mo2*, is a dominant gene that is not effective in practice for LMV control because it is overcome by most LMV isolates (25). Several pathotypic classifications of LMV isolates have been proposed based on the behavior of LMV isolates toward these resistance genes (4,7,25). The recent emergence in Europe of isolates that can overcome these resistance genes has caused serious lettuce yield losses (7) and indicates the clear possibility of increasing problems for LMV management.

Sequence analysis comparisons of coat protein (CP) genes can be used to identify and differentiate distinct potyviruses and their strains (33–35). Several studies have established phylogenetic relationships between strains of potyviruses based on CP gene sequences, e.g., sugarcane mosaic virus (11), potato virus Y (PVY) (36,37), plum pox virus (5), bean common mosaic virus (30), papaya ringspot virus (2,38), LMV (42), and zucchini yellow mosaic virus (6). The sequences of other regions of the potyvirus genome, such as the 3′ nontranslated region (3′NTR), also can be used to discriminate strains within a virus species (12,30,37). Several of these molecular analyses have shown a grouping of isolates correlated with either biological data (37) or geographic origin (2,38). Zerbini et al. (42) characterized 10 LMV isolates from California and demonstrated that variability existed in the CP N-terminal region.

However, in all of these studies, only one region of the potyvirus genome was analyzed and used to determine affinities between isolates. We recently have demonstrated that in some potyviruses the frequency of recombinant isolates is unexpectedly high (26). For example, almost one-third of PVY sequences present in the databases appears to result from recombination events located in the CP gene or the 3′NTR. Given such a high potential rate of recombination, determination of the phylogenetic affinities of virus isolates with a single, relatively short region of the genome may produce erroneous results. Because the comparison of entire genomic sequences currently is impractical, the use of several short regions scattered over the entire genome may be a simple and effective way to determine affinities or reconstruct phylogenies in potyviruses. Comparison of the results obtained with different short regions also may indicate the incidence of recombination within the set of isolates considered (26).

In this paper, we report the biological and molecular characterization of a collection of 10 LMV isolates selected to cover the biological and geographical diversity of LMV in Europe. The ability of these isolates to overcome the resistance genes present in a differential series of lettuce cultivars was evaluated in parallel experiments under standardized conditions. To study the molecular variability of these isolates, we used an immunocapture-reverse transcription-polymerase chain reaction (IC-RT-PCR) technique (40) coupled with direct sequencing to obtain nucleotide sequence data from three short regions of the LMV genome. Clus-

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tering analysis was performed with the sequence data, and the results of this analysis were compared to the behavior of the 10 isolates toward the lettuce resistance genes.

MATERIALS AND METHODS

Virus isolates. All 10 isolates, except 1, originated from Europe. The French isolates LMV-0, LMV-1, LMV-9, and LMV-13 and the Spanish isolate LMV-E, known to differ in their virulence and seed transmissibility, were described by Dinant and Lot (7). LMV-Aud was isolated in northern France in 1993 from lettuce cv. Audran, which contains the *mo1*^{1} resistance gene. The isolate from Yemen Arab Republic (LMV-Yar) was provided by D. Walkey (HRI, Wellesbourne, England) and described by Pink et al. (24). LMV-Gr4 and LMV-Gr5, two isolates from Greece that were studied by Bos et al. (4), were provided by R. van der Vlugt (IPO, Wageningen, Netherlands), and another isolate (LMV-GrB) was provided by C. Varveri (Benaki Institute, Athens, Greece). LMV-0, LMV-Yar, LMV-Gr4, LMV-Gr5, and LMV-GrB were maintained in susceptible butterhead lettuce cv. Trocadero, and LMV-1, LMV-9, LMV-13, LMV-Aud, and LMV-E were maintained in butterhead lettuce cv. Mantilia, which contains the *mo1*^{1} resistance gene.

Inoculation procedure and infectivity assays. Virus inoculum was prepared from leaves of infected lettuce plants 18 to 22 days after inoculation. The leaves were ground 1:4 (wt/vol) in a solution of 0.03 M Na_2HPO_4 containing 0.2% diethyldithiocarbamate (DIECA), and 100 mg of Carborundum and activated charcoal was added before rub-inoculation. Seeds of the differential lettuce cultivars were provided by B. Maisonneuve (Institut National de la Recherche Agronomique, Génétique et Amélioration des Plantes, Versailles, France). The five crisphead cultivars used were those described in Pink et al. (25). Six plants of each cultivar were inoculated with each isolate at the 4- to 6-leaf stage and maintained in insect-proof cages at 18 to 25°C. The experiment was repeated twice. Observations of symptoms were made at weekly intervals from 2 to 5 weeks after inoculation. The presence and concentration of virus were assessed by double-antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) 3 to 4 weeks after inoculation. Three leaves were sampled from each plant. A polyclonal antiserum raised against LMV-0 was used to test plants infected with all isolates, except LMV-Yar, for which a Yar-specific antiserum (provided by D. Walkey) was used because the LMV-0 reagents reacted very poorly with this specific isolate.

IC-RT-PCR. A protocol modified from Wetzel et al. (40) was used. Lettuce leaves were ground 1:3 (wt/vol) in PBS-Tween buffer (8 g of NaCl, 0.2 g of KH_2PO_4, 2.9 g of $Na_2HPO_4 \cdot 12H_2O$, 0.2 g of KCl, 0.2 g of NaN_3, and 0.5 ml of Tween 20 per liter) containing 2% polyvinylpyrrolidone K25, 20 mM sodium DIECA, and 0.1% $MgCl_2$. The plant extracts were incubated overnight at 4°C in 0.5-ml tubes precoated with anti-LMV immunoglobulins. After washing the tubes once with PBS buffer, 100 µl of RT-PCR reaction mix (10 mM Tris-HCl, pH 8.8, 1.5 mM $MgCl_2$, 50 mM KCl, 0.3% Triton ×100, 250 µM each of dNTPs, 1 µM each of the two primers, 0.25 units of AMV reverse transcriptase, and 0.5 units of *Taq* DNA polymerase) was added and overlaid by 100 µl of mineral oil. The tubes were incubated for 15 min at 42°C for reverse transcription, were incubated 5 min at 95°C for denaturation of RNA-DNA hybrids and reverse transcriptase, and were cycled as described for each primer pair in Table 1. Primer pairs P1 and P2, P3 and P4, and P5 and P6 were used to prime the amplification of the 5′ nontranslated region (5′NTR) together with the 5′ end of the P1 gene (region I), the 3′ end of the NIb gene together with the 5′ end of the CP gene (region II), and the 3′ end of the CP gene (region III), respectively (Fig. 1).

Direct sequencing of amplified fragments. A protocol modified from Hultman et al. (15) was used. About 400 ng of biotinylated PCR products (obtained using one of the PCR primers in a 5′ biotinylated form) were immobilized with 0.2 mg of magnetic beads containing covalently coupled streptavidin (Dynabeads M-280 streptavidin, Dynal International, Oslo, Norway). The immobilized biotinylated double-stranded DNA was denatured by incubation at room temperature with 0.15 M NaOH for 5 min. The magnetic beads retaining the single-stranded biotinylated DNA were washed once in 0.15 M NaOH and twice in H_2O and resuspended with 2 pmol of the sequencing primer in a buffer containing 40 mM Tris-HCl, pH 7.5, 20 mM $MgCl_2$, and 50 mM NaCl in a total volume of 10 µl. For sequencing of regions II and III, the sequencing primer was the primer used in its nonbiotinylated form in the PCR reaction. For region I, the sequencing primer was internal (5′GCCTAGCATCAGTTATGAAATC3′, positions 338 to 360) for LMV-1, LMV-9, LMV-13, LMV-Aud, LMV-Yar, and LMV-Gr5. For LMV-GrB and LMV-Gr4, the sequencing primer used to sequence region I was the nonbiotinylated PCR primer P2, because a shorter PCR product was obtained. The annealing mixture was heated to 65°C for 5 min and cooled to room temperature. Sequencing reactions were performed with a Sequenase, version 2.0, DNA sequencing kit (United States Biochemical Corporation, Cleveland). After extension, the supernatant was removed, and 4 µl of stop solution was added to the beads. Two microliters was loaded onto an 8 M urea, 6% polyacrylamide sequencing gel.

Cloning of PCR-generated fragments. The PCR fragments were electrophoresed in a 1% agarose gel in TAE buffer (40 mM Tris, 40 mM acetate, and 1 mM EDTA). Bands of the expected size were excised, and DNA was recovered with a Geneclean kit

TABLE 1. Primer pairs and cycling conditions used for amplification

Primer pair sequence[a]	Position[b]	Cycling conditions	
		1 cycle	35 cycles
P1 5′AAAATAAAACAACCCA-ACACAACTC3′	1	92°C 30 s	92°C 30 s
P2 5′GCAATAGTTGCAGTAG-TTCTGCCCTCCAACCTAGG3′	738	42°C 30 s + 72°C 1 min	54°C 30 s 72°C 1 min
P3 5′ATTCGAAAATTYTAYAA-RTGGTG3′	8825	92°C 20 s	92°C 20 s
P4 5′GCGTTBATGTCGTCGT-CYTT3′	9171	42°C 20 s + 72°C 40 s	56°C 20 s 72°C 40 s
P5 5′ACAAGAAGAAACCGTA-TATGCC3′	9588		92°C 20 s
P6 5′GCCAACACACGCCTTT-AGTG3′	9885		56°C 20 s 72°C 40 s

[a] Y = C or T; R = A or G; and B = T, G, or A. All primers were synthesized in two forms, 5′ biotinylated or not.
[b] Position on the genome of the 5′ most nucleotide of the primer.

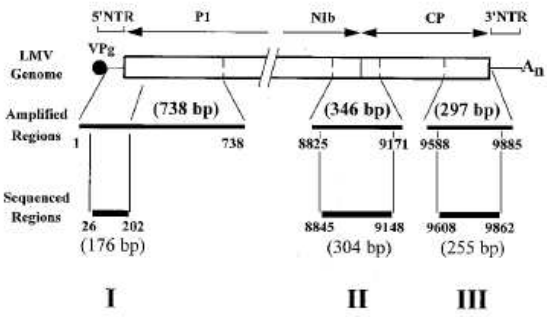

Fig. 1. Localization of regions I, II, and III along the lettuce mosaic virus (LMV) genome. The size in base pairs (bp) of the amplified (middle line) or sequenced (bottom line) regions are given in parentheses. The positions of the fragment along the LMV genome are given. The LMV genomic regions in which the studied fragments are included are labeled at the top (5′NTR [5′ nontranslated region], P1, NIb, CP [coat protein], and 3′NTR [3′ nontranslated region]).

II (Bio 101, Inc., La Jolla, CA). Purified DNA was blunted by the use of the Klenow fragment of *Escherichia coli* DNA polymerase I and was phosphorylated with T4 polynucleotide kinase. The DNA was ligated to *Hin*cII-linearized, dephosphorylated pBluescribe (Stratagene, La Jolla, CA) and used to transform competent *E. coli* XL1-blue cells (Stratagene). The recombinant plasmids were purified by alkaline lysis and digested with restriction enzymes. Plasmids containing fragments of the expected size were selected for sequencing. DNA templates were prepared as described by Zagurski et al. (41) and sequenced from both ends with Sequenase, version 2.0 (U.S. Biochemical).

Phylogenetic analysis. Multiple alignments of the amplified regions were obtained by the Clustal V program (14). Phylogenetic relationships were determined by distance methods implemented in the PHYLIP package, version 3.5 (10). Distance matrices were calculated by DNADIST with the Kimura two-parameter option (18), and distance trees were constructed from these matrices by NEIGHBOR, which implemented the neighbor-joining method (29). The trees derived were displayed by DRAWTREE to optimize the fitting of branch lengths to Kimura's distances. A bootstrap value for each internal node was calculated by performing 100 random resamplings using SEQBOOT (9) and synthesizing the resulting set of trees using CONSENSE. To confirm the topologies of the neighbor-joining trees, the maximum likelihood method, using DNAML, the parsimony method, using DNAPARS, and the FITCH method also were used.

RESULTS

Isolate reactions on different lettuce genotypes. The symptoms of LMV infection observed in most lettuce genotypes were not typical mosaic symptoms but rather vein clearing, yellow spotting, and stunting. The results of the pathogenicity tests, including symptoms and virus multiplication (Table 2), provide evidence that the isolates differed in virulence and aggressiveness. All isolates multiplied and produced symptoms on cv. Salinas. LMV-0 did not induce any symptoms in three genotypes, cvs. Malika, Salinas 88, or Vanguard 75, that possess one of the recessive genes, $mo1^1$ or $mo1^2$, even in plants in which viral multiplication was detected by ELISA. LMV-1 and LMV-9 caused severe symptoms in cvs. Ithaca and Malika, which carry the *Mo2* and $mo1^1$ genes, respectively, but did not multiply in cvs. Salinas 88 and Vanguard 75, which contain the $mo1^2$ gene. LMV-E and LMV-13, as well as the newly characterized isolates LMV-Aud and LMV-GrB, were virulent on all genotypes, including those containing the $mo1^2$ gene. The interactions of the LMV-Yar isolate and the two other Greek isolates did not fit with those of the other groups. They did not infect cvs. Ithaca and Vanguard 75, which possess the *Mo2* gene, but were able to overcome the two recessive genes, $mo1^1$ and $mo1^2$. Nevertheless, there were slight differences between the two Greek cultivars. In Malika, LMV-Gr4 infected fewer plants (8/11 versus 10/11 for LMV-Gr5 and 14/16 for LMV-Yar) and, like LMV-Yar, multiplied at a lower rate and was less aggressive when compared to LMV-Gr5. On Salinas 88, LMV-Gr4 induced milder symptoms than LMV-Yar and LMV-Gr5, which induced the most severe symptoms.

Amplification and sequencing of regions I, II, and III. To amplify the three short regions of the LMV genome, the IC-RT-PCR technique, developed by Wetzel et al. (40), was adapted, using LMV-infected lettuce leaves as the starting material. Capture of the virus particles, release of viral RNA, and RT-PCR were performed in a one-tube assay. Amplifications were performed twice, with either one of the primers in a biotinylated form, allowing sequence determination in both orientations. Single fragments of 738 bp (region I), 346 bp (region II), and 297 bp (region III) were generated successfully for nearly all the isolates tested (data not shown). However, RT-PCR products, in addition to the expected fragments, also were obtained in the case of the amplification of LMV-GrB and LMV-Gr4 region I and LMV-GrB, LMV-Gr4, and LMV-Gr5 region II.

A direct sequencing protocol was used to sequence the amplified fragments. However, because a mixture of amplified fragments was obtained after amplification of region II of LMV-GrB, LMV-Gr4, and LMV-Gr5, the product with the expected size was purified and cloned. For each of these three isolates, at least two independent clones were sequenced on both strands.

The three sequenced regions are presented in Figure 1. For region I, the size of the sequenced 5′NTR region was 87 nt for LMV-

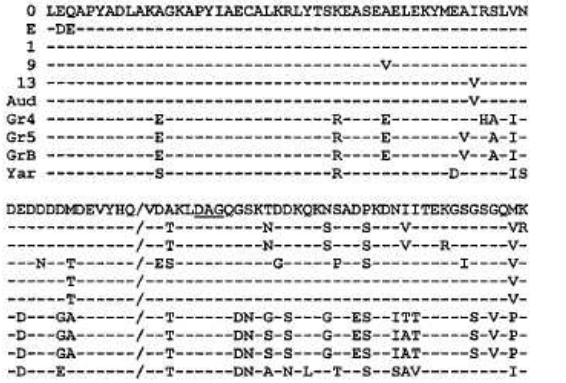

Fig. 2. Amino acid sequence alignment of region II of 10 lettuce mosaic virus (LMV) isolates. Amino acid differences are indicated with a single-letter code. Dashes indicate amino acids identical to the LMV-0 sequence. The DAG triplet is underlined. Slashes indicate the NIb/CP cleavage site.

TABLE 2. Interactions between lettuce mosaic virus isolates and lettuce differential cultivars

	Isolates							
	0		1 and 9		E, 13, Aud, and GrB		Yar, Gr4, and Gr5	
Cultivar[a]	Symptoms[b]	Vir. conc.[c]	Symptoms	Vir. conc.	Symptoms	Vir. conc.	Symptoms	Vir. conc.
Salinas	**	++++	**	++++	***	++++	* to ***	++++
Ithaca	**	++++	**	+++	***	++++	–	–
Malika	–	– to ±[d]	*	+++	* to **	++++	* to **	± to ++[d]
Salinas 88	–	++	–	–	* to **	++++	* to **	± to ++[d]
Vanguard 75	–	+	–	–	**	++++	–	–

[a] Ithaca and Vanguard 75 contain the *Mo2* gene (23). Malika contains the $mo1^1$ (formerly *g*) gene. Salinas 88 and Vanguard 75 contain the $mo1^2$ (formerly *mo*) gene (7).

[b] The number of asterisks (* to ***) scores symptom severity; symptoms included vein clearing, leaf deformation and stunting, and, occasionally, necrosis. – = no symptoms.

[c] The number of pluses indicates the relative virus concentration (vir. conc.) estimated by enzyme-linked immunosorbent assay: +, ++, and +++ indicate that the mean absorbance values were ~0.25, 0.5, or 0.75 of the absorbance on susceptible cv. Salinas (++++) respectively; ± indicates a low concentration of virus was detected; – indicates no virus was detected.

[d] In cases in which wide variability was observed in the virus content of individual plants, a concentration range is given.

Yar and 78 nt for all other isolates. The size of the sequenced P1 region was 98 nt for all isolates. The sequences obtained for regions II and III corresponded to the complete sequence of the amplified regions, excluding the primer sequences, and had the same number of nucleotides for all isolates. These nucleotide sequences have been submitted to the EMBL database and assigned the following accession numbers: Z78215 to Z78222 for region I, Z78223 to Z78230 for region II, and Z78231 to Z78238 for region III, respectively, for LMV-13, LMV-Aud, LMV-1, LMV-9, LMV-Yar, LMV-Gr5, LMV-Gr4, and LMV-GrB.

The amino acid sequences were deduced from the coding nucleotide sequences; for region I, they include the first 32 amino acids of the P1 protein; for region II, they include the last 62 amino acids of the NIb protein and the first 39 amino acids of the CP; and for region III, they include the last 85 amino acids of the CP (excluding the very last one). All LMV isolates tested contained in

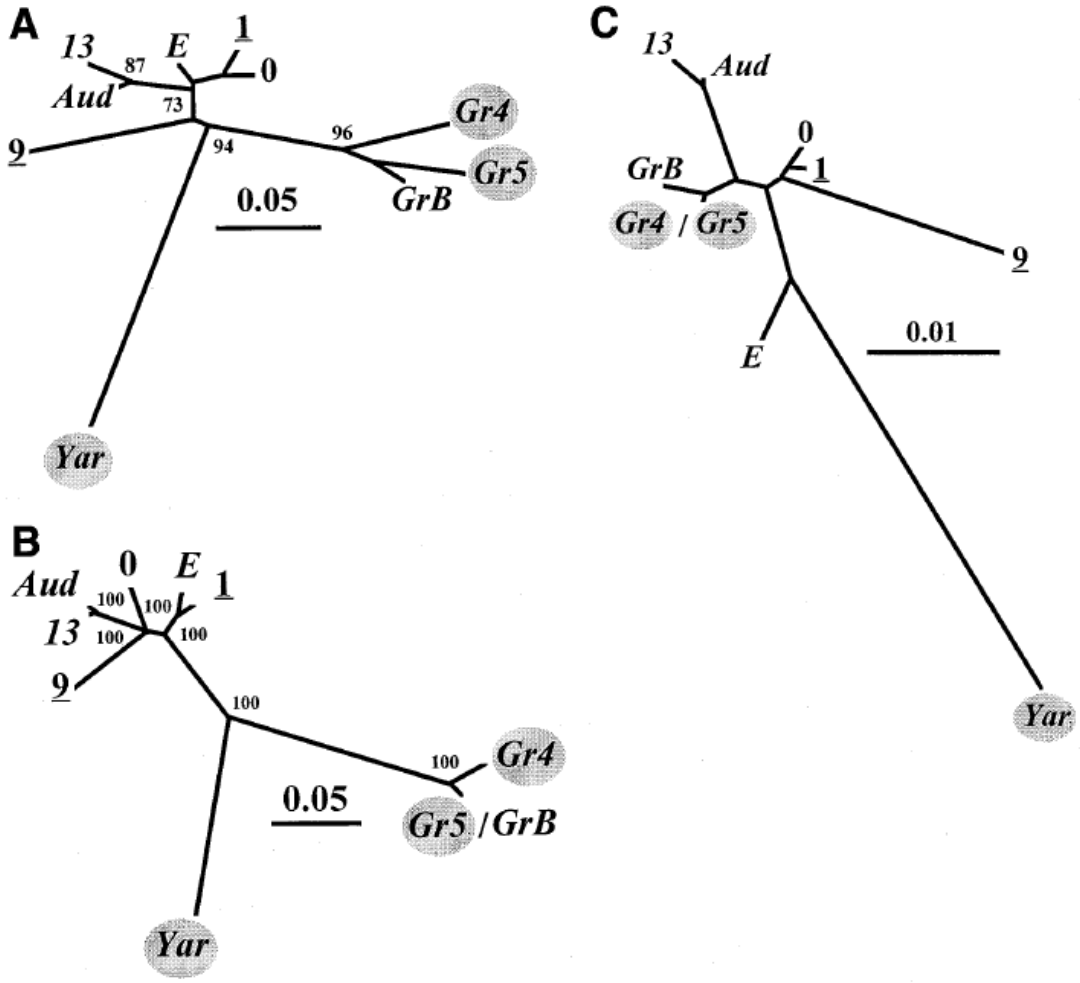

Fig. 3. Clustering neighbor-joining trees derived from regions A, I, B, II, and C, III of the lettuce mosaic virus (LMV) genome, as defined in text. The isolates that overcome only *mo1*[1] are underlined; the isolates that overcome both *mo1*[1] and *mo1*[2] are italic; the isolates that do not overcome *Mo2* are shaded. The percentage of bootstrap replicates in which each node was recovered is given when above 70%. The scale bar represents, for the branch lengths, Kimura distances of 0.05 (**A and B**) and 0.01 (**C**).

TABLE 3. Percentage of nucleotide sequence identity of regions I (above the diagonal) and II (below the diagonal) among the 10 lettuce mosaic virus isolates tested

	Isolate									
Isolate	0	E	1	9	13	Aud	Gr4	Gr5	GrB	Yar[a]
0		96.6	97.2	90.9	94.9	96.6	88.6	89.8	88.6	78.5
E	92.1		95.5	92.0	94.9	96.6	89.2	88.6	86.4	78.5
1	93.1	97.0		90.9	93.8	94.3	88.6	89.8	88.6	79.0
9	90.8	89.1	89.5		90.3	92.0	88.6	89.2	87.5	78.0
13	93.4	92.4	92.8	90.1		98.3	88.6	89.8	87.5	79.0
Aud	92.8	92.4	92.4	89.5	99.0		89.2	90.3	88.1	78.5
Gr4	74.3	75.0	75.3	73.4	73.4	74.3		92.6	94.3	77.3
Gr5	75.0	76.6	77.3	74.7	74.3	75.3	96.7		96.0	78.5
GrB	75.0	76.6	77.3	74.7	74.3	75.3	96.7	100.0		78.0
Yar	78.6	78.9	79.3	76.3	77.0	76.6	74.0	74.7	74.7	

[a] The nine positions at which insertions occur in the Yar sequence in the region I alignment were omitted to calculate the identity percentage.

their CP N-terminus sequence the DAG triplet involved in aphid transmission (1) and the NIb/CP cleavage dipeptide Q/V described for LMV-0 by Dinant et al. (8) (Fig. 2).

Sequence analysis. Multiple alignments of the nucleotide sequences were produced by the program Clustal V for each of the three analyzed regions. For LMV-0 and LMV-E, the corresponding regions derived from the complete sequence (27) were used. These three alignments have been submitted to the EMBL database and assigned the accession numbers DS26767 for region I, DS26768 for region II, and DS26770 for region III. The phylogenetic trees reconstructed for each of the three regions based on these data show the relationships among the various LMV isolates more clearly (Fig. 3). The general topologies of the various trees, obtained by the neighbor-joining method and supported by bootstrap analysis, are similar and show three subgroups of isolates, except in region III, for which only two subgroups can be distinguished. One subgroup included the western European isolates (LMV-0, LMV-1, LMV-9, LMV-13, LMV-Aud, and LMV-E), the second subgroup included the isolates from Greece (LMV-GrB, LMV-Gr4, and LMV-Gr5), and LMV-Yar (from Yemen Arab Republic) was the sole member of the third subgroup. The same subgroups were obtained by the maximum likelihood, parsimony, and FITCH methods (data not shown). Sequence variability was greater in regions I and II, with 77 to 98% and 73 to 100% identity, respectively, among the isolates (Table 3), whereas region III was much more conserved (94 to 100% identity). This range of variability agrees with what is known about other potyviruses (32). No specific nucleotide changes were related to any resistance-breaking properties of the LMV isolates.

The variability of the amino acid sequences was similar to that of the nucleotide sequences: identity levels in regions I and II of the 10 isolates ranged between 72 to 100% and 74 to 100% (Fig. 2), respectively, and the topologies of the phylogenetic trees constructed with amino acid sequences were similar to those obtained with the nucleotide sequence data (data not shown). All isolates had exactly the same predicted amino acid sequence in their region III (CP C terminus), except the last amino acid of LMV-Yar, which is a valine instead of a leucine in all other isolates. It is now well established that distinct potyviruses have CP amino acid identities <70%, whereas strains of a same potyvirus species have identities >90% (33,34). Our results, therefore, confirm that all of the isolates studied here are isolates of LMV. As for the nucleotide sequences, no specific amino acid change could be associated with any of the LMV pathotypes analyzed.

The CP sequences of the 10 LMV isolates used in this study were compared with those of the 10 California isolates determined by Zerbini et al. (42). Because the sequences determined by these authors began at nucleotide 26 of the CP gene and the N-terminal sequences determined in this work terminated at nucleotide 117, only the common 92 nt were available for comparison. The phylogenetic tree derived from a multiple alignment of this short region is presented in Figure 4. The California isolates clearly cluster with the western European group.

DISCUSSION

The emergence in Europe of new LMV isolates causing severe symptoms in commercial lettuce cultivars containing the *mo1*[1] resistance gene was reported recently by several authors (4,7,25). Because such cultivars are extensively grown in open fields, especially in France, resistance breaking was evoked (25). These data and the report in California of very aggressive isolates (42) have aroused interest in establishing a better understanding of the molecular interactions between lettuce (*L. sativa*) and LMV. The molecular basis for resistance breaking are known only for a few plant viruses. Resistance-breaking determinants have been located in the CP of tobacco mosaic virus (19,28), pepper mild mottle virus (3), and potato virus X (17,31) and in the replicase (21,23) or movement proteins (22,39) of the tobacco and tomato mosaic viruses. In potyviruses, such determinants are thought to be found in the 5′ end of the genome (13,16), as well as the VPg domain of the NIa protein in the pea seedborne mosaic potyvirus (E. Johansen, *personal communication*).

In an attempt to link phylogenetic affinities with LMV resistance-breaking properties, we carried out an analysis of the molecular diversity of 10 LMV isolates and compared the results to the virulence of these isolates on various LMV-resistance genes. We chose isolates that previously differed in virulence or aggressiveness. All 10 isolates originated from Europe, except LMV-Yar. Greek isolates were included because they had been reported to be very aggressive and had been studied by other authors (4).

The results obtained for LMV-0, LMV-1, LMV-9, LMV-E, and LMV-13 confirmed our previous work (7,20). We also confirmed that these five isolates overcame the dominant resistance gene *Mo2* identified by Pink et al. (24). According to these data, these isolates were grouped into three distinct pathotypes. The two previously uncharacterized isolates, LMV-Aud and LMV-GrB, had virulence characteristics similar to LMV-E and LMV-13. The results obtained for LMV-Yar, LMV-Gr4, and LMV-Gr5 on cvs. Ithaca and Vanguard 75 agreed with results presented by Pink et al. (24) and Bos et al. (4): they are unable to infect cultivars containing the *Mo2* gene. However, Pink et al. (24) described LMV-Yar as avirulent on the other resistant cultivars because it did not induce discernible symptoms, although it multiplied. We also found a low concentration of LMV-Yar in cvs. Malika and Salinas 88, as estimated with a homologous antiserum, but under our conditions, Malika and Salinas 88 exhibited symptoms in all cases when infected with LMV-Yar. The status of LMV-Gr4 and LMV-Gr5 in terms of virulence on differential cultivars was more difficult to interpret. The rate of infection was never 100%, and the virus concentration was rather low in the infected plants. This situation probably is not an experimental artifact caused by the low efficiency of LMV-0 antiserum in detecting these isolates, because our results fit with those of Bos et al. (4), who used an antiserum raised against LMV-Gr4.

Based on the results of our molecular analyses, at least three groups of LMV isolates can be discriminated. The largest group in our sample contains isolates from western Europe (France and Spain) that are closely related to isolates from California. This analysis does not seem to show evidence of any clearly recombinant LMV isolate. One possible recombination event might have occurred for

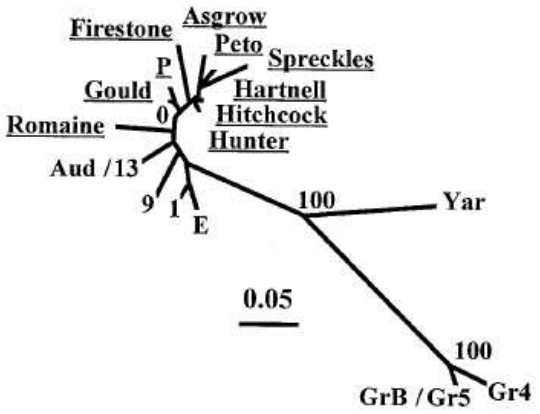

Fig. 4. Clustering neighbor-joining tree derived from the region of the N terminal of the coat protein gene discussed in text for California isolates of lettuce mosaic virus and the isolates studied here. The California isolates are underlined. The percentage of bootstrap replicates in which each node was recovered is given when above 70%. The scale bar represents, for the branch lengths, Kimura distances of 0.05.

the Greek isolates, with a recombination break point located between regions II and III (based on comparison of the position of the Greek isolates in the trees of regions I and II with that of region III). An alternate possibility is that the low intrinsic variability of region III simply may not allow discrimination of these Greek isolates from the pool of western European isolates. Indeed, the tree derived for region III is not statistically supported by significant boostrap values (Fig. 3). Sequencing of the entire CP gene of the Greek isolates is now underway and should allow us to settle this aspect of LMV phylogeny.

The three subgroups of LMV isolates correlate with the geographic origin of the isolates rather than with their virulence on differential cultivars. For example, the ability to overcome the resistance gene $mo1^2$ is shared by LMV-Yar, the three Greek isolates, and LMV-E, LMV-13, and LMV-Aud in the western European subgroup. However, these last three isolates cluster closer to LMV-0, LMV-1, and LMV-9, which are not virulent on $mo1^2$ (Fig. 3). Overcoming the $mo1^2$ gene is probably a relatively recent event (7). Therefore, it is reasonable to conclude that the common ancestor of the LMV isolates was unable to overcome this gene. A similar conclusion could be made about the $mo1^1$ and *Mo2* genes. Absence of evidence of recombination and failure to correlate differences in virulence properties and molecular variability among the LMV isolates suggest that the ability of these isolates to overcome the resistance genes may have appeared independently several times subsequent to the differentiation of the three LMV subgroups. Therefore, it would be interesting to determine the genomic determinants of resistance breaking and, particularly, to observe whether they are identical among isolates belonging to different subgroups.

Comparison of the isolates examined in this study with the partially sequenced California isolates (42) clearly showed that they are closely related to the isolates in the western European cluster. Of particular interest is the case of LMV-0: although it was originally isolated in France, it is more closely related to the California isolates than to the other western European isolates. In their study, Zerbini et al. (42) already showed that the LMV-0 sequence was 95 to 99% identical to those of the California isolates. These observations clearly indicate the possibility that LMV-0 may have been exchanged between the two continents in the recent past, perhaps via contaminated seed lots, because LMV-0 is seed transmissible.

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A l'occasion de cette étude de la variabilité moléculaire du LMV, j'ai mis au point au laboratoire une technique de séquençage direct des produits d'amplification, décrite dans la **publication 3** (cf ci-dessus) et qui a été aussi appliquée à l'étude de la variabilité d'un autre potyvirus étudié au laboratoire, le virus de la Sharka (PPV) qui a fait l'objet de la, **publication 5** pour laquelle je suis co-auteur.

Ce travail a donc permis de sélectionner deux isolats de LMV, LMV-0 et LMV-E, qui sur la base des 3 régions séquencées semblaient proches sur le plan moléculaire tout en étant différents par leurs propriétés biologiques. En effet, LMV-0 induit des symptômes peu sévères sur laitue sensible (ne portant pas de gène *mo1*), est incapable d'infecter les variétés de laitue porteuses des gènes de résistance *mo1* et est transmis par les semences de laitue alors que LMV-E provoque des symptômes sévères sur laitue sensible, contourne les résistances *mo1* mais n'est pas transmis par les semences de laitue. L'étape suivante à consister à séquencer le génome complet de ces deux isolats. Je me suis investi dans le séquençage de LMV-0 alors qu'un autre étudiant en thèse (Shujun Yang) ayant démarré sa thèse un an après moi s'est investi dans le séquençage de LMV-E. Ce travail a fait l'objet de la **publication 4** présentée ci-après.

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Comparison of the complete nucleotide sequences of two isolates of lettuce mosaic virus differing in their biological properties[1]

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Abstract

The complete nucleotide sequences of the genomic RNAs of the 0 and E isolates of lettuce mosaic potyvirus (LMV) have been determined. These two isolates differ by their behavior towards two lettuce resistance genes and by their seed transmission properties. LMV-0 is unable to induce disease in lettuce carrying either one of the *mo1*^{1} and *mo1*^{2} recessive resistance genes, whereas LMV-E is able to induce disease in the same plants. The genomes of these two isolates are 10 080 nucleotides (nt) in length, excluding the poly(A) tract, and encode polyproteins of 3255 amino acids (aa). The open reading frame is flanked by a 5′ non-coding region of 103 nt and a 3′ non-coding region of 212 nucleotides. Ten proteins were predicted. The P3 protein, with 377 aa, is the longest potyviral P3 protein characterized to date while the P1 protein, with 437 aa, is among the longest P1 proteins reported. Sequence comparisons between the two isolates demonstrated only limited sequence difference. The overall nucleotide and amino acid sequence identities between LMV-0 and LMV-E are 94 and 97% respectively. The greatest variability occurs in the P1 and in the variable N-terminal region of the coat protein, while the NIa protease domain, the NIb protein, the C-terminus of the helper component protease and the 3′ non-coding region are extensively conserved. While this sequence analysis does not allow direct identification of determinants involved in the resistance breaking or in the seed transmissibility properties, these data are a first step towards the characterization of these determinants. © 1997 Elsevier Science B.V.

Keywords: Lettuce mosaic virus (LMV); Potyvirus; Nucleotide sequences; Resistance breaking

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[1] The nucleotide sequence data reported in this paper have been submitted to the EMBL database and assigned the accession numbers X97704 for LMV-0 and X97705 for LMV-E.

[2] The authors request that F. Revers and S.J. Yang, who have contributed equal amounts of work to this project, be both considered as first authors.

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1. Introduction

Lettuce mosaic virus (LMV; Tomlinson, 1970), a potyvirus transmitted by seed and aphids, causes a major disease of commercial lettuce (*Lactuca sativa*) crops all over the world (Dinant and Lot, 1992). It has flexuous filamentous particles of 750 × 13 nm. In infected lettuce, the symptoms are dwarfing, defective heading, mottling, vein clearing and leaf distortion. Three resistance genes to LMV have been described and agronomically used to protect lettuce cultivars: the recessive genes $mo1^1$ and $mo1^2$ modify disease severity and virus accumulation to differing extents depending on the genetic background (Bannerot et al., 1969; Ryder, 1970; Pink et al., 1992a). The dominant gene *Mo2* affords immunity to the virus but is overcome by most LMV isolates (Pink et al., 1992a). Based on the ability to overcome these resistance genes, isolates of LMV have been classified into several pathotypes (Dinant and Lot, 1992; Pink et al., 1992a,b; Bos et al., 1994). These authors pointed out the emergence, in Europe, of a new pathotype, corresponding to severe isolates breaking all three resistance sources. The sequence of the LMV-0 3′ terminal 1651 nucleotides (nt) covering the coat protein (CP) gene has been reported previously (Dinant et al., 1991). The complete (LMV-R isolate) or partial sequence of the coat protein genes of ten isolates from the Salinas Valley in California has been also determined (Zerbini et al., 1995). As LMV-0, all these isolates belong to the same pathotype (pathotype II).

The complete nucleotide sequences of about 15 different potyviruses have now been determined (Shukla et al., 1994). The analysis of these genomes revealed the presence of a single open reading frame (ORF) encoding a large polyprotein, which is processed at conserved locations into mature viral proteins by three virus-encoded proteases (Riechmann et al., 1992; Shukla et al., 1994). The cleavage sites of the polyprotein are apparently similar in sequence and localization among the potyviral species.

Although they are the largest and most economically important virus group (Shukla et al., 1994), there is currently only very limited knowledge on potyvirus resistance breaking determinants (Hellman et al., 1990; Jayaram et al., 1992) or, more generally, on potyvirus symptoms determinants (Rodriguez-Cerezo et al., 1991; Atreya et al., 1992; Klein et al., 1994). We have undertaken the molecular characterization of resistance-breaking of common isolates of LMV with the aim of understanding better the molecular bases of such properties. As a first step, we have entirely sequenced two isolates of LMV which differ in their ability to overcome the two recessive $mo1^1$ and $mo1^2$ resistance genes. LMV-0 belongs to the pathotype II, described by Pink et al. (1992a), which provokes very mild or no symptom on plants harboring $mo1^1$ or $Mo1^2$ genes respectively. LMV-E (Dinant and Lot, 1992) belongs to the pathotype IV which causes severe symptoms on all cultivars carrying these genes or *Mo2* gene. In addition, LMV-0 and LMV-E differ in their seed transmission properties, since LMV-E is not seed transmissible while LMV-0 is seed transmitted at a 2–9% rate in susceptible lettuce (Dinant and Lot, 1992). This paper reports the cloning and complete sequencing of the genomic RNAs of these two isolates.

2. Methods

2.1. Virus propagation and viral RNA preparation

LMV-0 and LMV-E isolates were maintained and propagated under glasshouse conditions on lettuce (*Lactuca sativa*) plants cv 'Trocadero' (with no known resistance gene) and cv 'Mantilia' (harboring the $mo1^1$ gene) respectively. Virion purification was as described by Dinant and Lot (1992). Viral RNA was extracted from purified virions resuspended in 10 mM Tris, 5 mM EDTA, pH 7.6 using 50 mg/ml proteinase K and 0.5% SDS, followed by a phenol and a phenol/chloroform extraction.

2.2. Cloning of cDNA

Synthesis of the first strand of the cDNA was performed with oligo(dT) as primer and either the reverse transcriptase of avian myeloblastosis virus

(AMV RTase, Pharmacia) and purified RNA as the template (Le Gall et al., 1988) or the reverse transcriptase of moloney murine leukemia virus (M-MuLV RTase, BRL) and purified undissociated virus as the template (Fakhfakh et al., 1995). For the second strand synthesis, RNase H and *Escherichia coli* DNA polymerase I were used as described by Gubler and Hoffman (1983). The cDNA molecules were then size-selected by ultracentrifugation in 10–40% sucrose gradients (LMV-0) or by polyethylene glycol precipitation (LMV-E) (Le Gall et al., 1995). The largest cDNAs were ligated into *Sma*I–linearized, dephosphorylated pBluescribe (pBS) vector (Stratagene). The ligation mixture was used to transform competent *E. coli* XL1-Blue cells (Stratagene). Plasmids were characterized by restriction analysis after extraction by the alkaline lysis method (Maniatis et al., 1989). Overlapping cDNA clones were selected for the determination of the nucleotide sequence. For sequencing, a set of overlapping deletion subclones was prepared from various large cDNA clones, using either exonuclease III (as suggested by the manufacturer: Erase-a-Base System Kit, Promega, Madison, WI), or digestions with various restriction endonucleases.

2.3. Nucleotide sequencing

The nucleotide sequence was determined by the chain-termination method of Sanger et al. (1977) using the Sequenase™ kit (US Biochemical), [α-^{35}S]dATP (Amersham), and supercoiled plasmid DNA as the template. Primers complementary to either adjacent vector sequences or to LMV sequences were used for the sequencing reactions.

2.4. Direct RNA sequencing

The nucleotide sequence of the 5′ end of the LMV-0 and LMV-E genomic RNAs were determined by direct RNA sequencing (Fichot and Girard, 1990) using reverse transcriptase (AMV RTase, Pharmacia) and synthetic oligonucleotide primers with sequences 5′GCAATAGTTGCAGTAGTTCTGCCCTCCAACCTAGG3′ (LMV-211p; positions 704–738), 5′CCTCTGGCTTTGCGATGGTCC3′ (positions 570–590), 5′GCCTAGCATCAGTTATGAAATC3′ (positions 338–359) and 5′TGTGGTTGAAAATGTTGT3′ (positions 85–102).

2.5. Polymerase chain reaction (PCR) amplification

To confirm the 5′ end nucleotide sequence of LMV-0, PCR amplification was performed with the primers LMV-47 (5′AAAATAAAACAACCCAACACAACTC, positions 1–25) and LMV-211p (see above). One hundred microliters of reverse transcription–PCR (RT–PCR) reaction mix (Tris–HCl pH 8.8 10 mM; $MgCl_2$ 1.5 mM; KCl 50 mM; Triton X-100 0.3%; 250 mM each of dNTPs; 1 mM of each primer; 0.25 U of AMV RTase; 0.5 U Taq polymerase) were added to 1 ng of LMV RNA. Conditions for the RT–PCR were 42°C for 15 min for the RT, 95°C for 5 min for denaturation of the DNA–RNA hybrids and RTase inactivation, followed by 35 cycles of amplification by denaturing at 92°C for 30 s, annealing at 56°C for 30 s and extending at 72°C for 1 min. After electrophoresis and purification with Geneclean II kit (BIO101), the amplified cDNA was blunted by the use of the Klenow fragment of *E. coli* polymerase I, phosphorylated with T4 polynucleotide kinase, and ligated to *Hin*cII–linearized, dephosphorylated pBS vector. A cDNA clone corresponding to the 5′ region was selected for sequencing.

2.6. Sequence compilation and analysis

Analysis of the sequences was performed using the Microgenie (Beckman) and PC-GENE (Genofit) packages. A multiple alignment of 15 complete amino acid (aa) sequences of potyviruses currently available in the GenBank database, and of the two LMV sequences was obtained using the program Clustal W (Thompson et al., 1994).

3. Results and discussion

3.1. Nucleotide sequencing

Initial screening of the cDNA clones by restriction enzyme mapping, hybridization analysis and partial sequencing identified six overlapping cDNA clones for LMV-0 and at least ten overlapping cDNA clones for LMV-E, which were estimated to cover almost the entire genome of the two isolates (not shown). Sequence data was obtained mainly from ordered deletion subclones. To complete this data, primers complementary to already determined LMV sequences were used. Each position was sequenced at least once on each strand, most often from two independent cDNA clones (except for 550 and 630 nt, respectively for LMV-0 and LMV-E, for which only one clone was available).

The first 18 and 690 nt at the 5′ end of LMV-E and LMV-0 RNAs, respectively, were not represented in the cDNA banks and were thus sequenced directly from the RNA. For LMV-0, a PCR-amplified cDNA clone, corresponding to the first 5′ 738 nt, was sequenced to confirm the RNA sequencing results (not shown).

3.2. Genome organization

For both isolates, the genome was found to be 10 080 nt in length excluding the 3′ poly(A) tail. An alignment of the two nucleotide sequences showed that they are exactly co-linear, differing from each other only by point mutations (data not shown). The nucleotide positions indicated thereafter are thus valid for both LMV sequences. The RNA composition of the two isolates is 33% adenine (A), 24% guanine (G), 21% cytosine (C) and 22% uracil (U). Computer analysis revealed an ORF of 9768 nt encoding a polyprotein of 3255 aa. Two in frame AUGs were found at positions 104 and 140, but only the first one is in a context (ACAAAUGGC) described by Lütcke et al. (1987) as optimal for translation initiation in plants. The termination codon UAA is located at position 9869. The 5′ non-coding region (5′NCR) and the 3′ non-coding region (3′NCR) are respectively 103 and 212 nt long. The 3′NCR is 1 nt longer than that determined by Dinant et al. (1991) because for both isolates we found three uracil residues at positions 9925–9927 instead of two as reported by these authors. As is often the case for plant virus 5′ leader sequences (Gallie et al., 1987), the base composition of the 5′NCR has a low G-content with only 4% (24% C, 22% U and 50% A).

Within each isolate, a few nucleotide differences (4 for LMV-E, 6 for LMV-0) between independent cDNA clones were detected. For each of these differences, the nucleotide found most often if more than two independant clones were sequenced, or that identical between the two isolates, was selected and displayed in the final assembled sequence. Two replacements were silent in LMV-E ($U_{7327}A$ and $C_{7690}U$, where the first residue is the one from the assembled sequence at the position indicated and the second one the variant), and three were silent in LMV-0 ($C_{1423}G$, $G_{2047}A$ and $A_{7090}G$). Respectively, two and three replacements led to amino acid exchanges, $A_{1014}U$ and $U_{5712}C$ for LMV-E; $U_{4062}G$, $U_{5421}A$ and $A_{5718}G$ for LMV-0.

A multiple alignment of all complete potyviral amino acid sequences available, including the two LMV sequences, was performed (data not shown). The predicted cleavage sites of the polyproteins were aligned in each case, and at these positions possible cleavage sequences were also found in LMV (Table 1).

At least seven sites are putatively cleaved by the nuclear inclusion protein a (NIa) protease, as presented in Table 1. LMV NIa cleavage dipeptides are Q/A, Q/S, Q/G and Q/V. The latter is for the NIb/ CP cleavage as already described for LMV-0 (Dinant et al., 1991), and confirmed here for LMV-E. In addition, a consensual amino acid, valine, was found at position P4, as is the case for many potyviruses (Riechmann et al., 1992). As identified by Dougherty and Parks (1991), an internal cleavage site at an E/S dipeptide sequence was found in the NIa sequence in a similar context between the VPg and proteinase domains (Table 1).

The proteinase activity of the helper component (HC-Pro) of TEV has been shown to cleave at its carboxy-terminus between two glycine residues

Table 1
Amino acid sequence of the seven predicted cleavage sites of the NIa protease along the LMV polyprotein

Site		Amino acid position								
		P6[a]	P5	P4	P3	P2	P1	↓[a]	P′1	P′2
P3/6K1	(1268)[b]	E	E	V	E	H	Q		A	K
6K1/CI	(1320)	D	D	V	R	H	Q		S	V
CI/6K2	(1963)	D	V	I	Y	H	Q		S	K
6K2/VPg	(2016)	D	A/V[c]	V/I	R	H	Q		G	K
VPg/NIa-Pro	(2209)	I	P	V	K	H	E		S	K
NIa-Pro/NIb	(2452)	D	A	V	Q	F	Q		S	K
NIb/CP	(2972)	D	E	V	Y	H	Q		V	D
Consensus[d]		D	—	V	—	H	Q		S	K
		(E,I)		(I)		(F)	(E)		(G,A,V)	(V,D)

[a] P6–P1 and P′1–P′2 are the respective amino acid positions upstream and downstream of the cleavage site, numbered from this site (cleavage indicated with an arrow thus occurs between P1 and P′1).
[b] Locations within the polyprotein of amino acids P6.
[c] In the 6K2/VPg line, the first residue given at positions P5 and P4 is that occuring in LMV-0, and the second one is that of LMV-E.
[d] A consensus between the seven sequences is derived and displayed when more than four sequences have the same residue at a given position. Other amino acids also found at each position are given in parenthesis.

(Oh and Carrington, 1989; Carrington et al., 1989). A tyrosine at position P4 and a valine at position P2 are also required for this process (Carrington and Herndon, 1992). A sequence (QHYRVG/G) homologous to the TEV cleavage site exists in the LMV polyprotein at positions 890–896.

The third protease activity is harbored by protein P1 whose carboxy-terminal cleavage site has been identified by Mavankal and Rhoads (1991) in TVMV. A Y/S dipeptide has been located at positions 438 and 439 for LMV with a glutamine at position P2 as found in PeMV, PVY and PRSV P1/HC-Pro cleavage sites.

As observed in the multiple alignment, the P1 and P3 proteins of LMV are among the largest ones observed to date in sequenced potyviruses with respectively 437 and 377 aa. The other proteins are similar in size to those of other potyviruses.

A synthetic LMV polyprotein map is shown in Fig. 1. The predicted molecular masses (kDa) of the ten viral proteins are 50 (P1), 52 (HC-Pro), 42 (P3), 6 (6K1), 71 (CI), 6 (6K2), 22 (VPg), 27 (NIa), 60 (NIb) and 31 (CP).

Based on multiple alignments, as described above for the cleavage sites, all the conserved motifs described in potyviruses were identified in both LMV isolates. Amino acid sequences of the two isolates are presented in the Fig. 2. In the LMV P1 protein, conserved amino acids of the serine protease active site (Verchot et al., 1991; Riechmann et al., 1992) have been located in the carboxy-terminus at positions 345–389 with the sequence $Hx_8Dx_{31}GxCG$ (where x designates any unspecified amino-acid). In HC-Pro, the cysteine cluster, considered to be a zinc-finger-like metal binding motif and first noted by Robaglia et al. (1989) in the PVY sequence, occurs in the LMV polyprotein at positions 464–495 with the sequence $Cx_8Cx_{13}Fx_4Cx_2C$ (a phenylalanine replaces a consensual cysteine at position 487). Two other conserved amino acids, a cysteine and a histidine separated by 72 aa residues ($Cx_{72}H$), have been shown to be required for the HC-Pro protease activity of TEV (Oh and Carrington, 1989). In the LMV sequence, C_{781} and H_{854} are aligned with these residues and are thus likely to be their functional equivalents. The motif $EPYx_7SPx_2LxAx_2NxGx_2Ex_5F$ is found in the P3 region of LMV at positions 955–985, with a phenylalanine replacing the consensual C-terminal tryptophan. The presence of this potyviral sequence in the P3 region suggests that the P3

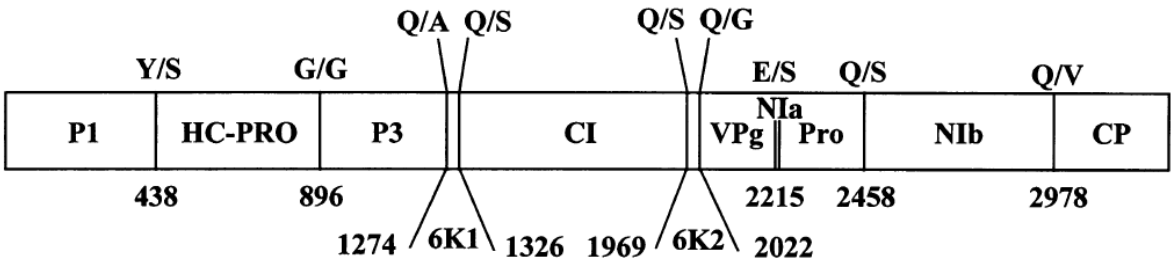

Fig. 1. Proposed genetic map of the LMV genome. The positions and the dipeptides at the putative cleavage sites in the polyprotein are shown below and above the map, respectively.

protein could be involved in the regulation of the proteolytic processing of the potyviral polyprotein, similar to what is seen with comoviruses (Riechmann et al., 1992). The nucleotide-binding motif of the cylindrical inclusion (CI) protein, for which a helicase activity has been shown for PPV (Lain et al., 1991), is also present in the putative CI protein of LMV at positions 1410–1418 near the amino-terminus with the sequence GGVGSGKST. In the carboxy-terminus of the NIa protease of LMV, the catalytic triad (histidine, aspartic acid and cysteine), which constitutes the active site residues of the NIa protease (Dougherty et al., 1989), has been located at positions H_{2260}, D_{2295} and C_{2365}. The protein NIb, which is the most conserved potyvirus protein, is thought to be the viral RNA polymerase because it contains a conserved motif present in all positive-stranded RNA virus RNA-dependent RNA polymerases (Kamer and Argos, 1984). This motif ($SGx_3Tx_3NTx_{30}GDD$) occurs at positions 2767–2810 in the LMV polyprotein. The exact location of the coat protein (CP) of LMV-0 has already been determined by Dinant et al. (1991) at the 3′ end of the genome. The tripeptide DAG, involved in aphid transmission as demonstrated for TVMV by Atreya et al. (1991), is found at positions 2983–2985 for both LMV isolates.

3.3. Sequence comparison between the two isolates

Sequence comparison between LMV-0 and LMV-E showed that the nucleotide and amino acid sequence identities are very high, reaching about 94 and 97%, respectively. These figures are higher than in the case of pea seed-borne mosaic virus (PSbMV), for which a similar analysis between isolates of different biological properties (but also different geographical origins) has been performed (Johansen et al., 1996).

In most of the LMV genome, the nucleotide identity between the two isolates varies from 92 to 96% (Fig. 3). However, the 5′ half of the region encoding the HC-Pro and the 3′NCR are distinct from the other regions because they have an identity level higher than 98% (211/212 in the 3′NCR and 685/700 in the 5′ region of the HC-Pro domain). As shown among other potyvirus species (Shukla et al., 1991), the P1 and the amino-terminus of the CP are the most variable regions of the polyprotein between the two isolates of LMV with only 93 and 82% aa sequence identities, respectively (Fig. 3). The largest number of non-conservative amino acid differences occurs in the P1 protein. These differences include four acido-basic residue changes: $E_{148}K$, $E_{245}K$, $K_{400}E$ and $E_{418}K$ (the first amino acid is from the LMV-0 sequence, the second one from the LMV-E sequence and the number indicates the amino acid position in the polyprotein; Fig. 2). The regions which show a higher amino acid variability usually also show a higher than average nucleotide variability (Fig. 3). However, some of the regions especially variable at the nucleotide level correspond to regions of amino acid conservation, due to a high proportion of silent nucleotide exchanges (about 80%). As illustrated in Fig. 3, the region located between the VPg and NIa protease domains has the most variable nucleotide sequence of the LMV genome with only 88% nucleotide identity, but has a very conserved

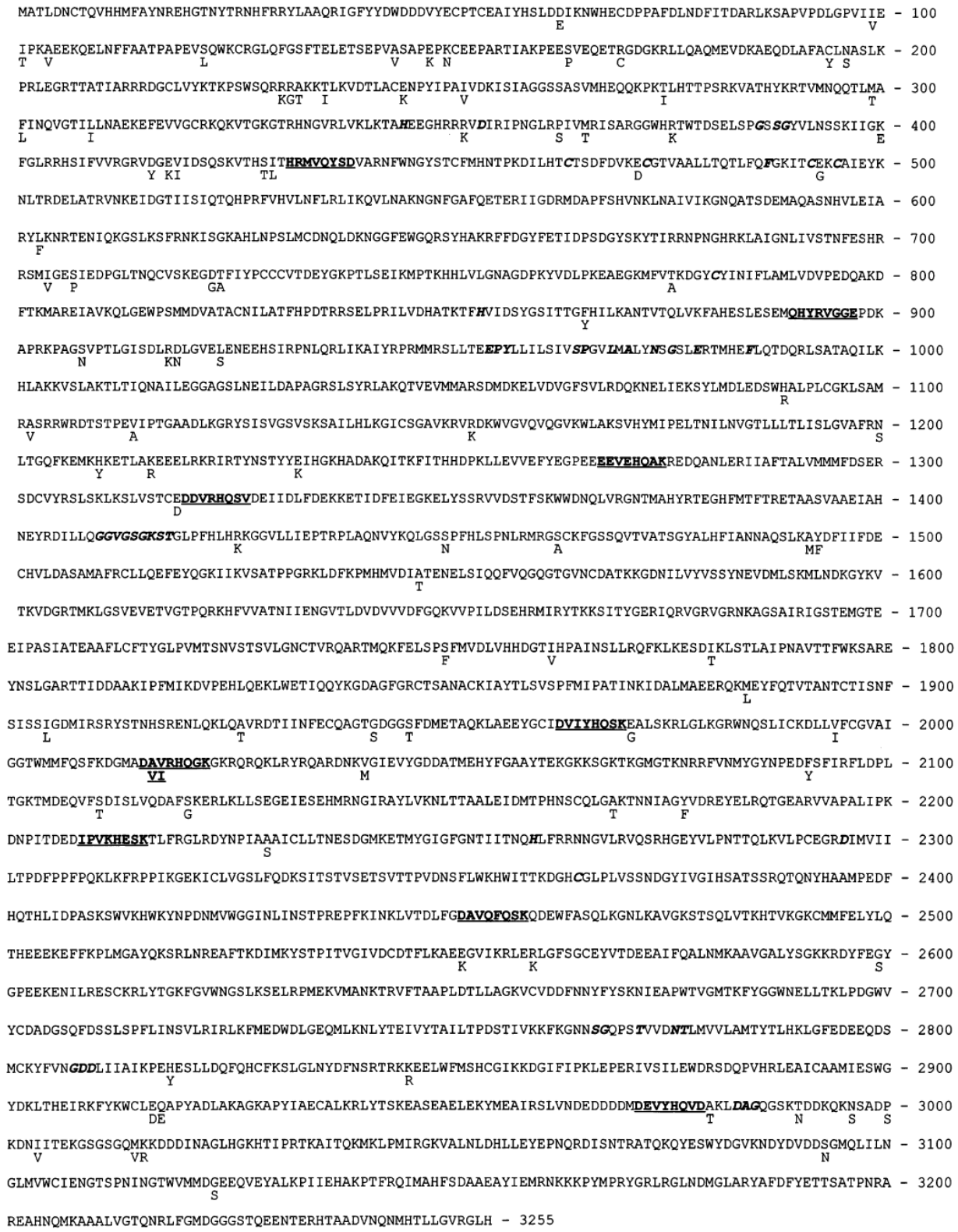

Fig. 2. Amino acid sequence of the LMV polyprotein. The sequence of LMV-0 is given and that of LMV-E, where different, is indicated below. The regions around the putative cleavage sites (from P6 to P′2) are underlined, and those of sequence signatures, as described in the text, are in italics. The amino-acid positions are indicated at the end of each line.

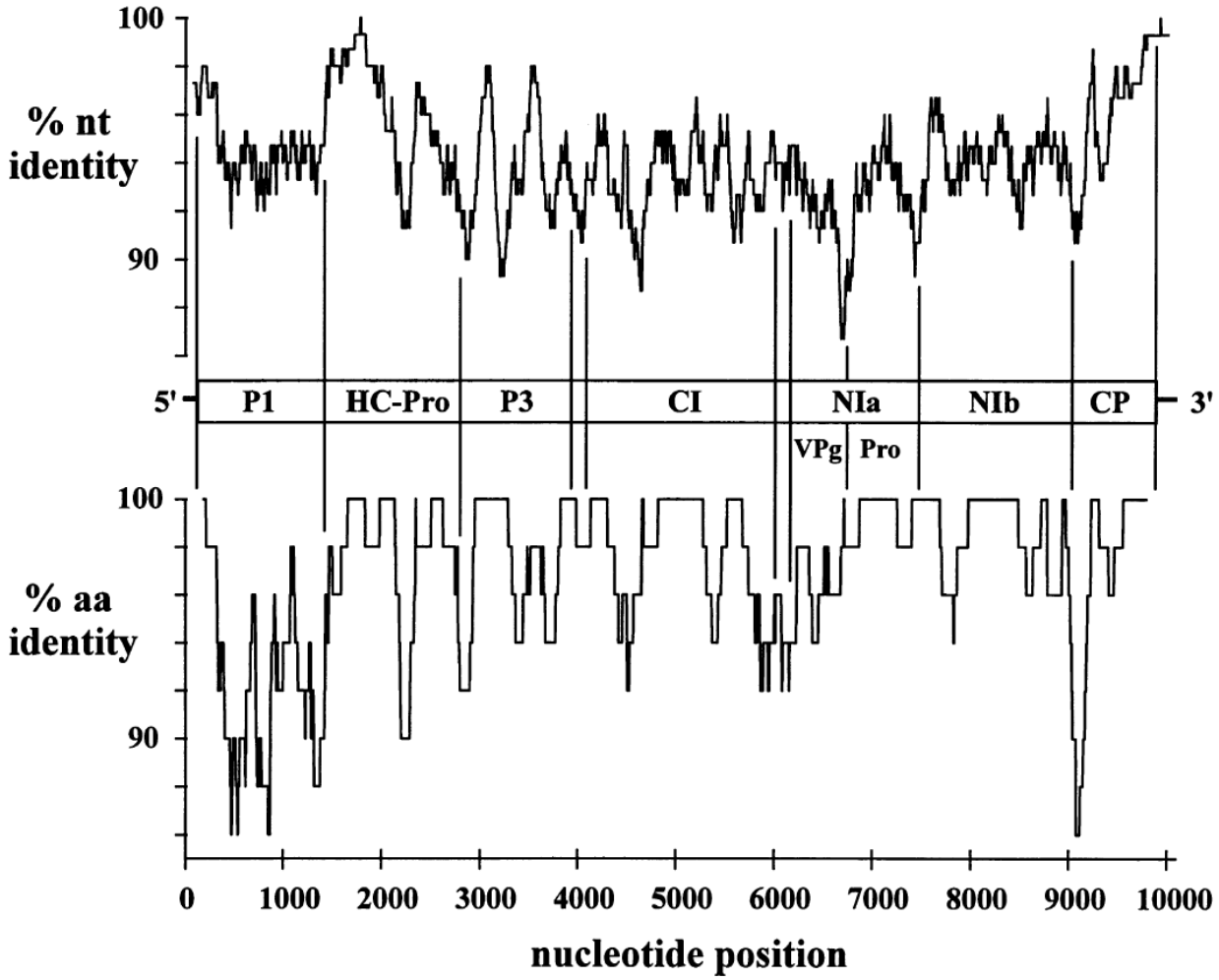

Fig. 3. Percentage of nucleotide (top) and amino-acid (bottom) identities between the two LMV isolates along their genome. The percentage of identity, measured in a window of 150 nucleotides or 50 amino-acids, was plotted at each position. The putative genetic map of LMV (see Fig. 1) is indicated between the two curves, and the nucleotide positions along the genome at the bottom of the figure.

amino acid sequence (about 98% aa identity). As compared to the PSbMV identity plot (Johansen et al., 1996), that of LMV is qualitatively characterized by additional divergent regions between the HC-Pro domains, as well as in the area around the 6K2 domain. In addition, the CP N-terminus is more divergent relative to the rest of the genome.

The molecular base for resistance breaking are known only for a few viruses. Several genomic determinants have been identified in the replicase proteins (Meshi et al., 1988; Padgett and Beachy, 1993) and in the movement protein (Meshi et al., 1989; Weber et al., 1993) of tobacco mosaic virus (TMV) and tomato mosaic virus (ToMV). Other genomic determinants have been mapped in the CP of TMV (Knorr and Dawson, 1988; Saito et al., 1987), pepper mild mottle virus (Berzal-Herranz et al., 1995) and potato virus X (PVX) (Kavanagh et al., 1992; Santa Cruz and Baulcombe, 1993). In these studies, changes in local net charge or structural changes between the proteins of virulent and avirulent strains have been proposed to result in the disruption of an

electrostatic interaction between the viral protein and a host resistance factor (Meshi et al., 1988; Padgett and Beachy, 1993).

In potyviruses, the 5′ terminal protein genes have been suggested to contain these determinants for TVMV (Hellman et al., 1990). Comparison of the nucleotide sequences of two strains of SMV differing in their resistance breaking abilities shows amino acid exchanges in the P1 and P3 proteins causing changes in local net charge (Jayaram et al., 1992). In LMV, sequence differences are mainly located in the P1 and CP proteins, but some significant changes are also present in other parts of the polyprotein such as in the 6K2 protein where 4 aa out of a total of 53 differ between LMV-0 and LMV-E. However, the hydropathy profiles of the polyprotein of the two isolates were not significantly different (data not shown).

Concerning seed transmission of potyviruses, only one study was performed for PSbMV, which showed a possible implication of the 5′ region of the genome. The implicated region contained the 5′NC, the P1 protein and two thirds of the HC-Pro (Johansen et al., 1994).

The sequences of isolates 0 and E of LMV provide the first basis for the characterization of specific determinants involved in resistance breaking. Identification of such determinants will be facilitated by the production of full-length infectious transcripts and by subsequent construction of recombinant viruses by exchanging genomic regions between the two isolates.

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Une fois ce séquençage réalisé qui ne permettait pas comme attendu d'identifier directement les déterminants moléculaires impliqués dans les propriétés biologiques du LMV, compte tenu de la répartition sur l'ensemble de la polyprotéine des différences en acide aminé entre les 2 isolats, nous avons décidé de 2 stratégies pour le production de clones infectieux de ces deux isolats : (1) construction d'un ADNc complet à partir de la banque d'ADNc produite pour le séquençage, stratégie classique utilisée à l'époque ; (2) production d'un ADNc complet par longue PCR, stratégie encore peu développé à ce moment là. C'est sur cette deuxième stratégie que je me suis investi.

De façon schématique, cette stratégie a consisté à amplifier l'ensemble du génome du LMV en une seule PCR à partir de l'ADNc produit après transcription inverse de l'ARN viral en utilisant des mégaprimers comme amorce. Ces mégaprimers étaient constitués en 5' du promoteur 70S fusionné à l'extrémité 5' du génome du LMV et en 3' du terminateur de transcription Nos fusionné à l'extrémité 3' du génome viral. Si plusieurs clones complets ont été obtenus par cette stratégie, aucun ne s'est révélé infectieux probablement du à des mutations introduites dans le génome du LMV lors des phases d'amplification.

Suite à ces travaux, Shujun Yang a pu obtenir par stratégie classique un clone infectieux de LMV-E, et Elise Redondo (qui a démarré sa thèse après la fin de la mienne) a obtenu par la même stratégie un clone infectieux de LMV-0 et a pu produire des clones recombinants entre ces deux isolats qui ont servi par la suite à cartographier les déterminants génomiques impliqués dans les différentes propriétés biologiques du LMV.

Mes travaux de thèse ont été valorisés dans les **Publications à comité de lecture [2], [3], [4], [5], [6], [8] et [9]** et **les Publications sans comité de lecture [18], [20], [22] et [24]**.

Je présente ci-après la **publication [9]** qui est une revue écrite à partir du travail de synthèse développé dans l'introduction du manuscrit de ma thèse. Elle fut écrite lors de mon séjour chez Andy Maule et publiée en 1999. Cette revue a été citée plus de 100 fois, ce qui représente la publication la plus citée parmi non seulement mes publications mais aussi toutes celles de l'équipe de virologie. Elle est encore aujourd'hui citée en moyenne tous les 2 mois et représente toujours une référence importante sur le plan international pour les équipes travaillant sur les potyvirus. Dix ans s'étant écoulés et de nombreuses données ayant été publiées sur ce sujet, nous envisageons d'écrire prochainement une nouvelle revue.

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Current Review

New Advances in Understanding the Molecular Biology of Plant/Potyvirus Interactions

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In recent years, researchers have adopted many new technologies to help understand potyvirus pathogenesis. Their findings have illuminated key aspects of the interactions between the host and the virus, and between the virus and its aphid vector. This review focuses on advances in our understanding of the molecular determinants of systemic infection, symptom expression, aphid and seed transmission, and natural and engineered resistance to potyviruses. Very recent developments in the area of post-transcriptional gene silencing indicate not only that the process is fundamental to engineered resistance, but may also underlie many aspects of the biology of plant viruses.

Additional keywords: cell-to-cell movement, HC-Pro, long-distance movement.

Potyvirus is the largest genus of plant viruses, with 180 definite or possible members causing significant losses in a wide range of crop plants (Shukla et al. 1994). The viruses are aphid transmitted in a nonpersistent manner and some of them are also seed transmitted (Johansen et al. 1994; Shukla et al. 1994). The organization of the potyvirus single-stranded RNA genome is shown in Figure 1.

Substantial advances have been made in recent years in our understanding of the molecular biology of the interactions between potyviruses and their hosts; the last major review of this topic was published in 1992 (Riechmann et al. 1992). These achievements have been made possible through knowledge of the complete nucleotide sequence of the viral genomes, through the generation of infectious molecules in vitro and in vivo from cloned viral cDNAs (for reviews see Boyer and Haenni 1994; Shukla et al. 1994), and through the techniques of mutagenesis and recombinant hybrid virus construction. Additionally, the use of genes for visible reporter molecules cloned into infectious viral genomes has been crucial. Notably, these have included the *uidA* gene encoding β-glucuronidase (GUS; e.g., Dolja et al. 1992) and the *gfp* gene from *Aequorea victoria* encoding the green fluorescent protein (GFP; e.g., Schaad et al. 1997a). Hence, the genes either have been cloned to give a fusion with a viral protein, or have been such that proteolytic cleavage releases the free reporter protein from the potyviral polyprotein. In the latter case, cloning the reporter gene and a new nuclear inclusion a protein (NIa)-specific cleavage site adjacent to the helper component-proteinase (HC-Pro; Schaad et al. 1997a, 1997b) has been particularly effective.

New technology has contributed significantly to the recent advances. Yeast two-hybrid systems for analyzing protein-protein interactions in the nucleus are commercially available, and the rapid expansion of genetic and bioinformatics resources has meant that the identification and characterization of host genes (particularly for *Arabidopsis thaliana*) is possible and will become routine within a few years.

This review focuses on these new advances, in particular on the host and viral molecular determinants shown to have a significant role during viral infection. Thus, we describe here the molecular determinants involved in systemic infection (genome amplification and cell-to-cell and long-distance movements), symptom expression, aphid and seed transmissions, and natural and engineered resistances to potyviruses.

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Systemic infection.

Systemic infection occurs when a virus is able to move, after a genome amplification step, from the primary infection focus to invade distal regions of the plant (Lucas and Gilbertson 1994; Carrington et al. 1996). This requires that the infectious unit should move locally from cell to cell through plasmodesmata, and then over longer distances through the phloem. For most plant virus groups, the movement process involves one or more specialized virus-encoded proteins, termed movement proteins (MPs). These proteins are usually characterized by mutagenesis when cell-to-cell movement from the primary infected cell is altered without affecting virus replication. Potyviruses do not encode a dedicated MP, but movement functions have been allocated to several proteins, including the coat protein (CP), HC-Pro, the cylindrical inclusion (CI) protein, and the genome linked protein (VPg). In some of these cases, the mutation-based definition has been supported by ultrastructural observations and microinjection experiments to demonstrate that the proteins influence a plasmodesmal function.

Genome amplification. Potyvirus genome amplification requires two fundamental processes, viral RNA translation for the synthesis of virus-specific proteins, including the viral replicase, and RNA replication itself. The interrelationship of

these two processes makes it difficult to assign particular functions uniquely to RNA replication. Hence, most potyvirus-encoded proteins affect the cellular level of virus accumulation. Superficially, the RNA-binding properties of P1 (Brantley and Hunt 1993; Soumounou and Laliberté 1994; Merits et al. 1998), HC-Pro (Maia and Bernardi 1996; Merits et al. 1998), CI protein (Eagles et al. 1994; Fernández et al. 1995; Merits et al. 1998), NIa (Daròs and Carrington 1997; Merits et al. 1998), nuclear inclusion b protein (NIb; replicase), and CP (Merits et al. 1998) may suggest that all these proteins function in the RNA replication process. However, the possibility of the involvement of viral proteins in the distinctive features of potyvirus RNA translation (reviewed in Riechmann et al. 1992) and the potential for cell-to-cell translocation of viral RNA (see below) make it difficult to allocate the functions precisely. Almost certainly, we will find that the processes of RNA replication and translation are spatially and temporally integrated.

Potyviral RNA differs from host mRNAs in the absence of a 5′-cap structure. In the virion this is replaced by the VPg; the 5′ structure of the polysome-associated potyviral RNA is not known. Both host mRNAs and potyviral RNAs are 3′-polyadenylated. Since efficient translation of most eukaryotic mRNAs requires that the 5′-cap and the 3′-poly(A) act in concert (Gallie 1998), some alternative mechanism must operate for potyviruses. Gallie et al. (1995) have shown for *Tobacco etch virus* (TEV) that the long 5′ nontranslated region (NTR) substitutes for the 5′-cap in the interaction with the 3′-poly(A) and can act as a translational enhancer when placed upstream of heterologous open reading frames. One role for the 5′-cap is the binding of translation factors before the recruitment of ribosomes. Hence, the 5′ NTR and/or VPg must provide this specific role. Some support for this comes from the demonstration of a yeast two-hybrid interaction between the VPg of *Turnip mosaic virus* (TuMV) and eIF(iso)4E from *A. thaliana* (Wittmann et al. 1997). For some members of the *Picornaviridae* family of viruses, which all have a 5′-VPg, ribosome entry has been linked to the presence of a complex RNA structure in the 5′ NTR termed the internal ribosome entry site (IRES; Pelletier and Sonenberg 1988). There is some evidence (Levis and Astier-Manifacier 1993; Basso et al. 1994) that potyvirus 5′ NTRs could act similarly, although no IRES-like structure has been identified. *Plum pox virus* (PPV) 5′ NTR presents an added complication in that it contains an additional in-frame AUG upstream of the polyprotein start codon. This appears to be handled through a mechanism of cap-independent "leaky scanning" translation (Simón-Buela et al. 1997a).

For RNA replication, the demonstration of RNA polymerase activity of the NIb (Hong and Hunt 1996), the association of TEV replication complexes with endoplasmic reticulum-like membranes, and the potential for the 6K protein to act as a membrane anchor (Schaad et al. 1997a) are important findings. Also, the *cis*-replicative function of NIa (Murphy et al. 1996; Schaad et al. 1996) and the physical interaction between NIa and NIb (Hong et al. 1995; Li et al. 1997; Fellers et al. 1998) might begin to define the components of the viral replicase complex. Mutational studies have also showed that P1, HC-Pro, and P3 are involved in potyviral genome amplification (Atreya et al. 1992; Klein et al. 1994; Kasschau and Carrington 1995; Verchot and Carrington 1995; Kasschau et al. 1997). Exceptionally, CP appears not to be required, although translation to a position between TEV CP codons 138 and 189, and a *cis*-active RNA sequence between TEV CP codons 211 and 246, are absolutely necessary (Mahajan et al. 1996). Recently, secondary structures involving both CP-coding and 3′ NTR sequences were also shown to confer replicative function (Haldeman-Cahill et al. 1998).

Cell-to-cell movement. Genetic evidence that CP is required for potyvirus movement was provided by Dolja et al. (1994, 1995). The potyviral CP is a three-domain protein with variable N- and C-terminal regions exposed on the particle surface and a conserved core domain that interacts with viral RNA (Allison et al. 1985; Shukla and Ward 1988). Dolja et al. (1994, 1995) produced mutants in the CP-core domain of TEV-GUS. All mutants were defective in cell-to-cell movement and in virion assembly. The effect of such mutations on assembly had been noted previously for *Johnsongrass mosaic virus* (Jagadish et al. 1993). This mutational analysis also showed that the N-terminal domain of the CP has an accessory role in this movement process since mutants with this domain removed exhibited slow cell-to-cell movement in inoculated leaves.

Several studies have implicated the CI protein, an RNA helicase required for genome replication (Laín et al. 1990; Eagles et al. 1994; Klein et al. 1994), in potyvirus cell-to-cell movement. By electron microscopy, CI protein is seen to form aggregates (called pinwheel or cylindrical inclusions [CIs]) in the cytoplasm of infected cells. These inclusions are frequently seen positioned over the plasmodesmal aperture

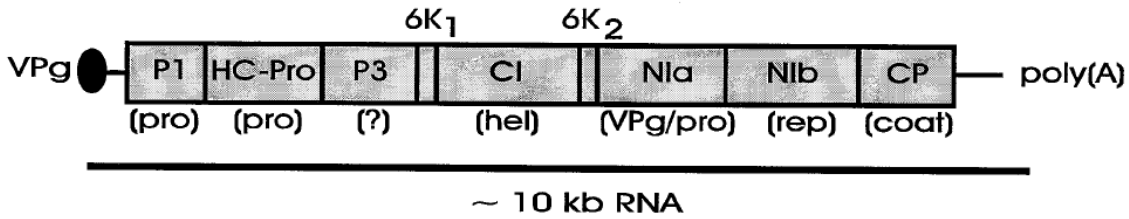

Fig. 1. Organization of the potyvirus genome. Potyviruses have single-stranded RNA genomes of approximately 10 kb. The RNA is polyadenylated at the 3′ end (poly(A)) and has a virus-encoded genome linked protein (VPg) covalently linked to the 5′ end. The RNA encodes a single polyprotein (shaded box) that is proteolytically cleaved by self-encoded proteases (pro) into functional proteins. The only structural protein is the coat protein (CP). Other identified functions include the aphid transmission helper component protease (HC-Pro), which also has a gene silencing suppressor activity, the cylindrical inclusion (CI) protein, which assists virus movement, the combined VPg-protease protein (NIa; nuclear inclusion a), and the RNA replicase (NIb; nuclear inclusion b).

(Lawson and Hearon 1971; Langenberg 1986). Mutational analysis of the TEV CI protein (Carrington et al. 1998) identified two mutants, altered in the N-terminal region, that were defective in cell-to-cell movement but still replicated to levels equivalent to that seen for the parental virus. Other TEV CI protein mutants failed to be, or were only weakly, replicated in infected protoplasts, supporting the view that CI protein also has an important role in the virus replication process.

Ultrastructural studies of tissues at an early stage of infection, combined with immunogold labeling of specific potyvirus proteins, have further supported the role of CI protein and CP in cell-to-cell movement (Rodríguez-Cerezo et al. 1997; Roberts et al. 1998). Observations of either young tobacco leaves infected with *Tobacco vein mottling virus* (TVMV; Rodriguez-Cerezo et al. 1997), or cells across an advancing infection front in pea cotyledons infected by *Pea seed-borne mosaic virus* (PSbMV; Roberts et al. 1998), showed that CIs immunolabeled for CI protein and CP were attached to the plasma membrane, close to or over the plasmodesmal opening. Most notable was the observation of a continuous channel through the center of the CIs and the plasmodesma. This channel contained CP and, in the case of tobacco cells, TVMV RNA (identified by in situ hybridization). Behind the PSbMV infection front, CIs were no longer associated with the cell wall, or with CP, and CIs accumulated as the characteristic pinwheel structures in the cytoplasm (Roberts et al. 1998). Both studies proposed that CIs could function transiently to transfer viral complexes from cell to cell through plasmodesmata.

It has also been suggested that VPg could have a role in potyviral cell-to-cell movement. Through site-directed mutagenesis and the construction of chimeric TVMVs, the viral resistance-breaking determinant for the *Nicotiana tabacum* cv. TN 86 resistance gene, *va,* has been identified as the VPg (Nicolas et al. 1997). The phenotypic expression of this recessive resistance is the confinement of the infection to initially infected cells, suggesting a restricted virus movement (Gibb et al. 1989). In co-inoculation experiments with virulent and avirulent TVMV strains, only the virulent strain was detected in systemically infected leaves. These data argue, first, against the elicitation of a general movement-limiting reaction in cv. TN 86 by the avirulent strain and, second, against a nonspecific mediation of transport through plasmodesmata by the virulent strain. However, the avirulent strain replicates in cv. TN 86 protoplasts to a lower level than in a susceptible cultivar of tobacco (Gibb et al. 1989), perhaps indicating that a plant defense response (see below) may limit the replication of this strain and reduce its ability to invade more than a few cells.

The genetic analyses of the involvement of HC-Pro during the cell-to-cell transport process are less clear (Klein et al. 1994; Cronin et al. 1995; Kasschau et al. 1997). Several HC-Pro mutants of TEV appeared to move from cell to cell less efficiently than the parental virus (Kasschau et al. 1997) and a TVMV HC-Pro mutant was unable to spread in inoculated leaves (Klein et al. 1994).

Since plasmodesmata in mesophyll cells have a size exclusion limit (SEL) of approximately 1,000 Da for the passive transport of molecules, some effects of the potyviral MPs on the plasmodesmal SEL might be expected. The effects of *Escherichia coli*–expressed *Bean common mosaic necrosis virus* and *Lettuce mosaic virus* proteins on plasmodesmal gating have been measured after microinjection into host cells (Rojas et al. 1997). Both CP and HC-Pro were shown to increase plasmodesmal SEL and to mediate viral RNA (approximately 1 kb encoding the viral CP) movement from cell to cell. Microinjection of CI protein or NIa (which contains the VPg domain) did not induce these effects. These are the first experiments designed to dissect the role of potyvirus MPs in the overall process of cell-to-cell movement, although they do not closely mimic natural potyvirus infections.

Collectively, these studies show that at least two potyviral proteins, CI protein and CP, could be considered MPs. From the model for potyvirus cell-to-cell movement proposed by Carrington et al. (1998), CI protein may direct intracellular translocation of a viral transport complex that includes the CP. Then, CP may interact with the plasmodesmata to increase the SEL, and CI protein may function to position the viral complex for translocation through the CI structures, into the plasmodesmata, and finally into the adjacent cells. A major question to address in the next years will be the nature of the viral transport complex. The strong correlation between competence for virion assembly and for cell-to-cell movement in the case of TEV (Dolja et al. 1994, 1995), and the fibrillar material (similar to PSbMV particles) observed within plasmodesmata in pea (Roberts et al. 1998), may be taken as an indication that potyviruses move from cell to cell as virions. However, direct evidence is still lacking.

Long-distance movement. Long-distance movement is the movement of the infectious agent from the mesophyll via the bundle sheath cells, phloem parenchyma, and companion cells into phloem sieve elements, passive translocation in the phloem, and unloading at a remote site to establish further infection foci (for review see Carrington et al. 1996). A specific example of this is *Pepper mottle virus* (PepMoV) long-distance movement in *Capsicum annuum* plants (Andrianifahanana et al. 1997). In this case, the virus was seen to follow the source-to-sink pattern route for translocation of photoassimilates. This involved descending transport via the external phloem in the stem from the inoculated leaf, entrance into internal phloem, and rapid ascending transport to young tissues of the plant. Generally, the complexity of the cell types and their connections, and the difficulty in analyzing long-distance movement independently of cell-to-cell movement, mean that the roles of potyviral proteins in this process are not well defined.

At least three potyviral proteins (CP, HC-Pro, and VPg) seem to be involved in long-distance movement. For CP, TEV-GUS mutants with deletions in the CP N- or C-terminal domains produced virions in vivo but the virus exhibited defects in long-distance movement in plants (Dolja et al. 1994, 1995). Also, mutational analysis demonstrated that changes to Ser_{47} of the PSbMV CP (Andersen and Johansen 1998) and Asp_5 in the DAG motif of the TVMV CP N-terminal domain (Atreya et al. 1995; López-Moya and Pirone 1998) can modulate the ability of the virus to move systemically in *Chenopodium quinoa* and tobacco plants, respectively.

Other analyses with TEV-GUS showed that HC-Pro also has a role in potyvirus long-distance movement (Klein et al. 1994; Cronin et al. 1995; Kasschau et al. 1997). The long-distance movement–defective TEV-GUS/CCCE mutant virus (substitution of the Cys_{293}, Cys_{294}, Cys_{295}, and Glu_{299}, highly conserved within the HC-Pro central region; Cronin et al.

1995) was analyzed for its ability to infect a series of grafted plants composed of various combinations of HC-Pro transgenic and nontransgenic scions and rootstocks (Kasschau et al. 1997). Systemic infection was only observed when both the stock and the scion could provide a complementing function from a wild-type HC-Pro transgene. This indicated that HC-Pro is required in both inoculated and noninoculated tissues for efficient long-distance movement and, hence, presumably for both entry into, and exit from, the host plant vascular system. Other mutations in the HC-Pro central domain were also analyzed and showed the same negative effect on long-distance movement. One mutant bearing insertions in the N-terminal part of TVMV HC-Pro also failed to result in systemic infection (Klein et al. 1994).

The involvement of VPg in long-distance movement was proposed from the analysis of TEV recombinants made between strains that differed in their capacity to invade *N. tabacum* cv. V20 systemically (Schaad et al. 1997b). TEV strain HAT shows restricted cell-to-cell movement phenotype in cv. V20 but a systemic infection in cv. Havana 425. TEV strain Oxnard is capable of systemic infection in both cultivars. To identify the TEV host-specific movement determinants, chimeric viral genomes were constructed between TEV-HAT and TEV-Oxnard. Chimeric viruses containing the TEV-Oxnard VPg domain were able to infect cv. V20 systemically.

In spite of the experiments described above, a detailed model for long-distance movement of potyviruses must be speculative. As CP is a structural protein and VPg is covalently linked to the viral RNA, these two proteins are probably included in a viral transport complex, and may interact with host factors for efficient movement through the plant. These host factors remain elusive although the complexity of the process means that many host proteins may be involved. At least three factors are suggested from host genetic analyses. Hence, segregation resulting from a cross between the V20 and Havana 425 *N. tabacum* cultivars (Schaad and Carrington 1996) suggested that TEV limitation in cv. V20 was due to two recessive genes. A monogenic, dominant locus (*RTM1*) of *A. thaliana,* conferring a restricted TEV infection phenotype (Mahajan et al. 1998), identifies the third factor. The cloning and molecular characterization of this dominant gene may be the more tractable experimentally and could provide the first detailed information about host proteins involved in potyvirus long-distance transport.

HC-Pro, a viral suppressor of gene silencing.

A recent exciting development in our understanding of the role of HC-Pro may mean that we need to view many aspects of long-distance transport in a different way. Analysis of synergistic infections involving potyviruses showed that HC-Pro may act as a general pathogenicity enhancer (Vance et al. 1995; Pruss et al. 1997; Shi et al. 1997) by interfering with a host defense response that normally limits viral infection. In several parallel pieces of work (Anandalakshmi et al. 1998; Brigneti et al. 1998; Kasschau and Carrington 1998), three groups have shown that HC-Pro has the capacity to suppress post-transcriptional gene silencing (PTGS), and that the consequence of this is enhanced virus replication. Essentially, two strategies led to this conclusion. Genetic crosses between transgenic lines expressing potyvirus P1/HC-Pro (a protein capable of self-cleavage to release free HC-Pro) and lines exhibiting PTGS of reporter genes (*uidA* or *gfp*) resulted in progeny in which the reporter gene function was restored (Anandalakshmi et al. 1998; Kasschau and Carrington 1998). Second, *Potato virus X* (PVX) vectors expressing HC-Pro replicated to a higher level than wild-type PVX and restored reporter gene expression on *uidA* or *gfp* silenced plants (Anandalakshmi et al. 1998; Brigneti et al. 1998). There was some evidence (Shi et al. 1997; Anandalakshmi et al. 1998) that it is the central region of HC-Pro that mediates synergistic effects in co-infection with other viruses and suppression of gene silencing, and that this property of HC-Pro was enhanced by the presence of the potyviral P1 protein (Kasschau and Carrington 1998).

There is increasing evidence that PTGS can be divided functionally into initiation and maintenance phases (Brigneti et al. 1998). In a comparative study between HC-Pro and the suppressor encoded by *Cucumber mosaic virus* (CMV; 2b protein), HC-Pro suppressed PTGS in somatic tissues, including the inoculated leaf, whereas CMV 2b was only active at the shoot apex. The interpretation was that CMV 2b only affected the initiation phase of PTGS while HC-Pro could affect either maintenance alone, or initiation and maintenance. Synergistic infections between CMV and PepMoV in *C. annum* cv. Avelar (Murphy and Kyle 1995) suggest that CMV 2b and HC-Pro are not functional antagonists.

We can view PTGS as a potential defense mechanism against viruses that could restrict virus accumulation in infected cells and, hence, delay virus movement (Baulcombe 1996; Pruss et al. 1997). Therefore, a complete block to long-distance movement could represent the indirect effect of PTGS in allowing extra time for other resistance mechanisms to come into play. This may explain why genetic studies of long-distance movement frequently appear to be difficult to interpret, as it reflects the balance between virus replication potential, the counteraction of PTGS, and further resistance responses.

Virus-induced symptomatology.

Most potyviruses induce conspicuous symptoms, often causing stunting and yield losses. They usually induce longitudinal chlorotic or necrotic streaks in the leaves of monocotyledonous species, and chlorotic vein banding, mosaic mottling, necrosis, and/or distortion of leaves in dicotyledonous species. Flowers, seeds, and fruits are also affected by numerous potyviruses (Shukla et al. 1994).

The nature and extent of symptoms for a specific host genotype depend upon the virus and particular virus strain, as well as upon environmental conditions, probably through their influence on host physiology and development. Symptoms are the result of complex cellular and supracellular interactions of the host with the virus. The effects may be the consequence of the diversion of plant resources into synthesis of virus-specific nucleic acids and proteins, or of the disruptive effects of virus-specific products on normal cellular processes (e.g., cell-to-cell communication). Generally, for any compatible host/virus combination, the severity of symptoms will reflect the level of virus replication and accumulation. As such, and from the preceding discussion, it is clear that the suppressors of PTGS (e.g., HC-Pro) could have a large impact on symptom expression in single infections, or combined infections with other viruses.

In recent years, several regions of the potyviral genome have been shown to have a role in symptomatology. Mutational analysis of the TVMV genome has shown that the P1/HC-Pro coding region, and particularly the 5′ coding region of HC-Pro, is involved in symptom expression on tobacco (Atreya et al. 1992; Atreya and Pirone 1993; Klein et al. 1994). By constructing recombinant hybrids of two PSbMV strains, Johansen et al. (1996) demonstrated that the genome segment encoding the PSbMV NIa and NIb has a major influence on symptom severity in *Pisum sativum*. Two separate TEV genomic segments, one encompassing the 3′ third of the P3 coding region and another encompassing the 3′ end of the CI, the 6K, and the 5′ end of the NIa coding regions, were responsible together for the wilting response of Tabasco pepper (Chu et al. 1997). Also, mutations introduced in the PPV P3-6K1 cleavage site sequence cause either symptom attenuation or more severe symptoms (Riechmann et al. 1995). A further study of PPV showed that a mutant lacking nucleotides 127 to 145 of the 5′ NTR induced only very mild symptoms on *N. clevelandii* (Simón-Buela et al. 1997b). In the 3′ NTR of TVMV, four repeats of 14 adenine and uracil residues were responsible for symptom attenuation on tobacco (Rodríguez-Cerezo et al. 1991). Unfortunately, despite all this information, it is still not possible to identify any unifying principles to explain symptom formation. Although for other virus genera there are examples of particular viral gene products acting as symptom inducers in the absence of infection (Balachandran et al. 1995; Olesinski et al. 1995; Cecchini et al. 1997), there is no such evidence for the potyvirus examples listed above.

To some extent our inadequate understanding of symptom expression reflects our lack of knowledge of host proteins that interact with viral proteins. Progress will certainly be made in future through the genetic analysis of differing symptomatic phenotypes. To date, only two potential interactions have been identified: (i) the interaction between TuMV VPg and eIF(iso)4E (Wittmann et al. 1997) described earlier; and (ii) with anti-idiotypic-antibodies, McClintock et al. (1998) identified a 37-kDa protein localized in chloroplast that interacts with the TuMV CP. The significance of these interactions for virus replication or symptom expression is not known.

Deducing what biochemical changes lead to symptom expression and distinguishing them from consequences of symptom production is a major challenge, complicated by the progressive nature of virus infections in plants where, in contrast to the period of several days to weeks being required to develop a complete systemic infection, the exponential phase of replication in individual cells may take no more than a few hours. Hence, from the catalog of biochemical changes recorded for systemically infected tissues (Zaitlin and Hull 1987) it is impossible to distinguish cause from consequence. An approach that may provide more information is to place the changes in a sequence of events following the onset of virus replication. Through spatial analyses of changes in host gene expression at the infection front, this approach has been applied to PSbMV infection of pea tissues. This revealed a progression of induced changes that included the downregulation of many host genes in a narrow zone behind the infection front, and a coincident but narrower zone where some other host genes (e.g., *hsp70, polyubiquitin*) were selectively induced (Wang and Maule 1995; Aranda et al. 1996). Some of these changes were akin to stress-induced changes seen in response to heat shock, but a related analysis of heat shock factor (*hsf*) expression showed that heat and virus stress utilized independent control pathways (M. A. Aranda and A. J. Maule, *unpublished*). How these effects lead to biochemical changes and symptom expression has not been studied for potyviruses.

The transient nature of virus replication and host responses seen in the PSbMV/pea experiments raises interesting questions about cells a long way behind the infection front. These cells contain massive amounts of progeny virus, no longer show the induced changes in gene expression, and may be susceptible to infection by heterologous viruses. Conceptually, this state is equivalent to latency in animal virus infections (Aranda and Maule 1998). It seems possible that this condition could be maintained by PTGS, which would prevent further replication but have no effect on encapsidated virus. The pool of encapsidated virus provides a reservoir for the establishment of subsequent infection mediated by insect vectors.

Potyvirus transmission.

Potyviruses are transmitted by aphids in a nonpersistent manner and some of them are seed transmitted. Viral determinants of aphid transmission are now well known (see reviews by Pirone 1991; Maia et al. 1996; Pirone and Blanc 1996, and references therein). Therefore, we will concentrate here only on the more recent data. New results have also been obtained for seed transmission.

Aphid transmission. Aphid transmission of potyviruses occurs during brief and superficial probes into the plant. Two potyviral-encoded proteins are involved in this process: CP and HC-Pro. In most cases, a conserved amino acid triplet, Asp-Ala-Gly (DAG) in the N terminus of the CP, and a lysine motif (KITC) located within the N-terminal conserved cysteine-rich domain of HC-Pro, have been shown to be essential for successful transmission. Another motif in HC-Pro, called PTK, seems also to be required (see reviews by Pirone 1991; Maia et al. 1996; Pirone and Blanc 1996). Recent experiments have confirmed this point since *Zucchini yellow mosaic virus* (ZYMV) mutants with mutation in the PTK motif failed to be transmitted by aphids (Peng et al. 1998).

Aphid transmission of potyviruses is dependent upon the acquisition of HC-Pro, prior to or together with the virus. Based upon these observations, it was suggested that HC-Pro acts as a bridge between virion CP and a putative receptor in the vector mouthparts (Pirone and Blanc 1996). Through transmission electron microscopy and light microscopic autoradiography, virions of TVMV were detected in the stylet food canal of aphids but not when CP DAG and HC-Pro lysine domains were mutated (Pirone and Blanc 1996; Wang et al. 1996). Direct evidence for the requirement of a specific interaction between CP and HC-Pro in aphid transmission was provided by Blanc et al. (1997) and Peng et al. (1998). Blanc et al. (1997) demonstrated in vitro binding between TVMV HC-Pro and a 7-amino-acid domain encompassing the DAG motif of the TVMV CP, whereas Peng et al. (1998) showed a binding of HC-Pro with dot-blotted ZYMV virions. The former binding was also shown for several other potyviruses. A good correlation between the efficiency of aphid transmission and HC-Pro/CP binding was also shown in both studies. In addition, nontransmitted mutants of ZYMV with an altered PTK motif failed to bind to virions, suggesting, first, that the

PTK motif is involved in this binding and, second, that the HC-Pro/virions interaction is necessary to transmit ZYMV infection. More recent experiments point to the N-terminal region of HC-Pro, including the KITC motif, as being involved in the interaction with the aphid stylet (Blanc et al. 1998). All these data support a "bridging" model for the function of HC-Pro in transmission by aphids, but the demonstration of a direct interaction of HC-Pro with a receptor in the aphid stylet is still lacking. Comparison of HC-Pro/vector combinations that differ in their ability to transmit potyviruses (e.g., with the aphid *Myzus ascalonicus,* which does not transmit; Wang et al. 1998), provides new opportunities to characterize an HC-Pro receptor in the aphid food canal.

Seed transmission. The seed transmission of potyviruses has been most extensively studied for the PSbMV infection of pea (Wang and Maule 1992, 1994; Johansen et al. 1996), an interaction in which the virus infects the immature embryo after fertilization rather than via infected gametes (Wang and Maule 1992). This mechanism is not universally true for all potyviruses, and some viruses may be transmitted by both routes simultaneously (Maule and Wang 1996). The failure of PSbMV to invade the pea meristem (Jones et al. 1998b) could explain why it is not transmitted through pollen. The precise tissue limitations of other potyvirus infections have not been studied.

For PSbMV in pea, multiple viral determinants (Johansen et al. 1996) and multiple host genes expressed in the maternal tissues (Wang and Maule 1994) seem to be involved in determining the level of seed transmission. Construction of hybrids between the PSbMV isolates DPD-1 (transmissible) and NY (not transmissible) localized the determinants to the 5′ NTR, HC-Pro, and CP regions of the genome. These regions have been shown to be important for virus replication and movement (Riechmann et al. 1992; Kasschau and Carrington 1995; Mahajan et al. 1996; see above). Based upon a spatial analysis of virus accumulation during the process of seed transmission, Wang and Maule (1992, 1994) proposed that the virus exploits the embryonic suspensor to invade the embryo. Since the suspensor is a structure programmed to degenerate early in development, it provides a structural window of opportunity to the transmission process, and, potentially, could indicate why the efficiency of virus movement (mediated directly or indirectly by HC-Pro) could be important for the invasion of the immature seed and, hence, for seed transmission.

Natural and engineered resistance.

Nonhypersensitive resistance to potyviruses may involve dominant, incompletely dominant, or recessive genes while hypersensitive resistance (HR) is controlled mostly by single dominant genes (Fraser 1992). Of all known potyvirus resistance genes, 40% are recessive (Provvidenti and Hampton 1992). This is higher than for other virus groups, where in only 20% of cases is resistance dependent upon recessive genes (Fraser 1992). While the molecular basis of hypersensitivity-associated resistance appears to have common features for a range of hosts and pathogens (Staskawicz et al. 1995), little is known about the nature of the recessive resistance genes. Two hypotheses could explain the role of recessive resistances: (i) the resistant host lacks a host function essential for particular steps in viral pathogenesis, and consequently the dominant allele encodes a host factor, which is required for the virus to replicate and/or move in the susceptible host; or (ii) the susceptibility allele encodes a dominant negative regulator of resistance. The recessive *mlo* gene, which confers resistance against powdery mildew in barley, seems to fit the latter model (Büschges et al. 1997). More complex scenarios are also possible in which a dose-dependent effect of the resistance gene could be involved or multiple, interacting loci could control resistance. An example of polygenic recessive resistance, active in pepper against several potyviruses, was reported recently (Caranta et al. 1997).

In several studies, recessive resistance genes have been functionally characterized through an analysis of their mechanism of action. The *C. annuum* y^a and *pvr3* recessive resistance genes have been shown to restrict movement of potato virus Y (Arroyo et al. 1996) and PepMoV (Murphy et al. 1998), respectively. An inhibition of TEV, PepMoV, and PSbMV RNA replication is a feature of the *C. annuum* et^a, *C. Chinense pvr1,* and *P. sativum sbm-1* recessive resistance genes, respectively (Deom et al. 1997; Keller et al. 1998; Murphy et al. 1998). In the case of the *N. tabacum* cv. TN 86 *va* resistance gene, it seems that both virus replication and movement of TVMV are restricted (Gibb et al. 1989).

Mechanisms of resistance associated with dominant genes have also been studied, particularly for potato resistance genes. In *Solanum brevidens* and the extremely resistant potato cultivars bearing the Ry_{sto} resistance gene, the movement of potyviruses such as PVY, PVA, and TEV was blocked after initial replication and movement triggered the HR response (Valkonen et al. 1991; Barker 1996; Valkonen and Somersalo 1996; Hinrichs et al. 1998).

More straightforward than the cloning of host resistance genes is the identification of the viral avirulence determinant. Through analysis of recombinant hybrid viruses between virulent and avirulent strains, progress has been made for the PSbMV/*sbm-1* (Keller et al. 1998), TVMV/*va* (Nicolas et al. 1997), and *Soybean mosaic virus* (SMV)/*Rsv* (Eggenberger and Hill 1997) interactions. PSbMV resistance-breaking determinants for the *sbm-1* gene were localized in the VPg coding domain, more specifically in a 15-amino-acid central domain of the VPg (Johansen and Keller 1997). Similarly, the TVMV-S VPg has been implicated in *va*-mediated resistance (Nicolas et al. 1997, see above). The important role for VPg in viral RNA replication (Schaad et al. 1996) might indicate an associated function for the products of the *Va* and *Sbm-1* dominant alleles. The interaction of VPg with the translational initiation factor eIF(iso)4E (Wittmann et al. 1997) provides at least one candidate function, although its role in recessive resistance has not been reported.

Chimeric SMVs were made between SMV-N and SMV-G7, strains that are avirulent and virulent, respectively, in soybean plants containing the dominant resistance gene *Rsv* (Eggenberger and Hill 1997). The resistance-breaking determinants were identified in the 3′ region of HC-Pro and the 5′ region of P3 coding domains. However, chimeras with either SMV-G7 HC-Pro or P3 domains alone were not able to overcome *Rsv*. This is probably the first example of a potyvirus for which two avirulence determinants are required to overcome a single resistance gene. Another explanation could be that resistance breaking is a response to viral RNA rather than to viral proteins.

Although it is too early to exploit these natural resistance genes with biotechnology, substantial progress has been made

in the generation of pathogen-derived resistance through the transgenic expression of potyvirus sequences such P1 (Pehu et al. 1995; Moreno et al. 1998), P3 (Moreno et al. 1998), CI protein (Wittner et al. 1998), VPg (Swaney et al. 1995), NIa (Maiti et al. 1993; Vardi et al. 1993), NIb (Audy et al. 1994; Guo and García 1997; Jones et al. 1998a), and CP (Lindbo and Dougherty 1992; Fitchen and Beachy 1993; Hackland et al. 1994) sequences. The resistance can be seen as either a failure of the infection to become established (extreme resistance), or a recovery phenotype in which, after an initial phase of susceptibility, the plants recover and remain resistant to subsequent challenge. In either case, the predominant mechanism is one based upon PTGS, which results in a degradation of transgene mRNA and viral RNA in the cytoplasm (reviewed by Baulcombe and English 1996; van den Boogaart et al. 1998). How this mechanism is triggered is not yet clear, although Jones et al. (1998b) and Guo et al. (1999) propose that a systemic signaling molecule and induced methylation of homologous transgenic sequences may be involved. A conundrum to be resolved in the future is understanding how a virus that encodes a PTGS suppressor (HC-Pro) can be resisted by a PTGS-based mechanism. For extreme resistance, this may reflect the degradation of the homologous RNA sequences before expression of HC-Pro. The induced resistance seen in the recovery phenotype may reflect the relative timing of HC-Pro expression and the remote signaling of PTGS ahead of the infection.

Conclusion.

This review illustrates the advances in our understanding of plant/potyvirus interactions over the last 6 years. By cloning and manipulation of the potyvirus genome, numerous viral determinants involved in the biological characteristics of the virus life cycle have been identified. The data obtained showed, first, that both coding and noncoding regions of the potyvirus genome have functions in these biological processes and, second, that most of the potyvirus proteins are multifunctional. A more complete understanding of the functions of these viral proteins will come from knowledge of the interacting host factors involved in susceptibility and defense.

Perhaps the most significant recent discovery in potyvirus research is the demonstration that HC-Pro can act as a negative regulator of a plant defense mechanism based upon PTGS. Since orthologs of HC-Pro are being discovered in other viruses (e.g., CMV; Béclin et al. 1998; Brigneti et al. 1998), it may be that viruses generally need to counteract PTGS to develop systemic infections. It will be intriguing to see whether PTGS and its regulation by viral proteins could be connected with latency in plants and animals, or even to the changes in host gene expression associated with virus replication.

The major challenge for the future will be to take advantage of the new opportunities offered by chip-based expression arrays, computer-assisted functional genomic analyses, and the rapidly accumulating knowledge of the host genome (particularly *A. thaliana*) to develop advanced studies of both fundamental and applied areas of potyviral biology.

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Aujourd'hui, le projet sur l'étude des interactions laitue/LMV est devenu l'un des plus importants de notre laboratoire sur lequel plusieurs chercheurs sont investis et dont les résultats majeurs de ces dernières années furent l'identification coté plante du gène de résistance *mo1* codant pour le facteur d'initiation de la traduction eIF4E et coté LMV de plusieurs protéines virales impliquées dans l'interaction avec eIF4E et le contournement de la résistance *mo1*.

Après avoir soutenue ma thèse le 14 mai 1997, j'ai passé le concours CR2 de l'INRA en juin 97 pour lequel je postulais pour un poste de virologie végétale à l'INRA d'Antibes et à l'INRA de Bordeaux dans mon laboratoire. C'est le poste à Bordeaux que j'ai obtenu.

…A LA PLANTE

2.1. Recherche menée durant le séjour postdoctoral

Durant les derniers 6 mois de ma thèse, je m'étais investi à rechercher un séjour postdoctoral. Après plusieurs contacts, je me suis entendu avec Andy Maule, responsable d`une équipe de recherche dans le département de virologie du Centre John Innes à Norwich (Angleterre). Ce dernier me proposait de travailler dans le cadre d'un projet européen grâce auquel il pouvait me financer pour une durée de 3 ans. Mon recrutement à l`INRA dés la fin de ma thèse a quelque peu modifié mes perspectives de séjour postdoctoral. Sachant cependant par avance qu'un séjour dans un laboratoire étranger, en particulier lorsqu`il est renommé, est une expérience très enrichissante et profitable, et au chercheur qui part, et au laboratoire qui l`envoie, nous avions décidé avec mon directeur de laboratoire de l'époque (Jean Dunez) que je parte faire ce séjour à Norwich. Je suis donc parti le 15 septembre 1997 pour une durée de 21 mois (retour à Bordeaux début juillet 1999). Mon départ dans le groupe d'Andy Maule avait été motivé par plusieurs raisons : (1) ce groupe avait une très bonne renommée et ses travaux publiés étaient très largement à la hauteur de cette réputation ; (2) tout en étant un laboratoire de virologie végétale, les projets développés étaient essentiellement basés sur l'étude de la plante hôte plutôt que sur les virus eux-mêmes ; (3) le modèle plante/virus plus particulièrement étudié dans ce groupe est le couple pois/*Pea seed born mosaic virus* (PSbMV, genre *Potyvirus*), très similaire en plusieurs points à notre modèle laitue/LMV ; (4) le laboratoire d'A. Maule fait partie d'un centre de recherche très dynamique dont les activités abordent un grand nombre de thématiques de recherche du domaine végétal ; (5) ce laboratoire est situé en Angleterre permettant donc de parfaire son anglais.

Ma recherche à Norwich a été d'initier un projet de caractérisation d'un gène de résistance récessif du pois au PSbMV, nommé *sbm-1*. A l'époque, aucun projet de ce type n'avait encore démarré ni à Bordeaux (pour identifier les gènes *mo1*), ni ailleurs à l'INRA (pour l'identification des gènes *pvr* chez le piment). Nous avons pour cela utilisé deux approches, un criblage d'une banque d'ADNc de pois par le système double-hybride en utilisant comme appât la VPg, produit du gène d'avirulence du PSbMV vis à vis du gène *sbm-1*, et la technique de cDNA-AFLP pour analyser l'expression différentielle de gènes de deux lignées de pois quasi-isogéniques obtenues après plusieurs rétrocroisements et différant par la présence du gène *sbm-1*.

Pour l'approche double-hybride, j'ai réalisé le clonage de la VPg du PSbMV dans les vecteurs double-hybride et j'ai surtout participé à la construction de la banque d'ADNc de pois. Un autre postdoc recruté sur le financement initialement prévu pour moi a réalisé le crible de la banque de pois qui a permis d'identifier une dizaine d'interacteurs dont la plupart ne présentait aucune homologie ni avec des protéines connues ni avec le facteur de traduction eIF4E (à cette époque nous savions seulement qu'eIF4E interagissait directement avec la VPg d'un potyvirus). Avec l'aide de généticiens travaillant sur le pois, nous avons pu cartographier ces candidats pour le gène *sbm-1* mais aucun ne s'est révélé être un marqueur lié au gène de résistance recherché. J'avais aussi obtenu en parallèle un ADNc partiel du facteur eIF4E de pois mais celui-ci n'était pas non plus lié au gène *sbm-1* (on saura plus tard qu'en fait *sbm-1* codait non pas pour eIF4E mais pour une autre isoforme, eIFiso4E !!).

L'approche basée sur la technique d'AFLP, sur laquelle je me suis davantage investi, a été utilisée pour tenter d'identifier un marqueur lié au gène *sbm-1*, voire le gène lui-même. Après une période d'apprentissage et d'optimisation, j'ai pu obtenir à la fin de mon séjour une méthode fiable et reproductible pour analyser par « cDNA-AFLP » les deux lignées de pois quasi-isogéniques disponibles. Une dizaine de marqueurs potentiels ont été identifiés après cartographie sur le génome du pois. Mais suite à mon départ, cette approche n'a pas été poursuivie.

Ce séjour à Norwich a été pour moi très bénéfique puisqu'il m'a permis de me familiariser avec des techniques de pointe utilisées pour identifier des facteurs de plante impliqués dans les interactions plante/virus, d'élargir mon réseau de contact au niveau international et de perfectionner mon anglais.

A mon retour à Bordeaux, j'ai continué à collaborer avec A. Maule sur ce projet qui a été poursuivi par deux autres postdocs. Nous avons notamment montré qu'une des protéines qui interagissait fortement avec la VPg du PSbMV, nommée VIP1 (pour Vpg-Interacting Protein), interagissait avec la VPg du LMV et d'autres potyvirus mais semblait aussi jouer un rôle dans le mouvement à longue distance des potyvirus. Ce travail a fait l'objet de la **Publication [12]** présentée ci-dessous.

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A Cysteine-Rich Plant Protein Potentiates *Potyvirus* Movement through an Interaction with the Virus Genome-Linked Protein VPg

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We have identified a cellular factor that interacts with the virus genome-linked proteins (VPgs) of a diverse range of potyviruses. The factor, called *Potyvirus* VPg-interacting protein (PVIP), is a plant-specific protein with homologues in all the species examined, i.e., pea, *Arabidopsis thaliana*, and *Nicotiana benthamiana*. The sequence of PVIP does not identify a specific function, although the existence of a "PHD finger" domain may implicate the protein in transcriptional control through chromatin remodeling. Deletion analysis using the yeast two-hybrid system showed that the determinants of the interaction lay close to the N terminus of VPg; indeed, the N-terminal 16 amino acids were shown to be both necessary and sufficient for the interaction with at least one PVIP protein. From a sequence comparison of different potyvirus VPg proteins, a specific amino acid at position 12 was directly implicated in the interaction. This part of VPg is distinct from regions associated with other functional roles of VPg. Through mutation of *Turnip mosaic virus* (TuMV) at VPg position 12, we showed that the interaction with PVIP affected systemic symptoms in infected plants. This resulted from reduced cell-to-cell and systemic movement more than reduced virus replication, as visualized by comparing green fluorescent protein-tagged wild-type and mutant viruses. Furthermore, by using RNA interference of PVIP in *Arabidopsis*, we showed that reduced expression of *PVIP* genes reduced susceptibility to TuMV infection. We conclude that PVIP functions as an ancillary factor to support potyvirus movement in plants.

The *Potyvirus* group is the major genus of the family *Potyviridae*. Potyviruses infect a broad range of monocot and dicot plants and can be responsible for severe damage to crops. Despite the economic importance of potyviruses, relatively little is understood about the detailed molecular interactions that occur in infected tissues that lead to the exploitation of cellular machinery and the accumulation of high virus titers. Notably, only the translation factors eIF4E (28, 30) and eIF(iso)4E (14, 15) have been identified as susceptibility factors supporting virus replication in potyvirus hosts. These proteins have been identified variously through mutational analysis of model host plants, such as *Arabidopsis thaliana* (hereafter referred to as *Arabidopsis*) (14), and through protein-protein interaction studies, initially in *Saccharomyces cerevisiae* (15, 30). We have also applied the latter approach to identify host proteins that interact with the potyvirus genome-linked protein, VPg.

VPg forms a covalent linkage to the 5′ end of the viral RNA and is one of the diagnostic features of the picornavirus supergroup, which includes the family *Potyviridae* (13). For potyviruses, it is one of 10 mature proteins that are proteolytic products of the primary translated protein product encoded by the viral RNA (6, 27). VPg is a multifunctional protein with several suggested roles in the viral infection cycle. It may act as a primer for RNA replicase during virus multiplication, possibly through direct interaction with the viral RNA polymerase (7, 11, 16). It has also been implicated indirectly in cell-to-cell movement of the virus through plasmodesmata and, more directly, through mutagenesis in the long-distance translocation of the virus (18, 22, 23, 31, 33). Additionally, the potyvirus VPg protein has been shown to be the avirulence factor for recessive resistance genes in various plants (12, 18).

Previous reports have identified eIF(iso)4E (37) and eIF4E (30) as host proteins that interact with VPg from *Turnip mosaic virus* (TuMV) and *Tobacco etch virus* (TEV), respectively. The location of VPg at the 5′ end of the viral RNA, in place of the mRNA cap structure, points to a role for VPg in the initiation of translation of the viral RNA. Competition for these translation factors has been proposed as a mechanism for reducing host gene expression during potyvirus infection (2). In addition to its covalent association with viral RNA, VPg or its immediate precursor protein, NIa, shows intracellular targeting to the nucleus (3, 25, 32). Whether or how this is related to the functional roles of VPg is unclear.

In this paper, we describe the identification, using the yeast two-hybrid system (YTHS), of a cellular factor that interacts with the VPg proteins of a diverse range of potyviruses. Initially identified from pea, the protein named PVIP (for *Potyvirus* VPg-interacting protein) has homologs in *Nicotiana benthamiana* and in *Arabidopsis* and interacts with the VPg proteins from potyviruses that infect one or more of these hosts. We have assessed the structural limitations of the interaction and used the information to obtain evidence to support a role for PVIP in vivo. Finally, using RNA interference (RNAi)-based silencing of PVIP in transgenic *Arabidopsis*, we confirmed that the protein acts as an ancillary factor to support potyvirus infection and movement.

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MATERIALS AND METHODS

Virus and plant material. The source clones for the VPg proteins of *Pea seed borne mosaic virus* (PSbMV) (isolate DPD1), TEV (HAT strain), *Grapevine fan leaf virus* (GFLV) (F13 strain), *Tomato black ring virus* (TBRV) (L strain), and *Cowpea mosaic virus* (CPMV) were gifts from E. Johansen, J. Carrington, C. Ritzenthaler, C. Fritsch, and G. Lomonossoff, respectively. Infectious clones, p35Tunos and pCB-TuMV-GFP, of TuMV UK1 (29) were gifts from F. Ponz. The latter plasmids give rise to infectious viral RNA in planta after expression from the upstream transcriptional promoters. The p35Tunos expression cassette was inserted into the binary vector pGreenII (10) as follows. The *Sac*I site was deleted from pGreenII by digestion with *Sac*I, polishing of the ends with Klenow fragment, and religation. After excision using flanking *Sma*I and *Apa*I sites, the complete expression cassette was inserted into similarly digested modified pGreenII to obtain pGreen-TuMVwt. This was transformed into *Agrobacterium tumefaciens* strain GV3101. The plasmid pCB-TuMV-GFP is a binary plasmid and was transformed directly into *A. tumefaciens* strain GV3101. Virus infections on *N. benthamiana* and *Arabidopsis* were established by stabbing the leaf tissue to introduce the bacteria. Alternatively, *Agrobacterium* was pressure infiltrated into the leaf lamina using a syringe, following established protocols (36), except when discrete foci of infection were required, in which case bacterial cultures (optical density at 600 nm, 1) were diluted 1 in 5,000 before infiltration. *Arabidopsis* ecotype Columbia (Col-0) plants were grown at 20 ± 3°C with a 10-h photoperiod. After inoculation, *Arabidopsis* plants and plants of *N. benthamiana* were grown at 25 ± 3°C with a 16-h photoperiod. Green fluorescent protein (GFP) expression was monitored with a handheld UV light (UV Products, Upland, Calif.) or under a Nikon SMZ1500 dissecting microscope coupled to a 100-W epifluorescence module (Nikon). A band pass filter allowed the removal of chlorophyll fluorescence.

Yeast two-hybrid analysis. (i) Yeast two-hybrid library. A custom library from *Pisum sativum* L. cv. 'Scout' was made in the vector HybriZap 2.1 (Stratagene, La Jolla, Calif.). Excision of the library resulted in a yeast two-hybrid library in pAD-GAL4-2.1. This library was screened, using PSbMV P1 VPg as the bait, in pBD-GAL4 Cam in the yeast strain YRG-2 following Stratagene protocols. The strengths of the protein-protein interactions were measured using a chemiluminescent detection assay (Galacto-light kit; Applied Biosystems).

(ii) Yeast two-hybrid mating assay. A yeast two-hybrid screen was performed using either the Matchmaker GAL4 or Matchmaker LexA two-hybrid system (Clontech, Palo Alto, Calif.) for detecting protein-protein interactions in yeast (4, 8). Bait constructs were cloned into pGBT9 and transformed into the yeast strain CG1945 or cloned into pLexA and transformed into the yeast strain EGY48[p8opLacZ], resulting in expression as a DNA-binding domain fusion protein. Prey constructs were cloned into pGAD424 and transformed into the yeast strain Y187 or cloned into pB42AD and transformed into the yeast strain YM4271, resulting in expression as an activation domain fusion protein. Protein-protein interactions were identified by yeast mating experiments. A positive interaction was indicated by the ability of cotransformed yeast to grow on synthetic medium lacking leucine, tryptophan, and histidine and containing 5 mM 3-aminotriazole in the case of the GAL4 YTHS or lacking leucine, tryptophan, histidine, and uracil and containing 5 mM 3-aminotriazole and X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) in the case of the LexA YTHS. A positive control was provided by the interaction between a murine p53 protein and simian virus 40 large T antigen. A human lamin cDNA-binding domain fusion provided a negative control for the extraneous interaction of the binding domain with the prey. Methods were carried out as described in the Clontech Matchmaker and Matchmaker LexA manuals.

(iii) Protein extraction and Western blot analysis. Yeasts containing pGBT-VPg constructs or pGAD424-VIP1 constructs were grown overnight in synthetic medium lacking tryptophan or synthetic medium lacking leucine, respectively. Yeast culture and urea-sodium dodecyl sulfate protein extraction were performed as described in the Clontech Yeast Protocol Handbook. Equalized loads of protein extracts (optical density at 600 nm, ~0.8) were electrophoresed on a sodium dodecyl sulfate–12.5% polyacrylamide gel and blotted to Hybond-C (Amersham Pharmacia Biotech). The immunoblot was incubated with antibodies raised in rabbits against *Potato virus A* (PVA) VPg (21) at a dilution of 1/5,000 or antibodies raised in rabbits against a PVIP-specific synthetic peptide, SDQEPRRESPAESASS (Eurogentec), at a dilution of 1/500 and washed, and the specific reactivity was visualized using alkaline phosphatase-conjugated goat anti-rabbit serum and nitroblue tetrazolium as a colorimetric substrate, following standard techniques.

Deletion, site-specific mutagenesis, and mutant virus construction. Site-directed mutagenesis was carried out by overlap extension PCR with specific mutagenic primers (sequences available upon request) and high-fidelity *Pfu* polymerase (Stratagene). The mutated sequences were cloned into the relevant vectors, and the mutations were confirmed by sequence analysis. The F12M point mutation was inserted into the TuMV cDNA expression plasmids pGreen-TuMVwt and pCB-TuMV-GFP as follows. pGreen-TuMVwt was digested with *Sac*I, and the *Sac*I-*Sac*I fragment covering the VPg cistron was replaced with the corresponding mutant fragment obtained by overlap extension PCR to give pGreen-TuF12M. For pCB-TuMV-GFP, an *Nco*I-*Mlu*I fragment covering cistrons P3 to NIb was inserted into similarly digested pGemT to give pGemT-TuGFP. In turn, this was digested with *Cla*I to replace the *Cla*I-*Cla*I fragment covering the VPg region with the corresponding mutant fragment to give pGemT-TuF12MGFP. An *Nco*I-*Mlu*I fragment from pGemT-TuF12MGFP containing the F12M VPg was mobilized into similarly digested pCB-TuMVwt-GFP to give pCB-TuMVF12M-GFP.

RNAi transgene constructs and *Arabidopsis* transformation. To generate *PVIP1* and *PVIP2* RNAi transgenic lines (*pvip1* and *pvip2*), gene-specific 0.7-kb cDNA fragments were amplified using high-fidelity *Pfu* polymerase and the following primer pairs: *PVIP1* forward primer (5′-TATACCCGGGGGCGCGCCACCTGCTGATGATTGCTACTG-3′) (*Xma*I and *Asc*I sites underlined) and reverse primer (5′-TATATCTAGAATTTAAATCTGAAACTTCCTTGGCTTTGTC-3′) (*Xba*I and *Swa*I sites underlined) and *PVIP2* forward primer (5′-TATATCTAGAGGCGCGCCGCTTGCCAAAACGAACTCCC-3′) (*Xba*I and *Asc*I sites underlined) and reverse primer (5′-TATAGGATCCATTTAAATACCTCTCTCTCGCATTCCTC-3′) (*Bam*HI and *Swa*I sites underlined). *PVIP* cDNA fragments were first cloned in the sense orientation between the *Asc*I and *Swa*I sites of pFGC5941 (http://www.chromdb.org/fgc5941.html), after which the antisense *PVIP* cDNA fragment was inserted between *Xma*I and *Xba*I sites for *PVIP1* and between *Xba*I and *Bam*HI for *PVIP2*. *A. tumefaciens* strain GV3101 carrying these constructs was used to transform *Arabidopsis* (ecotype Col-0) plants by floral dipping (5). Seeds from these plants were sown, and transgenic plants were screened for resistance to the herbicide BASTA. Semiquantitative duplex reverse transcription (RT)-PCR was used to determine the efficiency of RNAi on *PVIP1* and *PVIP2* mRNAs. cDNA was synthesized using Expand reverse transcriptase (Roche Biodiagnostics GmbH, Mannheim, Germany) and used in a duplex PCR containing oligonucleotides specific for *ubiquitin* and *PVIP1* or *PVIP2* mRNA. Semiquantitative PCR of cDNA derived from the equivalent of 1 μg of total RNA was performed under the following conditions: 94°C for 2 min for 1 cycle; 94°C for 30 s, 52°C for 40 s, and 72°C for 1 min for 21, 26, and 31 cycles; and 72°C for 10 min for 1 cycle for each sample. The linear phase of DNA amplification (26 cycles) was determined by electrophoresing and ethidium bromide staining of the PCR products separated in a 1.5% agarose gel. Oligonucleotides to *PVIP1* and *PVIP2* were designed to detect *PVIP1* or *PVIP2* mRNA and not the *pvip-1* or *pvip-2* RNA sequences produced by the RNAi transgene.

RNA extraction and dot hybridization. Total RNA was extracted from frozen plants using TriReagent (Sigma) according to the manufacturer's instructions. For infected plants, leaf tissue was pooled from at least three independent plants. For quantifying virus replication in infiltrated *N. benthamiana* leaves, at each time point, RNA was extracted from four replicate leaves from different plants and duplicate aliquots of 2.5 μg of total RNA were spotted to Hybond N (Amersham Pharmacia Biotech) before hybridization with virus-specific probes. Radiolabeled probes for detection of TuMV RNA were made in the presence of [α-^{32}P]dCTP using either random hexanucleotides as primers for cDNA synthesis in the Prime-a-gene labeling system (Promega) to measure total viral RNA or TuMV strand-specific primers (sequences corresponding to nucleotides [nt] 5681 to 5700, 5903 to 5921, and 6469 to 6502) to synthesize a minus-strand viral RNA-specific probe using the Klenow fragment of DNA polymerase I. Dot blot quantification was carried out using AIDA software associated with the Fuji FLA500 phosphorimager, using a probe for *actin* RNA as an invariant standard for normalizing the data.

Nucleotide sequence accession numbers. The nucleotide sequences of *PVIP* from *P. sativum* (pea; *PVIPp*) and its homolog from *N. benthamiana* (*PVIPnb*) have been deposited in GenBank under accession no. AY271743 (PVIPp) and AY271742 (PVIPnb).

RESULTS

A novel host factor interacts with potyvirus VPg. Viruses infect and multiply in their hosts through the deployment of a relatively small number of multifunctional gene products. To explore the diverse roles of potyvirus VPg (26), we screened plant cDNA libraries in yeast using VPg as the bait. Since our initial interest lay in the characterization of resistance genes

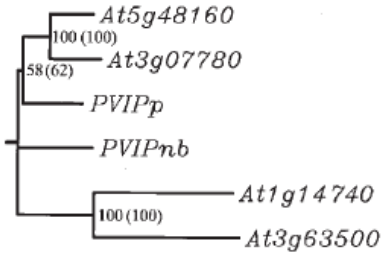

FIG. 1. Cluster analysis of the PVIP-related genes from pea, *N. benthamiana*, and *Arabidopsis*. *Arabidopsis* proteins translated from *At5g48160* (PVIP1; 573 aa), *At3g07780* (PVIP2; 566 aa), *At1g14740* (protein 760 aa), *At3g63500* (protein 1162 aa), and *PVIPp* (protein 503 aa) from *P. sativum* and *PVIPnb* (protein 549 aa) from *N. benthamiana* were aligned and clustered using the Clustal X program. Bootstrap values within and outside brackets correspond to calculations without and with inserted gaps, respectively, to optimize the alignment. The absence of a value indicates that the branch link is not supported by the analysis.

TABLE 1. Analysis of the breadth of the interaction between VPgs and PVIP in yeast

Bait	Growth[d] of yeast transformed with pGAD424 containing prey[c]:						
	AD[b]	At1	At2	At3	At4	PVIPp	PVIPnb
BD[a]	−	−	−	−	−	−	−
PSbMV	−	+	+	−	−	+	+
TuMV	−	+	+	−	−	+	+
LMV	−	+	+	−	−	+	+
TEV	−	−	−	−	−	−	−
CPMV	−	−	−	−	−	−	−
TBRV	−	−	−	−	−	−	−
GFLV	−	−	−	−	−	−	−

[a] Empty bait vector (confirming that PVIP was unable to autoactivate transcription).
[b] Empty prey vector (confirming that VPg was unable to interact with the activation domain).
[c] At1, At2, At3, At4, At5g48160, At3g07780, At1g14740, and At3g63500, respectively.
[d] Growth of the yeast on selective media. Extent of the growth was equivalent for all positive interactions. −, no growth.

from pea, we first used the VPg protein from PSbMV and a cDNA library constructed from pea leaf mRNA. PSbMV VPg was fused to the GAL4 DNA-binding domain, whereas the pea cDNA library was fused to the GAL4 activation domain. From $\sim 7 \times 10^6$ independent yeast transformants, 10 classes of potential interacting proteins were identified. Among them, one protein interacted more than twice as strongly as the positive control. This protein was studied further. The protein was named PVIPp (for *Potyvirus* VPg-interacting protein from pea). Based on nucleotide sequence and BLAST searches, PVIPp appeared to be related to the products of a small gene family in *Arabidopsis* (the genes *At5g48160*, *At3g07780*, *At1g14740*, and *At3g63500*). None of these proteins showed significant homology with proteins of known function. PVIPp showed the highest homology with At5g48160 and At3g07780 (Fig. 1).

To determine whether the interaction with PVIP was specific for pea and PSbMV VPg, other plant virus VPgs and other plant PVIPs were tested using the YTHS. The VPg coding sequences for the potyviruses *Lettuce mosaic virus* (LMV) (strain 0) (24), TuMV, and TEV or related members of the family *Comoviridae*, CPMV, TBRV, and GFLV, were cloned into pGBT9. They were assayed for interaction with the products of *At5g48160*, *At3g07780*, *At1g14740*, and *At3g63500*; *PVIPp*; and a homologue of *PVIPp* isolated from *N. benthamiana* (*PVIPnb*), all cloned into pGAD424. The results (Table 1) showed that VPg proteins of PSbMV, LMV, and TuMV, but not TEV, interacted with products of *At5g48160* and *At3g07780* (named PVIP1 and PVIP2) and of *PVIPp* and *PVIPnb*. In contrast, interactions with PVIP were not observed for the VPg protein from CPMV, GFLV, or TBRV or for potyvirus VPg with products of *At1g14740* and *At3g63500*. We also found that while the interaction between potyvirus VPg and PVIP was readily detected when transferred to the LexA YTHS, reversing the fusion partners so that VPg and PVIP were fused to the activation and DNA-binding domains, respectively, abolished the interaction (data not shown).

PVIP-VPg interaction in yeast depends on sequences within the N terminus of VPg. Deletion analysis was used to determine the sequence limitations of the interaction between VPg and PVIP. Two series of deletions for TuMV VPg were constructed and tested as fusions to the GAL4 DNA-binding domain in the YTHS, using pGAD-*PVIP1* and -*PVIP2* as the interaction partners. The first series contained deletions collectively spanning the entire VPg sequence (Fig. 2). The N-terminal 66 amino acids (aa), the central region (aa 67 to 138), or the C-terminal 54 aa, respectively, were deleted from the proteins encoded by pGBT-*Tu*Δ1, pGBT-*Tu*Δ2, and pGBT-*Tu*Δ3. The second deletion series (Fig. 2), pGBT-*Tu*Δ4, pGBT-*Tu*Δ5, pGBT-*Tu*Δ6, pGBT-*Tu*Δ7, and pGBT-*Tu*Δ8, removed aa 1 to 16, 1 to 31, 27 to 42, 32 to 192, and 17 to 192, respectively. Growth of the yeast transformants on selective media showed that the interaction with PVIP1 and -2 was absolutely dependent upon the first 66 aa of VPg (Fig. 2, compare TuΔ1 with TuΔ2 or TuΔ3). From the second deletion series, this region could be subdivided to identify aa 27 to 42 (TuΔ6) as not being required for the interaction while the N-terminal 16 aa (TuΔ4 and TuΔ5) were essential for the interaction with either PVIP1 or -2. Interestingly, the deletions TuΔ7 and TuΔ8, encoding only the first 31 and 16 aa, respectively, exhibited interaction with PVIP2 but not PVIP1. This showed that in the case of PVIP2, the first 16 aa of TuMV VPg were not only necessary but also sufficient for the interaction. A similar positive interaction was seen with PVIPp (data not shown). Comparison of the interaction assay for PVIP1 with

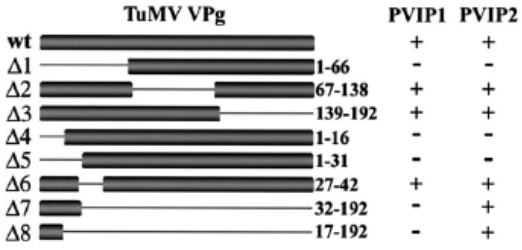

FIG. 2. Deletion analysis of the interaction of TuMV VPg with PVIP1 and PVIP2. TuMV wt VPg and two series of deletion mutants for TuMV VPg (Δ1 to Δ3 and Δ4 to Δ8) were tested for their interactions with PVIP1 and PVIP2 by yeast two-hybrid analysis. The positive (+) interactions were all equivalent, and the data were scored qualitatively.

those for TuΔ1 (aa 1 to 66) and TuΔ6 (aa 27 to 42) suggests that an additional component between aa 42 and 66 is required for the VPg-PVIP1 interaction to succeed.

Similarly, we tried to identify regions within PVIP that were necessary for the interaction with VPg. Using PVIP1, a series of four deletion mutants, together covering the entire PVIP1 sequence, were constructed in the pGAD-*PVIP1* vector. Mutants pGAD-*PVIP1*Δ1, -*PVIP1*Δ2, -*PVIP1*Δ3, and -*PVIP1*Δ4 had aa 3 to 157, 157 to 283, 283 to 403, and 403 to 574 deleted, respectively. No growth was detected with these yeast strains in the presence of pGBT-*TuMV VPg*. Hence, we were unable to identify a functionally discrete PVIP domain for the interaction between VPg and PVIP1.

It is possible that the lack of detectable interaction reflected the stability and accumulation of the fusion proteins rather than a structural defect in either partner. Using antibodies to PVIP and VPg, we attempted to detect the respective fusion proteins in yeast expressing the Gal4 fusion proteins. These proteins accumulate at very low levels (Clontech instruction manual) and may not always be detectable. Nevertheless, both PVIP and VPg fusions were detected for most of the expressed constructs, but there was no correlation between detection of the fusion protein and a positive interaction (data not shown).

A single amino acid differentiates the abilities of two VPgs to interact with PVIP2. Sequence comparisons of TuMV and three other potyviruses (PSbMV, TEV, and LMV) were made for the two domains (VPg aa 1 to 16 and 42 to 66) that controlled the interactions with the PVIP1 and PVIP2 proteins (Fig. 3A). The comparison shows that both regions are highly conserved among these potyviruses. To identify a residue(s) critical for the interaction with PVIP, it was useful to compare the sequence of TEV VPg, which showed no interaction, with the other sequences. Within the N-terminal 16 aa, which was sufficient for the interaction of TuMV VPg with PVIP2, 5 residues are identical and 2 are closely related for all four viruses (Fig. 3A). Among the nine remaining residues, the only consistent differences between the VPg proteins of PSbMV, TuMV, and LMV and that of TEV were a deleted glycine at aa position 3 in TEV; position 12, where an aromatic amino acid (F/Y) in TuMV, PSbMV, and LMV was replaced with methionine in TEV; and position 14, where TEV contained an acidic (E) rather than an uncharged (Q/N) residue. Site-directed mutagenesis was used to individually insert each of these changes into the sequence of TuMV VPg, and the mutants (G3-, F12 M, and N14E, respectively) were assessed for their interaction with PVIP1, PVIP2, PVIPnb, and PVIPp in yeast. A reciprocal mutant, TEV VPg M12F, was also constructed and tested. Mutations at positions 3 and 14 were benign with respect to the interaction with all PVIP proteins (Fig. 3B). In contrast, the F12M mutation in TuMV VPg abolished interactions with all PVIPs, whereas the reciprocal M12F mutation in TEV VPg conferred the interaction with PVIP2 and PVIPp. These results showed that F12 plays a crucial role in the physical interaction between VPg and PVIPs and that for TEV, M12 is the only lesion that prevents interaction with PVIP2 and PVIPp.

Mutations in VPg that block the interaction in yeast correlate with reduced virus movement in planta. Interaction of two proteins in the YTHS is not proof of a biological role in vivo. We have also obtained evidence that the interaction is important for virus infection. Based upon the results obtained from the YTHS experiments, we tested the VPg F12M mutation in the complete TuMV virus genome for its effect upon virus multiplication. After stab inoculation to introduce *Agrobacterium* and the potential infection into *N. benthamiana* plants, the plants were observed for 15 days. At 7 days postinoculation (p.i.), all eight of the plants infected with the wild-type (wt) TuMV showed systemic chlorosis, some necrotic flecking, leaf distortion, and stunting (Fig. 4A). By 15 days p.i., all the plants showed extensive necrosis or had died. In contrast, all the plants inoculated with pGreen-TuF12M remained symptomless for 12 days p.i., developed weak chlorosis after 15 days p.i., and showed little growth reduction (Fig. 4A). The accumulation of viral RNA in these plants was assessed using dot hybridization of RNA samples extracted from leaves harvested over 13 days (Fig. 4B). Viral RNA was detected readily in inoculated and systemically infected leaves of plants inoculated with pGreen-TuMV as early as 7 days p.i. (Fig. 4B). In contrast, in plants inoculated with pGreen-TuF12M, viral RNA could not be detected in inoculated leaves and was first detected in systemically infected leaves of plants only at 13 days p.i. Since viruses show high efficiency in the selection of sequence variants, we checked that the virus appearing in pGreen-TuF12M-inoculated plants had retained the inserted mutation. Progeny viral RNA was analyzed by direct sequencing of RT-PCR products that spanned the VPg coding region between nt 5718 and 6538 of the TuMV genome. In all eight plants, the mutation had been retained, and no other muta-

A

```
TuMV   1  AKGKRQRQKLKFRNAR  16
LMV    1  GKGKRQRQKLRYRQAR  16
PSbMV  1  --GKSKAKTLRFRQAR  14
TEV    1  GK-KNQKHKLKMREAR  15
             :▲*      *:▲*▲**

TuMV  42  KGKSKGRTRGIGHKNRKFINMYGFD  66
LMV   42  KGKKSGKTKGMGTKNRRFVNMYGYN  66
PSbMV 40  KGKKSGKARGMGVKTKKFVNVYGFD  64
TEV   41  KGKRKGTTRGMGAKSRKFINMYGFD  65
          ***  *▲::*:*  *  ::*:*:**::
```

B

		1	2	p	Nb
Tu wt	AKGKRQRQKLKFRNAR	+	+	+	+
Tu G3-	AK-KRQRQKLKFRNAR	+	+	+	+
Tu F12M	AKGKRQRQKLKMRNAR	-	-	-	-
Tu N14E	AKGKRQRQKLKFREAR	+	+	+	+
Te wt	GK-KNQKHKLKMREAR	-	-	-	-
Te M12F	GK-KNQKHKLKFREAR	-	+	+	-

FIG. 3. Analysis of amino acid determinants of VPg-PVIP interactions. (A) VPg sequences from TuMV, LMV, PSbMV, and TEV were aligned using the Clustal W program. Only regions 1 to 16 and 42 to 66 are shown. Identical (*) and related (:) amino acids for the four sequences are indicated. Amino acid positions where TEV differed from the other three with an unrelated residue are also identified (▲). (B) Mutational analysis of TuMV and TEV VPg proteins targeting aa 3, 12, or 14. The TuMV point mutants Tu G3-, F12M, and N14E and the TEV point mutant Te M12F (shaded) were tested for interaction with PVIP1 (column 1), PVIP2 (column 2), PVIPp (column p), and PVIPnb (column Nb) using yeast two-hybrid analysis. Interactions in yeast were scored qualitatively; all the positive (+) interactions were seen as equivalent yeast growth on selective media.

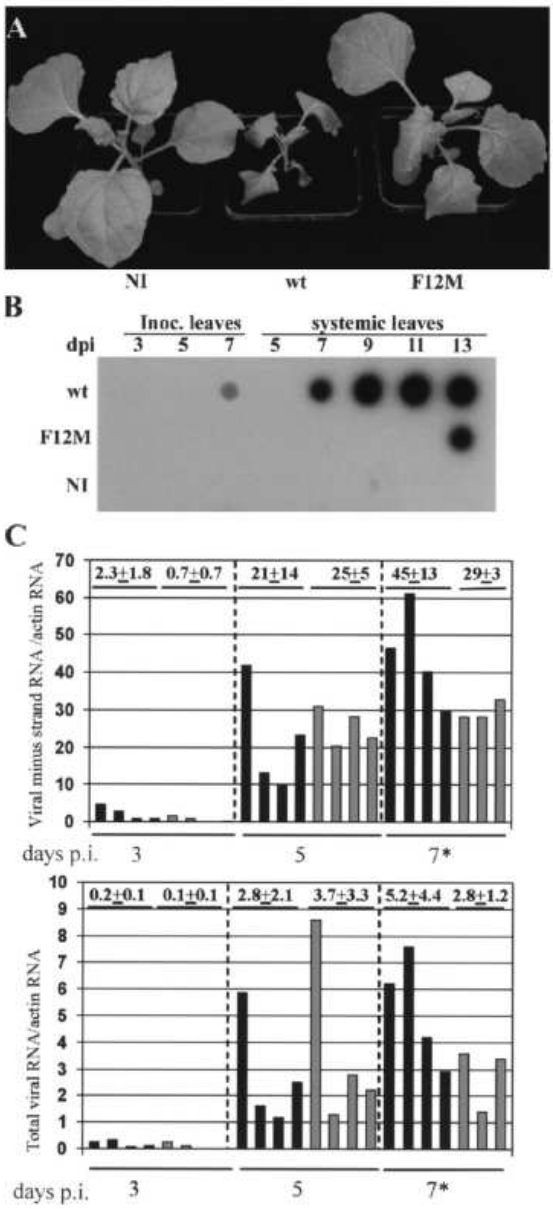

FIG. 4. Phenotypic assessment of the VPg F12M mutation for TuMV multiplication in *N. benthamiana*. (A) Noninoculated (NI) *N. benthamiana* plants and plants stab inoculated with TuMV wt and TuMV F12M were compared. At 12 days p.i., plants infected with TuMV F12M showed significantly fewer symptoms than those infected with the wt TuMV. (B) Hybridization analysis of total viral RNA accumulation in plants illustrated in panel A. Stab-inoculated (Inoc.) and systemically infected (systemic) leaves were harvested 3, 5, and 7 and 5, 7, 9, 11, and 13 days p.i., respectively. Viral RNA accumulation was substantially less for the TuMV F12M mutant than for wt TuMV. (C) Hybridization analysis of minus-strand (top) and total (bottom) viral RNAs in patches of *N. benthamiana* leaves infiltrated to give infections with wt TuMV (solid bars) or TuMV F12M (shaded bars). Individual data points are expressed as arbitrary units relative to hybridization with a probe for *actin* RNA as an invariant standard. The mean data points for all the leaf samples are presented. The overall mean value ± standard deviation is given above each set. Because of the different specific activities of the probes for different viral RNAs, the upper and lower panels are not quantitatively comparable. *, only three samples were available for mutant infections at 7 days p.i.

tions were present in the VPg. These results showed that the VPg F12M mutation directly or indirectly restricted TuMV multiplication and/or spread.

To assess quantitatively the impact of the F12M mutation on virus replication, infections with pGreen-TuMV and pGreen-TuF12M were established after infiltrating the cultures of *Agrobacterium* into patches on the leaf lamina of *N. benthamiana*. This strategy provides high-efficiency transient expression of the transferred agrobacterium T-DNA in a large proportion of the leaf cells (36). Leaf samples were harvested 3, 5, and 7 days p.i., and the extracted RNA was analyzed for total or minus-strand viral RNA using dot hybridization (Fig. 4C). Minus-strand viral RNA is an essential and unique component of the replication mechanism for single-stranded viral RNAs. Total viral RNA measurements could feasibly include progeny viral RNA and RNA transcribed directly from the input cDNA expression cassette. For the wt and mutant virus infections, total and minus-strand viral RNAs were detected 3 days p.i. and increased substantially by 5 days p.i., but no further at 7 days p.i. Although there was variation among the individual samples at different times, there was no significant difference between wt and mutant virus infection for any of the times analyzed.

To visualize directly the impact of the F12M mutation on virus movement, the wt and mutant virus constructs were tagged with GFP. Leaf laminal tissues of *N. benthamiana* were infiltrated with suspensions of *Agrobacterium* at high dilution so that isolated foci of infection developed. The spread of the foci was monitored 2, 3, 4, 5, and 6 days p.i. as GFP fluorescence (Fig. 5). No fluorescence was visible 2 days p.i. Over days 3 to 5, GFP lesions were visible for both the wt and mutant virus, but the size and rate of expansion of the mutants were significantly less (Fig. 5A to H illustrate typical infection foci). At 6 days p.i., the wt infection had spread through virtually all of the infiltrated leaf area and showed significant fluorescence in the veinal tissues, including the petiole (Fig. 5I and J). At the same time, foci of infection for the F12M mutant were visible as abundant small patches of fluorescence, subjectively as bright as for the wt infection. Some of these patches overlapped the leaf veins, but there was no evidence of spread along the veins (Fig. 5L and M). Systemically infected tissue at 6 days p.i. showed extensive tissue invasion of the veins and lamina for the wt virus but no detectable spread of the mutant virus (compare Fig. 5K and N).

Reduced PVIP expression in planta reduces symptom production and virus accumulation. The results presented above strongly suggest that VPg-PVIP interaction is important for potyvirus multiplication. However, as potyviral VPgs are multifunctional proteins, it remained possible that the F12M mutation could also have pleiotropic effects on one or more VPg functions. To assess directly the importance of PVIP for potyvirus infection, we generated *Arabidopsis PVIP1* and *PVIP2* mutant lines (designated *pvip1* and *pvip2*, respectively) using

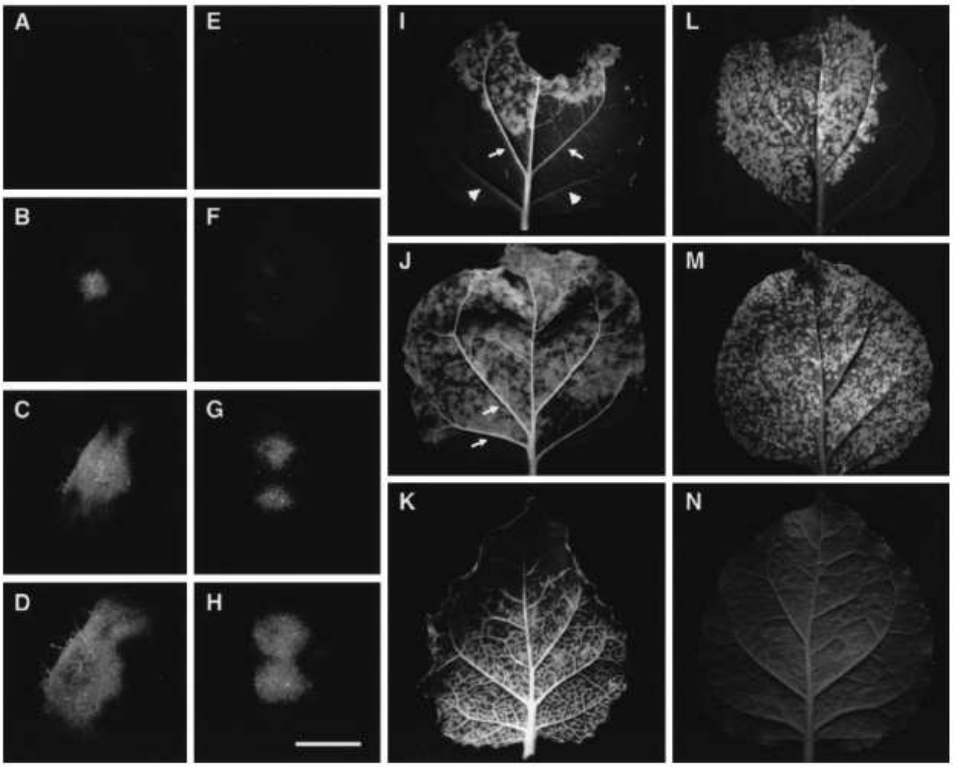

FIG. 5. Differential local and systemic spread associated with the F12M mutation in TuMV VPg. Virus inocula pCB-TuMV-GFP (A to D and I to K) and pCB-TuMVF12M-GFP (E to H and L to N) were introduced into infiltrated leaf patches with *Agrobacterium* at a dilution appropriate to give isolated infection foci. The leaves were photographed at 2 (A and E), 3 (B and F), 4 (C and G), 5 (D and H), and 6 (I, J, L, and M) days p.i. Additionally, systemic leaves from infected plants were examined 6 days p.i. (K and N). Passage of the virus in vascular tissues (arrows) is seen for the wt virus only (compare panels I and J with L and M) as fluorescence along the veinal tissues within and beyond the infiltrated area (I and J); compare these with veins from the same leaf (I) without fluorescence (arrowheads). Photographs taken under UV light and printed as greyscale show bright (green) fluorescence against dark tissue background. Bar for panels A to H = 3 mm.

RNAi (35) and challenged them by virus inoculation. *Arabidopsis* plants were transformed with an inverted-repeat construct specific for either *PVIP1* or *PVIP2* RNA and capable of forming a hairpin RNA after expression from the Cauliflower mosaic virus 35S promoter. Activation of RNAi by the double-stranded RNA leads to the targeted degradation of homologous plant RNAs. Since RNAi is functionally dominant, we expected all lines containing the transgene to show a depletion of the target transcript.

The expression of PVIP1 or PVIP2 transcripts was examined in 20 randomly chosen independent *pvip1* or *pvip2* RNAi lines by duplex RT-PCR, using *ubiquitin* RNA as an invariant control. *PVIP1* or *PVIP2* RNA was depleted to various extents in the RNAi lines tested, whereas our internal control, the *ubiquitin* transcript, showed no variation. We chose for further analysis two independent *pvip1* and *pvip2* lines that were most efficiently silenced for the corresponding *PVIP*, i.e., lines in which there was the least transcript accumulation (Fig. 6A). These lines showed no major morphological differences from wt plants except that they exhibited slightly slower growth (data not shown). Three plants of each of these RNAi lines were infected with pGreen-TuMV by stab inoculation. As for infection of *N. benthamiana*, TuMV-infected, untransformed *Arabidopsis* showed leaf necrosis and stunting. In contrast, no symptoms were visible on either the *pvip1* or *pvip2* RNAi lines (Fig. 6B). The level of progeny viral RNA in these plants was assessed by dot hybridization. Viral RNA was readily detected in systemic leaves of wt *Arabidopsis* at 23 days p.i. In contrast, at the same time, viral RNA accumulation in *pvip1* and *pvip2* RNAi lines was not detectable (Fig. 6C) unless the autoradiograph was exposed for at least 20 times as long, when a faint hybridization signal above background could be detected (data not shown). In inoculated leaves, we were able to detect progeny viral RNA in infected *pvip1* and *pvip2* plants 5 days p.i., although at a significantly lower level than in wt plants (Fig. 6C). Since all of the leaves received equal bacterial inocula, it is likely that most of the RNA detected for the wt inoculum resulted from virus replication rather than transcription of the input cDNA expression cassette.

DISCUSSION

We have identified an interaction in the YTHS between the VPg proteins of a range of potyviruses and a plant protein,

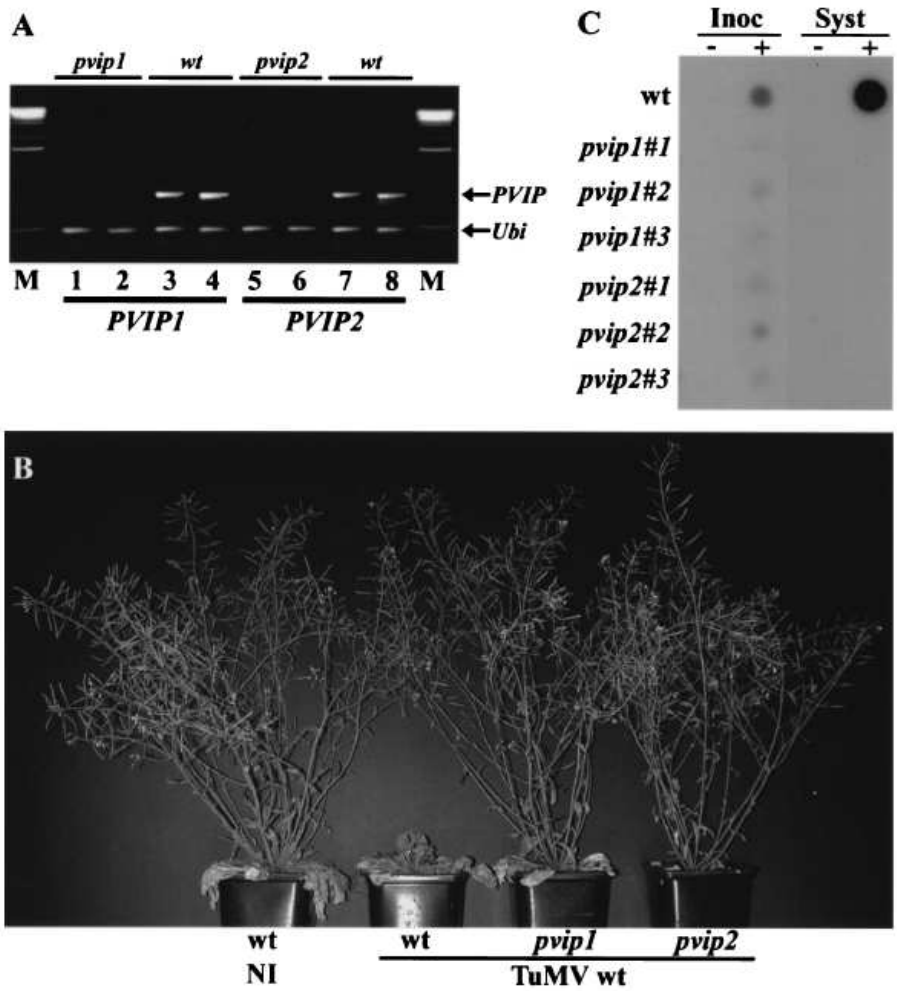

FIG. 6. TuMV infection phenotypes on plants with reduced *PVIP1* or *PVIP2* expression. *Arabidopsis* Col-0 plants were transformed with RNAi constructs to target *PVIP1* or *PVIP2* expression. (A) Duplicate samples from *pvip1* and *pvip2* transgenic plants and wt plants were assessed for the expression of *PVIP1* and *PVIP2* using semiquantitative RT-PCR. Products from duplex RT-PCRs with primers for *PVIP* RNAs and *ubiquitin* RNA (*Ubi*; as a control) were analyzed by agarose gel electrophoresis. Plant lines selected for inoculation (illustrated in lanes 1 and 2 and lanes 5 and 6) showed no detectable transcript accumulation compared with their wt parent plants (lanes 3 and 4 and lanes 7 and 8). Lane M shows DNA size markers. (B) wt uninfected (NI) plants and wt, *pvip1*, and *pvip2* plants stab inoculated with wt TuMV. In contrast to the severe stunting seen with TuMV infection of wt plants, no disease symptoms were seen on the *pvip1* and *pvip2* plants. (C) Hybridization analysis of viral RNA for the plants illustrated in panel B. Leaves from noninfected (−) and infected (+) plants were analyzed for the presence of TuMV RNA. TuMV RNA was readily detected in inoculated (Inoc; 5 days p.i.) and systemically infected (Syst; 23 days p.i.) leaves of wt *Arabidopsis* plants. Analysis of triplicate samples from *pvip1* and *pvip2* plants showed reduced accumulation in inoculated leaves and no detectable accumulation in systemically infected leaves. However, exposure of the autoradiograph for at least 20 times as long showed a low level of accumulation in systemically infected leaves (data not shown).

PVIP. This interaction has also been confirmed in vitro (our unpublished data). PVIP appears not to be essential for virus replication but functions more as an ancillary factor for potyvirus movement and the development of disease. PVIP is part of a small gene family in *Arabidopsis* and has counterparts in pea and *N. benthamiana*. Alignment of all the PVIP-related sequences (Fig. 1) shows that At1g14740 and At3g63500 form a group discrete from PVIPs, data that reflect our YTHS interaction analysis, in which no interaction between VPg and these proteins could be demonstrated. Unfortunately, database searches using the PVIP sequences failed to identify any associated biological function. Furthermore, the phenotype of the uninfected *pvip1* and *pvip2* plants did not indicate an interruption in any particular stage of growth and development that might indicate a candidate function. Analysis of amino acid sequence motifs did, however, identify a "PHD finger-like" domain (34) present in all PVIPs analyzed. This domain is characterized as a series of histidine and cysteine residues in the order C4HC3 and is frequently associated with proteins that have roles in transcriptional regulation through chromo-

some remodeling (1). If this was true for PVIP, it would point to a role for VPg in the nucleus. VPg has a nuclear targeting sequence and has been shown to accumulate in the nucleus, at least in the form of its immediate proteolytic precursor molecule, nuclear inclusion a, or NIa, protein (3). Since potyvirus infection can be associated with altered host gene expression (17), the potential for the PVIP-VPg interaction to modulate host transcription following infection is intriguing.

The impact of breaking the PVIP-VPg interaction using a virus mutant or RNAi plants, *pvip1* and *pvip2*, was to reduce disease symptoms significantly and to almost prevent virus accumulation in systemic uninoculated leaves. However, virus multiplication was not abolished, making PVIP an unlikely candidate as a complete resistance factor (e.g., in genetic knockout plants). Following the initial identification of PVIPp, we mapped the chromosomal position of *PVIPp* relative to that for the resistance genes *sbm-1* and *sbm-3* in pea. The map positions for all three loci were completely different (unpublished data).

VPg is attached to the 5′ end of the viral RNA through a covalent linkage to the tyrosine residues at position 64. Either as VPg or as part of NIa, it has also been functionally implicated in viral RNA replication and local or systemic virus movement and as an avirulence determinant. During viral RNA replication, VPg may act as a primer, but there is evidence (16) that NIa also interacts with the NIb polymerase. This interaction is dependent upon the C-terminal protease domain of NIa rather than the N-terminal VPg region. The structural determinants of virus movement have been determined for *Tobacco vein mottling virus* and PVA and have been shown to be associated with aa 110 to 115 and 116 or 118, respectively (18, 22, 23). In the latter case, the specific amino acids appeared to be a determinant of the host range (22, 23). Although our deletion analysis identified a different (N-terminal) region of VPg as the location of determinants of the interaction with PVIP, there was little effect of the TuMV F12M mutation on virus replication in infiltrated leaf patches and a stronger effect on the infection resulting from virus movement. Hence, the local and systemic infection of *N. benthamiana* with TuMV F12M was substantially delayed. Systemic infection of *pvip1* and *pvip2* plants by wt TuMV was also much delayed. Although replication in infiltrated leaf patches was unaffected, there was a marked reduction in the accumulation of the mutant virus (or wt TuMV on mutant plants) following stab inoculation. On balance, we conclude that the VPg-PVIP interaction has a predominant effect on virus cell-to-cell movement, with longer-term consequences for systemic movement. Using GFP-tagged wt and mutant virus, this effect was visualized as a slower expansion of isolated infection foci and reduced phloem loading, leading to systemic infection.

PVIP constitutes a small gene family in *Arabidopsis*. In addition to generating *pvip1* and *pvip2* RNAi plants, we also made *pvip1-pvip2* double mutants (our unpublished data). These plants were unsuitable for virus inoculation due to their extremely small size, a phenotype not seen in either single-mutant line. While this suggests that PVIP1 and PVIP2 have redundant functions, it raises the question of why the single-mutant lines did not support virus multiplication and movement through complementation. We interpret this as indicating that in *Arabidopsis*, VPg interacts with a PVIP1/2 heterodimer. PVIP1 and PVIP2 self- and cross-interact in the YTHS (our unpublished data), and VPg can interact with itself in yeast (9, 19, 38).

A putative three-dimensional structure for the potyvirus VPg has been published (20). The VPg amino acid sequence showed the greatest structural homology to the folded structure for the enzyme malate dehydrogenase. This placed the N-terminal 16 aa on the surface of the folded VPg (20). In this location, F12 would be available for a surface interaction with PVIPs. From a comparison of VPg sequences for several potyviruses with TEV, we showed that F12 was crucial for interaction with PVIPs. Comparison of the VPg sequences for all four potyviruses for the region aa 42 to 66 revealed that only position 49 showed a consistent difference between PSbMV, TuMV, and LMV and TEV. Here, a basic amino acid (K or R) in TuMV, PSbMV, and LMV is replaced with a polar uncharged amino acid (T) in TEV. We speculate that a basic amino acid at position 49 might also be needed to achieve an interaction with PVIP1, although this has not been tested. This raises an important biological question about TEV VPg, which did not interact with any PVIP proteins. If PVIP makes a significant contribution to virus infection, how and why has TEV avoided a requirement for this interaction? From an alignment of the VPg sequences from 39 potyviruses (data not shown), it appears that the F/Y-M transition at position 12 in TEV is uncommon, and may be unique, among the potyviruses. Whether TEV has found an alternative means to generate the supportive potential of VPg-PVIP for infection or whether the selection pressure to force the M12F change is too weak in this virus remains to be determined.

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2.2. Recherche menée depuis 1999 suite au séjour postdoctoral

Lorsque j'ai réintégré l'équipe de virologie à Bordeaux en rentrant de mon séjour à Norwich, je me suis attaché dans un premier temps à m'imprégner des différents projets en cours, qui avaient bien entendu évolué depuis la fin de ma thèse, afin de me concentrer sur les aspects qui nous paraissaient les plus prometteurs. Dès 2000, j'ai centré mon activité de recherche sur d'une part, la production de nouveaux clones de LMV recombinant (projet initié par Elise Redondo durant sa thèse soutenue au printemps 2001) afin de préciser la région du génome du LMV impliquée dans le contournement des gènes de résistance récessifs *mo1*, et d'autre part, l'analyse du pathosystème *Arabidopsis thaliana*/LMV dont les premières expériences d'infection avaient été conduites par T. Candresse. Ces deux aspects adhéraient complètement aux projets d'identification des déterminants viraux et de plante impliqués dans les interactions moléculaires plante/potyvirus proposés par notre équipe, en particulier dans le cadre de la création de la Jeune Equipe INRA (associant en plus des chercheurs de l'équipe, V. Decroocq, chercheur GAP, et V. Schurdi-Levraud, maître de conférence). De plus, mes expériences passées m'avaient permis d'aborder successivement l'étude du virus lui-même (pendant ma thèse) et celle de facteurs de plante (pendant mon postdoc) impliqués dans les interactions plante/potyvirus et il nous a semblé tout à fait logique que je m'intéresse et m'investisse sur un projet de caractérisations génétique et moléculaire des interactions Plante/Potyvirus

La construction de clones recombinants entre les deux isolats de LMV étudiés pendant ma thèse a été initiée par Elise Redondo pendant sa thèse démarrée peu de temps après mon départ à Norwich. Elle a pu montrer que les déterminants viraux du contournement des gènes *mo1* étaient localisés dans une région comprise entre la fin du gène codant pour la protéine CI et la région 3' non codante (Figure 1 pour voir l'organisation génomique des potyvirus). Afin d'affiner la localisation de ces déterminants, j'ai poursuivi à partir de la fin 1999 ce travail avec l'aide d'une technicienne de l'équipe. Après quelques mois, et au fur et à mesure que le projet "*Arabidopsis*" prenait de l'ampleur, nous avons décidé que mon investissement dans ce projet se limiterait au suivi des expériences réalisées par notre technicienne. Nous avons produit plusieurs recombinants dont ceux échangeant la VPg (domaine N-Ter de la NIa) et ainsi augmenté la panoplie de LMV recombinants disponibles au laboratoire. Ces outils ont notamment servi de base pour la thèse de Thomas Guiraud soutenue fin 2004.

2.2.1. Développement et premières analyses du pathosystème LMV/*Arabidopsis thaliana*

Des expériences préliminaires réalisées par H. Lot (Pathologie végétale, INRA Avignon) et T. Candresse avaient révélé que le LMV pouvait infecter *Arabidopsis* mais sans développement de symptôme et cela, quel que soit l'isolat de LMV utilisé. Lorsque j'ai pris ce projet en main, T. Candresse avait réalisé un premier criblage d'une cinquantaine d'accessions d'*Arabidopsis* en utilisant un isolat de LMV (LMV-E) marqué avec le gène GUS et avait mis en évidence des accessions sensibles et résistantes au LMV.

Le fait que le LMV puisse infecter *Arabidopsis* nous ouvrait des voies prometteuses pour aller identifier des facteurs de plante impliqués dans les interactions plante/virus, facteurs encore mal connus à l'heure actuelle. Comme de plus les mécanismes d'interactions entre plante et potyvirus semblent relativement bien conservés, les données fournies par ce pathosystème devaient pouvoir être facilement transférables à des plantes d'intérêt agronomique. Arabidopsis offre en effet plusieurs avantages dont ceux d'avoir un cycle de développement court et être de petite taille. Mais surtout, il existe un nombre important

d’accessions qui ont été collectionnées au sein de centres de ressources génétiques et de nombreuses populations issues de croisements entre différentes accessions d'*Arabidopsis*, notamment sous forme de population F2 et de lignées recombinantes (RIL) ont été produites. Ces dernières sont produites à partir d’au moins 6 autofécondations d’une population F2 et génotypées avec un ensemble de marqueurs moléculaires polymorphes distribués sur l’ensemble du génome. Elles sont ainsi des outils puissants pour réaliser la cartographie génétique de gènes. L'ensemble de ces matériels permet relativement aisément l'identification de gènes par des approches de génétique classique. Complémentaire des approches de génétique, la génomique d’Arabidopsis a aussi été particulièrement bien développée. En effet, non seulement son génome a été séquencé, mais les techniques récentes de génomique (microarrays) permettent d'envisager des analyses à haut débit du transcriptôme de cette plante en réponse à des situations variées. Enfin des banques de mutants « knock-out » (KO) sont aussi disponibles et il est devenu simple de se procurer de tels mutants pour une grande proportion des gènes d’Arabidopsis.

Afin d'avoir des données les plus larges possibles sur ce nouveau pathosystème, j'ai entrepris le criblage de quelques dizaines d’accessions avec les isolats de LMV étudiés dans notre équipe, LMV-0, LMV-E mais aussi LMV-AF199, isolat brésilien présentant des propriétés biologiques différentes de celles de LMV-0 et LMV-E et introduit au laboratoire en 1999. La révélation du virus dans les plantes s'est fait soit par un test histochimique GUS lorsque les isolats LMV-0 et LMV-E marqués avec le gène GUS étaient utilisés, soit par ELISA pour les tests avec LMV-AF199. Pour chacun des trois isolats testés, trois catégories d'accessions ont été révélées:

- une première catégorie où le LMV n’est pas détecté sur feuilles inoculées (résistance qualifiée de résistance locale liée au blocage de la réplication ou du mouvement de cellule à cellule du virus),

- une deuxième où le LMV est détecté sur feuilles inoculées mais pas dans le reste de la plante (résistance qualifiée de résistance systémique liée au blocage du mouvement à longue distance du virus),

- et enfin une troisième où une infection de toute la plante est observée (infection systémique).

De façon intéressante, ce travail a aussi permis de révéler des différences d'infection d'accessions d'un isolat de LMV à un autre et de classer les accessions d'Arabidopsis en 7 groupes de sensibilité. Ces données très prometteuses non seulement nous montraient qu’une espèce végétale n’ayant pas co-évolué avec un pathogène donné (espèce « naive ») et n’ayant pas été domestiquée par l’homme (maintien d’une large diversité génétique) pouvait réagir très différemment à ce pathogène et ouvraient des perspectives importantes pour aller identifier des gènes de plante contrôlant ces phénotypes.

Dans le but d'identifier la base génétique de quelques phénotypes de résistance identifiés lors de ce criblage et de cartographier les gènes impliqués, j'ai entrepris l'analyse de populations F2 et de RILs issues de croisements entre accessions sensible et résistant au LMV. Je me suis tout d’abord attaché à analyser des populations issues du croisement entre l'écotype Columbia (Col), qui présente une résistance locale à LMV-0 et une résistance systémique à LMV-AF199, et l'écotype Niederzenz (Nd), présentant une résistance systémique à LMV-0 et une sensibilité totale à LMV-AF199. Cette analyse a permis, après avoir déterminer le phénotype d’infection de ces populations après inoculation avec ces deux isolats de LMV, de déterminer la base génétique de ces 2 types de résistance mais aussi de cartographier un des gènes de résistance. Ainsi, la résistance locale à LMV-0 semble être contrôlée par un gène de résistance dominant, que nous avons nommé *LLM1* et cartographié

sur le chromosome I à proximité du marqueur nga280. La résistance à LMV-AF199 semblait être contrôlée par au moins deux gènes de résistance dominants, dont la cartographie génétique n'a pas pu être établie précisément du fait du caractère polygénique de cette résistance. Mais nous reviendrons plus loin sur cette résistance suite à une découverte intéressante à son sujet. Une partie de ce travail a fait l'objet d'un projet de recherche de DEA que j'ai encadré au cours de l'année universitaire 2000-2001 (DEA de Thomas Guiraud).

La richesse du pathosystème Arabidopsis/LMV s'illustre aussi dans les différences de comportement entre isolats de LMV. Ainsi, grâce à la panoplie de recombinants de LMV construits au laboratoire, j'ai pu montrer l'implication de la protéine VPg dans la différence de phénotypes observés sur Nd entre les isolats LMV-0 (bloqué pour le mouvement à longue distance) et LMV-E (non bloqué).

L’ensemble de ces résultats ont fait l'objet d'une publication dans la revue MPMI (**Publication [11]**) présentée ci-dessous.

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Multiple Resistance Phenotypes to *Lettuce mosaic virus* Among *Arabidopsis thaliana* Accessions

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With the aim to characterize plant and viral factors involved in the molecular interactions between plants and potyviruses, a *Lettuce mosaic virus* (LMV)–*Arabidopsis thaliana* pathosystem was developed. Screening of *Arabidopsis* accessions with LMV isolates indicated the existence of a large variability in the outcome of the interaction, allowing the classification of *Arabidopsis* accessions into seven susceptibility groups. Using a reverse genetic approach, the genome-linked protein of LMV, a multifunctional protein shown to be involved in viral genome amplification and movement of potyviruses, was established as the viral determinant responsible for the ability to overcome the resistance of the Niederzenz accession to LMV-0. Preliminary genetic analyses from F2 and recombinant inbred lines available between susceptible and resistant *Arabidopsis* accessions revealed the existence of at least three resistance phenotypes to LMV with different genetic bases. One dominant resistance gene, designated *LLM1*, involved in blocking the replication or cell-to-cell movement of the LMV-0 isolate in the Columbia accession, was mapped to chromosome I and shown to be linked to the marker nga280. At the same time, genetic analyses of segregating F2 populations were consistent with the restriction of the systemic movement of the LMV-AF199 isolate in Columbia being controlled by two dominant genes and with the complete resistance to all tested LMV isolates of the Cape Verde islands (Cvi) accession being conferred by a single recessive resistance gene. Sequencing of the eukaryotic translation initiation factor 4E genes from the different LMV-resistant *Arabidopsis* accessions showed that these factors are not directly involved in the characterized resistance phenotypes.

The interaction between a plant and a virus may result in a number of biological situations, ranging from complete systemic invasion of the plant, usually accompanied by symptoms (full susceptibility), to the inability of the virus to mount a productive replication in the initially inoculated cells. A number of intermediate situations in which the virus is blocked more or less early in the plant invasion process also can be observed (Hull 2002). Knowledge of the plant and viral factors involved in the outcome of these interactions is crucial to further our understanding of the molecular determinism of key biological processes such as viral host range or plant resistance. Important advances have been made in recent years in the understanding of the molecular biology of the interactions between potyviruses and their hosts (Revers et al. 1999). The genus *Potyvirus* is the largest of the plant virus genera and its members cause severe losses to many crops (Shukla et al. 1994). Potyviruses are aphid transmitted and some of them are also seed borne. Their positive single-stranded RNA genome of approximately 10,000 nucleotides is polyadenylated at its 3′ end and covalently linked at its 5′ end to an approximately 25-kDa virus-encoded protein (VPg). The genome encodes a polyprotein which is matured by three virus-encoded proteinases into approximately 10 mature viral proteins (Riechmann et al. 1992). In several host–potyvirus pathosystems, the VPg has been shown to be involved in overcoming host resistance genes or in the ability to infect specific host plants (Borgstrøm and Johansen 2001; Masuta et al. 1999; Rajamäki and Valkonen 1999, 2002; Revers et al. 1999). This protein also has been shown to interact with the eukaryotic translation initiation factor eIF4E or its isoform eIF(iso)4E (Léonard et al. 2000; Schaad et al. 2000; Wittmann et al. 1997), which recently were shown to play an important role in the potyviral cycle and to be involved in recessive resistances to potyviruses in several hosts (Duprat et al. 2002; Lellis et al. 2002; Ruffel et al. 2002; V. Nicaise and S. German-Retana, *unpublished data*). Together, these results suggest that, in different potyvirus–plant pathosystems, common viral and host factors repeatedly seem to be involved in determining the outcome of the interaction, underlying a conserved infection strategy in the genus *Potyvirus,* and support the hypothesis that plants carrying recessive resistance genes against viruses lack a function essential for a particular step of the viral cycle (Fraser 1992). This conserved strategy may, in turn, explain why recessive resistance genes represent approximately 40% of the known resistance genes to potyviruses (Provvidenti and Hampton 1992), whereas they represent only approximately 20% for other virus groups (Fraser 1992). However, up to now, only the *eIF4E* genes have been identified to have a direct role in the plant–potyvirus interactions.

Among the numerous other resistance genes controlling potyvirus infection which have been characterized from a genetic standpoint, only two dominant resistant genes, *RTM1* and *RTM2,* restricting long-distance *Tobacco etch virus* (TEV) movement, have been cloned in *Arabidopsis thaliana* and shown to encode a jacalin-like protein (Chisholm et al. 2000) and a small heat shock-like protein (Whitham et al. 2000), respectively. These resistant genes, specifically expressed in a sieve element (Chisholm et al. 2001), were shown to be specific for TEV because other potyviruses such as *Potato virus Y* (PVY), *Tobacco vein mottling virus* (TVMV), and *Turnip mosaic virus* (TuMV) are able to infect *Arabidopsis* accessions bearing these two genes (Martín Martín et al. 1999; Whitham et al. 2000). Whether the products of these *RTM* genes act directly or indirectly to block the long-distance movement of TEV still remains to be elucidated.

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In order to identify new viral and host factors involved in potyvirus–host interactions, the high genetic variability of *A. thaliana* (Alonso-Blanco and Koornneef 2000) and the molecular and biological variability of *Lettuce mosaic virus* (LMV) isolates (Krause-Sakate et al. 2002; Revers et al. 1997a) have been exploited to identify *Arabidopsis* or LMV genetic determinants governing the interactions between these two partners. LMV is the causal agent of lettuce mosaic, one of the most devastating viral diseases in lettuce (*Lactuca sativa*) worldwide (Dinant and Lot 1992). Genetic resistance against LMV in lettuce is based on the use of either of two allelic recessive resistance genes, $mo1^1$ and $mo1^2$ (Dinant and Lot 1992), recently shown to correspond to allelic variants of the *eIF4E* gene (V. Nicaise and S. German-Retana, *unpublished data*). The recent emergence of LMV isolates, collectively named LMV-Most (Krause-Sakate et al. 2002), which combine resistance-breaking and seed-transmission properties, has raised the question of the durability of such recessive resistance genes in crops and created the need for the identification of other resistance sources against this virus.

In this study, screening of *Arabidopsis* accessions using three LMV isolates revealed the existence of a large variability in the outcome of LMV–*Arabidopsis* interactions. Viral and plant determinants of these interactions then were analyzed using reverse or forward genetic approaches.

RESULTS

Variability in LMV-AF199 susceptibility identified among *Arabidopsis* accessions.

In order to determine whether *A. thaliana* is a host for LMV, 35 accessions were inoculated manually with LMV-AF199, an isolate belonging to the LMV-Most group (Krause-Sakate et al. 2002). After 3 weeks, no visible symptoms were observed on plants, either on the inoculated leaves or on noninoculated parts such as other rosette leaves or inflorescence tissues. In order to evaluate the possibility of symptomless infections, inflorescence tissues of all inoculated plants were analyzed by enzyme-linked immunosorbent assay (ELISA) using a rabbit antiserum raised against purified LMV (Table 1). Twenty-eight accessions presented high ELISA values (at least 10-fold the value of the mock-inoculated controls) and the presence of LMV in the systemically infected inflorescence tissues was confirmed both by back inoculation to susceptible lettuce plants and by reverse transcriptase-polymerase chain reaction (RT-PCR) amplification of the coat protein region of the LMV genome from total RNA extracts (*data not shown*). In contrast, seven accessions presented ELISA values similar to those of the mock-inoculated controls even when the ELISA was repeated 4 to 6 weeks postinoculation (wpi). Back-inoculation from all the ELISA-negative accessions to susceptible lettuce plants confirmed the absence of systemic LMV accumulation in these plants.

In all accessions showing systemic LMV accumulation, infection by LMV-AF199 was detected at 25 days postinoculation (dpi) and even as early as 15 dpi in all plants of the accessions La-0, La-er, Ler, Ler-0, Pog-0, Mh-0, Shahdara, C24, Ws, Bay-0, Sh-0, Tsu-0, and RLD.

The possibility of seed transmission of LMV in *Arabidopsis* spp. also was examined. Approximately 200 15-day-old seedlings of the RLD, Ler, and Nd-1 accessions produced from seeds obtained from LMV-infected plants were analyzed by ELISA. All the ELISA were negative, demonstrating that LMV either is not seed borne in *Arabidopsis* spp. or is transmitted only at a very low rate, despite the fact that the virus was easily detected in seed pod tissues by ELISA (*data not shown*).

In order to further characterize the resistance phenotype of the seven accessions for which LMV was not detected in upper, noninoculated parts, ELISA were performed on inoculated leaves at 15 dpi (Table 1). Two accessions, Cvi-0 and Cvi-1, seemed to be unable to accumulate LMV in the inoculated leaves because the ELISA values obtained for these plants were similar to those from mock-inoculated leaves. Upon back-inoculation from the inoculated leaves of these two accessions to susceptible lettuce, no symptoms were observed. In contrast, five accessions, Col-0, Col-3, Col-5, Wt-1, and Ll-0, were ELISA positive in inoculated leaves. Therefore, these results suggest that two levels of resistance control LMV-AF199 infection in the *Arabidopsis* accessions tested. The first level of resistance, observed in the two Cvi accessions, seems to inhibit LMV multiplication or cell-to-cell movement and will be referred to as "local resistance", whereas the second level, observed in accessions Wt-1, Ll-0, Col-0, Col-3, and Col-5, corresponds to an inhibition of long-distance viral movement and will be referred to as "systemic resistance".

Variability in susceptibility to other LMV isolates among *Arabidopsis* accessions.

In order to test whether the two levels of resistance observed against LMV-AF199 also were active against other LMV isolates, selected *Arabidopsis* accessions were inoculated manually with LMV-0 and LMV-E, two well-characterized LMV isolates differing in their biological properties in lettuce (Revers et al. 1997a, 1997b), and with LMV-0-GUSclvHC and LMV-E-GUSHC, two GUS-tagged recombinant LMV isolates derived from LMV-0 and from LMV-E infectious cDNA clones, respectively (German-Retana et al. 2000; S. German-Retana, *unpublished data*). Systemic LMV accumulation in inflorescence tissues was determined by ELISA 3 wpi and local LMV accumulation in the inoculated leaves was determined by GUS histochemical staining assays 10 dpi. A global analysis of these results showed that *Arabidopsis* accessions could be classified into seven classes, based on their behavior upon inoculation with the three LMV isolates used (Table 2). Both LMV-0 and LMV-E were able to infect a more restricted set of accessions than LMV-AF199, and all accessions showing resistance against LMV-AF199 also showed resistance to LMV-0 and to LMV-E, with the exception of Col accessions, in which a small percentage of plants repeatedly were observed to systemically accumulate LMV-E (Table 1). On the other hand, LMV-AF199

Table 1. Susceptibility of *Arabidopsis thaliana* to infection by *Lettuce mosaic virus* (LMV)-AF199[a]

Accession or mutant	Local accumulation[b]	Systemic accumulation[c]
La-0, La-er, Ler, Ler-0, Pog-0, Mh-0, Shahdara, WS, Bay-0, Sh-0, Gre-0, C24, Chi-0, RLD, Tsu-0, Kas-1, Oy-0, Kin-0, Jl-1, St-0, In-0, Nd-0, Nd-1, Ge-1, Di-0, Vi-0, Zü-0, Aa-0	+	+
Wt-1, Ll-0, Col-0, Col-3, Col-5, Col-0 npr1-1, Col-0 pad4-1, Col-0 eds4-1, Col-0 ndr1-1, Col-0 etr1-1, Col-0 jar1-1	+	–
Cvi-0, Cvi-1	–	–

[a] + = LMV accumulation and – = no accumulation detected.
[b] LMV accumulation in inoculated leaves as detected 10 days postinoculation by enzyme-linked immunosorbent assay (ELISA).
[c] LMV accumulation in non inoculated inflorescence tissue as detected 3 weeks postinoculation by ELISA.

appears to be able to overcome local or systemic resistance active against LMV-E or LMV-0 in a number of accessions. Among the accessions tested, only Nd-0 and Nd-1 differed clearly in their susceptibility towards LMV-E and LMV-0, in that systemic infection by LMV-E was detected 3 wpi in both accessions, whereas LMV-0 systemic infection was never detected, even up to 6 wpi by ELISA, back-inoculation to susceptible lettuce, or RT-PCR. The patterns of GUS histochemical staining of leaves of these Nd accessions after inoculation with LMV-E-GUSHC and LMV-0-GUSclvHC (Fig. 1) gave similar results, indicating that resistance to LMV-0 in Nd-0 and Nd-1 was not related to a decrease in replication or to slow cell-to-cell movement of this isolate. In the LMV-0- and LMV-E-resistant accessions Di-0, Vi-0, Zü-0, Aa-0, Col-0, Col-3, Col-5, Cvi-0, and Cvi-1, no GUS activity was detected in inoculated leaves with either tagged virus, even upon stereomicroscope examination, which should have allowed the detection of single-cell infection foci resulting from a blockage of cell-to-cell movement (subliminal infection) (Fig. 1). Similarly, no hypersensitive-like lesions were observed at the macroscopic level (*data not shown*).

Analysis of the LMV-GUS inoculated leaves from susceptible accessions revealed other differences, mostly in the number and size of the infection foci. Indeed, some accessions such as Ler presented very few GUS spots or GUS spots of relatively small size, whereas in other accessions, such as Mh-0, GUS spots were consistently both more numerous and larger (Fig. 1). However, systemic infection was detected with a similar timing in all types of accessions (*data not shown*), suggesting that the number and size of infection foci in inoculated leaves does not directly control the kinetics of systemic infection.

The LMV VPg is involved in systemic infection in the Nd accessions.

To further analyze the molecular interactions between *Arabidopsis* spp. and LMV, *Arabidopsis* accessions belonging to some of the classes described above were studied. The LMV molecular determinant responsible for the difference between LMV-E and LMV-0 in the Nd-1 accession first was analyzed by a reverse genetic approach using recombinants constructed from infectious LMV-0 and LMV-E cDNA clones (Redondo et al. 2001; Yang et al. 1998). The pair of recombinants, 0xbaE and Exba0 (Redondo et al. 2001), first was used to show that the 3′ half of the LMV genome was involved in overcoming resistance to systemic invasion (Fig. 2). Because no LMV recombinants were available for this part of the genome, new recombinants were constructed (Fig. 2). Infectivity of these recombinant clones was assayed in susceptible lettuce and the recombinant regions were confirmed by RT-PCR and sequencing. Then, lettuce plants infected with the recombinants were used as source of inoculum to test the ability of these recombinants to systemically infect Nd-1 plants. For each recombinant, ELISA were performed 3 wpi using inflorescence tissues from three inoculated Nd-1 plants tested individually. Each experiment was repeated at least twice. ELISA-positive plants were analyzed by RT-PCR coupled with restriction fragment length polymorphism (RFLP) analysis of the amplification products to confirm the identity of the recombinants (*data not shown*). Only 0stafE, a LMV-0 clone carrying the LMV-E VPg, was able to overcome the Nd-1 resistance to LMV-0, whereas the symmetrical recombinant, Estaf0, and the other recombinants were not detected systemically in Nd-1.

Similarly, an LMV-0 recombinant in which the VPg coding region was replaced by that from LMV-AF199 also was able to overcome the blockage in systemic movement observed in accession Nd-1 (*data not shown*), demonstrating that, for both LMV-E and LMV-AF199, the resistance-breaking determinant is localized in the viral VPg.

Amino acid sequence alignment of the VPg of the three LMV isolates (GenBank accession numbers X97704 for LMV-0, X97705 for LMV-E, and AJ278854 for LMV-AF199) showed only three positions (2,121, 2,169, and 2,177) at which the LMV-0 sequence simultaneously differed from those of both LMV-E and LMV-AF199 (Fig. 3).

Local resistance to LMV-0 in the Columbia accession is controlled by *LLM1*, a dominant resistance gene linked to the genetic marker nga280 on chromosome I.

The Columbia accessions showed a local resistance against LMV-0 (Table 2). A number of resources for genetic mapping are available for this accession; therefore, the genetic analysis of this resistance was undertaken, using an F2 population and F9 recombinant inbred (RI) lines produced from a cross between Col-5 and Nd-1 (Holub and Beynon 1997) available from the Nottingham Arabidopsis Stock Center (NASC).

To characterize the genetic basis of the LMV-0 local resistance in Columbia, 96 plants of the F2 population were inoculated with LMV-0-GUSclvHC and the inoculated leaves were analyzed 10 dpi by a GUS histochemical assay. Similarly, 71 F2 plants were inoculated with LMV-0 and the inoculated leaves were tested by ELISA at 15 dpi. In both experiments, LMV-0 resistance segregated in a manner consistent with the

Table 2. Comparison of susceptibility of *Arabidopsis* accessions and mutants to infection by various *Lettuce mosaic virus* (LMV) isolates

	LMV-AF199		LMV-E		LMV-0	
***Arabidopsis* accession**	**Local[a]**	**Systemic[b]**	**Local[c]**	**Systemic[b]**	**Local[c]**	**Systemic[b]**
La-0, La-er, Ler, Ler-0, Pog-0, Mh-0, Shadara	+	+	+	+	+	+
Nd-0, Nd-1	+	+	+	+	+	–
Ge-1	+	+	+	–	+	–
Aa-0	+	+	–	–	–	–
Wt-1	+	–	+	–	+	–
Col-0, Col-3, Col-5	+	–	±	±	–	–
Col-0 mutants[d]	+	–	nt	nt	–	–
Cvi-0, Cvi-1	–	–	–	–	–	–

[a] LMV accumulation in inoculated leaves as detected 10 days postinoculation by enzyme-linked immunosorbent assay (ELISA); + = LMV accumulation and – = any LMV accumulation detected.

[b] LMV accumulation in noninoculated inflorescence tissue as detected 3 weeks postinoculation by ELISA; + = LMV accumulation; – = no LMV accumulation detected; ± = indicates a situation in which viral accumulation was detected but only in a small fraction of inoculated plants; nt : non tested.

[c] LMV accumulation in inoculated leaves by GUS assay following inoculation with a GUS-tagged recombinant virus; + = LMV-GUS accumulation; – = no LMV-GUS accumulation detected; ± = indicates a situation in which viral accumulation was detected but only in a small fraction of inoculated plants; nt = not tested.

[d] The Col-0 mutants are *npr1-1*, *pad4-1*, *eds4-1*, *ndr1-1*, *etr -1*, *jar1-1*.

presence of a single dominant gene (71 resistant for 96 plants with LMV-0-GUSclvHC, $\chi^2 = 0.555$ for a 1:3 segregation; 56 resistant for 71 tested plants with LMV-0, $\chi^2 = 0.568$). This resistance locus was named *LLM1* (local resistance to LMV).

In order to map *LLM1*, plants from the RI population were inoculated with both LMV-0-GUSclvHC and LMV-0 and tested in the same way as the F2 population. Of the 89 RI lines tested, 45 were resistant and 44 were susceptible. These data correspond to a ratio of the number of resistant versus susceptible lines of 1.02, which, again, is in agreement with a single gene model as suggested by the F2 population analysis. Using the molecular markers released on the NASC website and additional markers from J. Beynon (*unpublished*), a recombination analysis revealed that marker nga280 located on chromosome I was linked to *LLM1* with a recombination rate of 12.5% between these two loci (Table 3).

To analyze the mechanism of this LMV-0 resistance, Col-0 mutants with altered defense responses in systemic acquired resistance (SAR) or *R* gene-mediated signaling defense pathways (Dangl and Jones 2001; Glazebrook 2001) were tested for their resistance to LMV-0 (Table 2). These included Col-0 plants with a mutation in the NPR1 gene (*npr1-1*), which are unable to activate SAR and present enhanced disease susceptibility (Glazebrook et al. 1996), and Col-0 plants with mutations in genes belonging to the two known signaling pathways downstream of nucleotide binding site-leucine rich repeat (NBS-LRR) resistance genes NDR1 (*ndr1-1),* PAD4 (*pad4-1*), and EDS1 (*eds4-1*) (Glazebrook et al. 1996). In addition, Col-0 jasmonate-resistant *jar1-1* plants (Staswick et al. 1992) and Col-0 ethylene-insensitive *etr1-1* plants (Bleecker et al. 1988) also were tested. Four plants of each mutant were inoculated with LMV-0 and LMV-0-GUSclvHC. GUS activity and ELISA were performed at 10 and 15 dpi for LMV-0-GUSclvHC- and LMV-0-inoculated leaves, respectively. Local resistance to LMV-0 was not compromised in any of these mutant plant lines; whereas, as a positive control in these experiments, the Ler plants inoculated in parallel all were infected successfully. These results support the hypothesis that the mechanism of local LMV-0 resistance in the Columbia accession differs from those controlled by the well-characterized defense pathways of *Arabidopsis* spp.

To further evaluate the stability of the local LMV-0 resistance in Columbia, Col-5 plants previously infected with *Cucumber mosaic virus* (CMV) R strain, a virus belonging to the genus *Cucumovirus* and able to suppress posttranscriptional gene silencing (PTGS) (Lucy et al. 2000), were inoculated with LMV-0. As a control, CMV-infected plants of the Ler and Nd-1 accessions also were inoculated with LMV-0. At 15 dpi, no systemic LMV-0 infection was detected in the CMV-infected Col-5 plants, whereas LMV was detected in CMV-infected Ler and Nd-1 plants (*data not shown*). Thus, CMV infection did not suppress resistance to LMV in the Columbia accession. Identical results were obtained when plants were inoculated simultaneously with LMV and CMV on the same or on different leaves (*data not shown*). To determine whether the block in local LMV-0 accumulation results from the induction of a general antiviral response, LMV-infected Col-5 and Ler plants were inoculated with CMV 15 days after LMV inoculation, and CMV accumulation was evaluated by ELISA 10 days later. All plants were found to accumulate CMV, indicating that LMV resistance does not affect the susceptibility of the Columbia accession to CMV.

Given the LMV-0 infection phenotypes observed in the Col (local infection blockage) and Nd (systemic infection blockage) parents, the possibility existed that fully susceptible plants could be recovered in the F2 or RI lines populations. In all, 10 F2 plants and 10 RI lines, for which LMV-0 was detected by ELISA in inoculated leaves, were analyzed 3 wpi for systemic LMV-0 accumulation in inflorescence tissues. None of these plants were found to be systemically infected by LMV-0.

Preliminary genetic analysis of the LMV-AF199 systemic resistance in the Columbia accession and of the LMV local resistance in the Cvi accession.

The Columbia accession showed a systemic resistance to LMV-AF199, whereas the Cvi accession showed a local resistance to this isolate as well as to the other LMV isolates tested. As a preliminary genetic analysis, two F2 populations produced from these accessions were analyzed. From the F2 population (Col-5 × Nd-1) described above, 308 plants were inoculated with LMV-AF199 and inflorescence tissue was analyzed by ELISA 3 wpi. Of these, 23 plants (7.5%) showed systemic accumulation of LMV-AF199, whereas the remaining 285 plants (92.5%) were found resistant. These data are consistent with a segregation ratio expected for two nonlinked dominant loci independently conferring resistance ($\chi^2 = 0.76$ for a 15:1 segregation). In addition, this systemic resistance was evaluated in the Col-0 mutant lines described above but was not affected in any of these lines (Table 1).

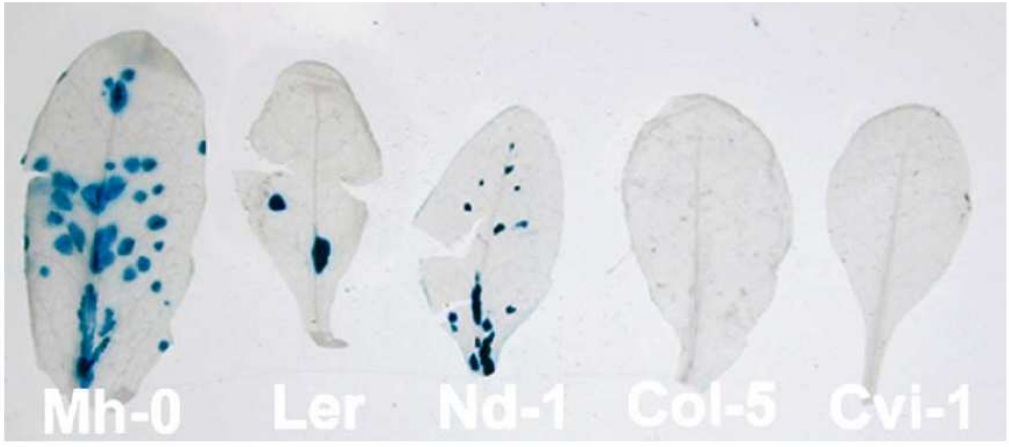

Fig. 1. *Lettuce mosaic virus*-0-GUSclvHC multiplication in inoculated leaves of *Arabidopsis* accessions. GUS assays were performed 8 days postinoculation. Accession names are indicated under each leaf.

Plants of an F2 population produced by a cross between Cvi-1 and Ler, an LMV-susceptible accession, were inoculated with LMV-AF199 and analyzed by ELISA at 3 wpi. Of 194 plants tested, 131 were susceptible (67.5%), suggesting that, in Cvi, the local resistance is controlled by a single recessive locus ($\chi 2$ = 5.78 for a 3:1 ratio). Mixed infections with CMV produced and analyzed as described above showed that the systemic resistance in Columbia and the local resistance in Cvi are not suppressed by a heterologous virus able to suppress PTGS, and that LMV-AF199 inoculations in Columbia and Cvi do not trigger a general antiviral defense effective against CMV (results not shown).

Eukaryotic translation initiation factors eIF4E and eIF(iso)4E of *Arabidopsis* spp. are not directly involved in the resistance mechanisms against LMV identified in the Nd, Col, and Cvi accessions.

Several recent studies have shown that the eukaryotic translation initiation factors eIF4E and eIF(iso)4E interact with the VPg of potyviruses (Léonard et al. 2000; Schaad et al. 2000; Wittmann et al. 1997) and have implicated these genes in recessive resistance mechanisms active against potyviruses in *Arabidopsis* spp., pepper, and lettuce (Duprat et al. 2002; Lellis et al. 2002; Ruffel et al. 2002; V. Nicaise and S. German-Retana, *unpublished data*). In order to investigate the possibility that the *eIF4E* genes are involved in the LMV resistance in Nd-1, in which the VPg is a resistance-breaking determinant or in the LMV recessive resistance in Cvi-1, the Nd-1 and Cvi-1 *eIF4E* and *eIF(iso)4E* gene sequences were determined and aligned with those of other *Arabidopsis* accessions (GenBank accessions NM_117914 [Columbia *eIF4E*], AY086496 [WS/Ler *eIF4E*], Y10548 [Ler *eIF4E*], NM_122953 [Columbia *eIF(iso)4E*], AY056630 [WS/Ler *eIF(iso)4E*], and Y10547 [Ler *eIF(iso)4E*]). Alignment of all these sequences showed 100% nucleotide sequence identity for each one of these genes. This absence of allelic variation suggests that the *eIF4E* and *eIF(iso)4E* genes from the Nd-1, Cvi-1, and Col-0 accessions are not the basis for the various forms of LMV resistance identified in this study.

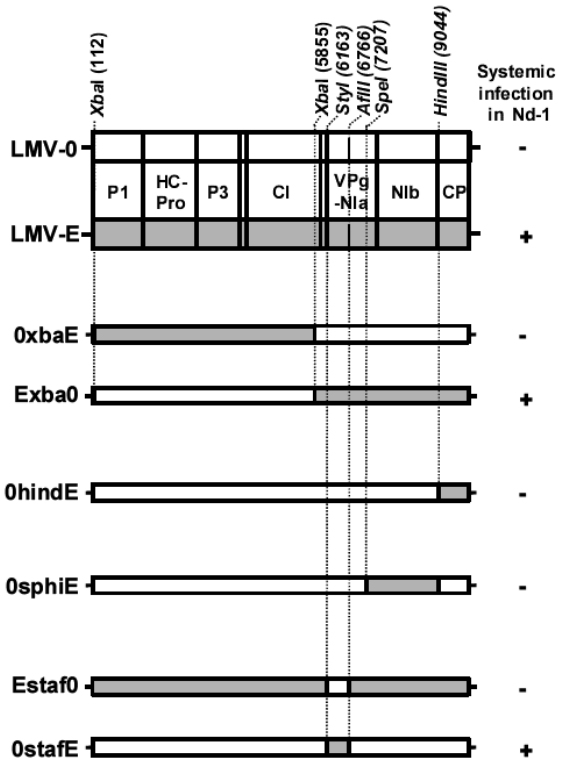

Fig. 2. Schematic representation of the genome of *Lettuce mosaic virus* (LMV) and of the LMV recombinants constructed between LMV-0 and LMV-E. Coding regions are indicated in white and gray boxes for LMV-0 and LMV-E, respectively. The positions of cleavage sites between the viral proteins are indicated by vertical lines. The name of each protein is indicated between the two LMV genomes. Restriction sites used to construct the recombinants are indicated at the top, with their position along the LMV genome. The ability of each recombinant to infect systemically accession Nd-1 is indicated on the right; + indicates a positive systemic infection and – indicates no systemic infection detected.

DISCUSSION

In this study, the high natural genetic variability of *A. thaliana* was exploited to establish an LMV–*Arabidopsis* pathosystem. Indeed, the screening of *Arabidopsis* accessions with three well-characterized LMV isolates, LMV-AF199, LMV-E, and LMV-0, revealed variability in the susceptibility to these isolates of the accessions tested. Approximately 80% of the accessions tested with LMV-AF199 were found to be fully susceptible; however, in all cases, the resulting systemic infections were found to be symptomless and to develop more slowly than infection in susceptible lettuce cultivars. Symptomless and slower-infection phenotypes also were observed in *Arabidopsis* spp. with TEV, another member of genus *Potyvirus* (Mahajan et al. 1998). In order to explain these differences in symptom expression and infection timing, several hypotheses can be proposed. Because viruses need a panel of host factors for the different steps of their cycle in plants, one hypothesis is that the affinity between hosts and viral factors provides a basis for genetic variation in the timing and extent of viral infection. A second hypothesis is that one or some active defense responses are overcome less efficiently by the virus in different plant species, resulting in a delayed systemic infection. With regard to this second hypothesis, RNA silencing has been shown to be an antiviral defense system in higher plants against which various viruses, including potyviruses, have developed viral-encoded suppressors (Voinnet 2001). In the case of symptomless LMV and TEV infections in *Arabidopsis* spp., suppression of RNA silencing may be less efficient, resulting in the delayed and symptomless infection phenotype. This hypothesis, however, is weakened by the mixed LMV–CMV infections reported here, in which no beneficial effect of the presence of CMV could be noticed despite the known ability of CMV to suppress the RNA silencing defense pathway (Lucy et al. 2000).

Among the resistant accessions, at least two different resistance phenotypes were identified. The first phenotype (local resistance) was characterized by an absence of detectable viral accumulation in inoculated leaves and the second (systemic resistance) by a failure of LMV to accumulate in noninoculated tissues, likely to result from a blockage in long-distance viral movement.

By a reverse genetic approach, the LMV VPg was identified as the viral determinant involved in overcoming the blockage in long-distance movement of LMV-0 in accession Nd-1. Among the three amino acid positions which differ between LMV-0 and the two others LMV isolates, LMV-E and LMV-AF199, position 2,121 (Serine in LMV-0, Glycine in LMV-E and LMV-AF199) is located in the central region of VPg, in which several mutations associated with the ability to over-

come recessive resistances have been identified for several potyviruses, such as TEV (Schaad et al. 1997), TVMV (Nicolas et al. 1997), *Pea seed-borne mosaic virus* (Keller et al. 1998), PVY (Masuta et al. 1999), and *Potato virus A* (PVA) (Rajamäki and Valkonen 1999, 2002). As in the observations reported here, no effect on the cell-to-cell movement efficiency accompanied the systemic blockage of TEV in tobacco (Schaad et al. 1997) or of PVA in *Nicandra physaloides* (Rajamäki and Valkonen 1999) and in *Solanum commersonii* (Rajamäki and Valkonen 2002). However, the precise function of the VPg in these resistance mechanisms remains to be determined, as well as the identity of the host factor or factors involved. In the case of *N. tabacum*, segregation analysis in an F2 population indicated involvement of two nonlinked recessive genes in the TEV resistance which have not yet been cloned (Schaad and Carrington 1996). In the Nd-1 accession, the genetic basis for LMV-0 resistance has not yet been established, although the absence of systemic accumulation of LMV-0 in Col-5 × Nd-1 F2 and RI line plants suggests that this resistance could be a complex trait controlled by at least two genes. Sequence determination of the *eIF4E* and *eIF(iso)4E* genes and comparison with other *Arabidopsis* accessions suggested that these host genes are not involved in this resistance to LMV-0.

With the aim to identify the genetic bases and the resistance genes which control LMV infection in the various *Arabidopsis* accessions, genetic analyses of the local and systemic resistance phenotypes observed were performed. For the local resistance phenotype observed in Columbia with LMV-0, the genetic analysis revealed the involvement of one locus designated *LLM1*. The LMV-0 resistance locus *LLM1*, identified in Col-5, was mapped to chromosome I using Col-5 × Nd-1 RI lines, and was found to be linked to marker nga280. In the region between markers GAPB and genea, which flank nga280, several putative disease resistance genes were identified. Because of the dominant nature of *LLM1* and of the local resistance phenotype it confers, it is tempting to speculate that *LLM1* corresponds to one of the NBS-LRR genes (Dangl and Jones 2001) belonging to the *R* gene cluster identified in this region of chromosome I. This hypothesis is strengthened by two observations: i) that this cluster contains *RPP7* as well as homologues of *RRP8* (locus AT1G53350) and *RPP13* (locus AT1g59218), three resistance genes whose function is known to be independent of the well-known *Arabidopsis* resistance pathways controlled by *PAD4, NDR1,* and *EDS1* (Glazebrook 2001); and ii) that the function of *LLM1* was shown here to be independent of these genes as well as of a number of other genes (*NPR1*) or mediators (salycilate, ethylene, and jasmonate) involved in *Arabidopsis* resistance mechanisms. However, unambiguous identification of *LLM1* as a classical *R* gene acting through the *RPP7* signaling pathway clearly will require its identification through positional cloning or through a candidate gene approach.

Concerning the local resistance to all LMV isolates tested in Cvi, the F2 population analysis suggests that a single recessive locus is involved in the resistance phenotype. In addition, sequence comparisons of both *eIF4E* genes in several *Arabidopsis* accessions, including Cvi, revealed a complete identity between all alleles, therefore excluding the role of these initiation factors in the resistance to LMV in Cvi. These results suggest that the susceptibility allele at this locus is either another host factor required for the successful completion for the potyvirus cycle or a negative regulator of resistance. Alternatively, it cannot be ruled out at this point that transcriptional, translational, or posttranslational defects of one or the other of the eIF4E isoforms could be involved in LMV resistance. Analysis of RI lines produced from a cross between the LMV-susceptible accessions Ler and Cvi (Alonso-Blanco et al. 1998) should confirm the involvement of such a recessive resistance gene and facilitate its mapping and cloning.

The genetic basis of the systemic resistance to LMV-AF199 in Columbia seems more complex because a two-gene model is proposed. This resistance does not involve a macroscopically visible hypersensitive response or a known resistance pathway, and is not affected by CMV infection, an unrelated virus which is able to suppress RNA silencing (Lucy et al. 2000). This resistance system appears to be similar to the one which inhibits long-distance movement of TEV in Col-3 (Mahajan et al. 1998, Whitham et al. 2000). In this accession, at least two dominant genes, *RTM1,* which encodes a jacalin-like protein (Chisholm et al. 2000), and *RTM2,* which encodes a small, heat shock-like protein (Whitham et al. 2000), are involved in TEV resistance. *RTM1* was mapped near the top of chromosome I (Mahajan et al. 1998) and *RTM2* near the top of chromosome V (Whitham et al. 1999). A more detailed genetic analysis of the systemic resistance to LMV-AF199 in Columbia should clarify whether the *RTM* loci are involved in this resistance.

To conclude, the data reported here support the idea that screening of *Arabidopsis* accessions with different potyviruses can reveal different sets of genes for which co-evolution between each virus and *Arabidopsis* accessions has led to the establishment of a virus-specific infection phenotype. Indeed, LMV interactions with *Arabidopsis* accessions are different from those of two other well-studied potyviruses, TEV (Mahajan et al. 1998) and TuMV (Martín Martín et al. 1999), in this plant species. Thus, genetic and molecular analysis of such pathosystems, coupled with the wealth of genetic and genomic resources now available for this plant, provide a very powerful means to identify viral and host factors governing potyvirus–host interactions.

```
LMV-0      GKGKRQRQKLRYRQARDNKVGIEVYGDDA
LMV-E      --------------------M---------
LMV-AF199  ----------------------------

LMV-0      TMEHYFGAAYTEKGKKSGKTKGMGTKNRR
LMV-E      ----------------------------
LMV-AF199  ----------------------------K

LMV-0      FVNMYGYNPEDFSFIRFLDPLTGKTMDEQ
LMV-E      ----------------------------
LMV-AF199  ----------------------------

LMV-0      VFSDISLVQDAFSKERLKLLSEGEIESEH
LMV-E      ----------G----------------
LMV-AF199  -----G------G----------------

LMV-0      MRNGIRAYLVKNLTTAALEIDMTPHNSCQ
LMV-E      ----------------------------
LMV-AF199  --S-------------------------

LMV-0      LGAKTNNIAGYVDREYELRQTGEARVVAP
LMV-E      --T-------F------------------
LMV-AF199  --I-------F------------------

LMV-0      ALIPKDNPITDEDIPVKHE
LMV-E      -------------------
LMV-AF199  -------------------
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Fig. 3. Amino acid sequence alignment of the genome-linked protein of isolates *Lettuce mosaic virus* (LMV)-0, LMV-E, and LMV-AF199. Bold and italicized amino acids are positions at which the sequences of both LMV-E and LMV-AF199 differ from that of LMV-0. Hyphens indicate identical amino acids.

MATERIALS AND METHODS

Plant materials, virus, and inoculation.

Seed of all *Arabidopsis* accessions, RI lines, and mutants were obtained from NASC, except Bay-0 and Shahdara (gift of O. Loudet, INRA, Versailles, France), RLD (gift of C. Robaglia, CEA, Cadarache, France), the *ndr1-1* mutant (gift of B. Staskawicz, University of California, Davis, U.S.A.), the F2 Col-5 × Nd-1 seed (gift of Y. Marco, INRA, Toulouse, France), and the F1 Cvi × Ler seed (gift of M. Koornneef, Wageningen University, The Netherlands). The lettuce cv. Trocadéro, turnip (*Brassica rapa*), and *Nicotiana tabacum* cv. Xanthi were used to propagate LMV isolates, TuMV, and CMV, respectively. All plants were grown under greenhouse conditions (16-h day length, 18 to 25°C) and maintained in insect-proof cages after inoculation. Mechanical inoculation of 4- to 5-week-old *Arabidopsis* plants was performed as previously described (Revers et al. 1997a). Plants were re-inoculated on different leaves 3 to 5 days later to ensure infection. The LMV isolates LMV-0, LMV-E, and LMV-AF199 used in this study have been described by Revers and associates (1997a and b) and Krause-Sakate and associates (2002).

ELISA, RT-PCR, and GUS assay.

ELISA was performed as described by Revers and associates (1997a). An ELISA result was considered as positive when its value was at least three times the healthy controls value. RT-PCR was performed using the primers NIb and P4 as described by Revers and associates (1999), from semipurified total RNA preparations (Bertheau et al. 1998).

Histochemical GUS staining was performed as described by German and associates (2000).

Construction of LMV recombinants.

Construction of the two recombinants 0xbaE and Exba0 has been described by Redondo and associates (2001). For the recombinants 0hindE, 0sphiE, Estaf0, and 0stafE, restriction sites (Fig. 2) were used to exchange the different regions between full-length infectious clones of LMV-0 and LMV-E. Particle bombardment was used to infect lettuce seedlings with these full-length cDNA constructs, as described by Redondo and associates (2001). Infected lettuce plants were further used as the source of inoculum for the inoculation of *Arabidopsis* plants. Identity of each recombinant was checked by RT-PCR (Revers et al. 1997a), using semipurified total RNA preparations as described above and primers chosen to flank the junction regions of the exchanged fragments. The identity of the recombinants finally was determined by RFLP analysis or sequencing of these amplified fragments.

Cloning of the eIF4E and eIF(iso)4E cDNA from the Cvi and Nd-1 accessions.

Total RNAs were extracted from 100 to 200 mg of rosette leaf tissues using TRI Reagent (Sigma-Aldrich, St Louis). Total cDNAs were synthesized from 5 µg of total RNAs using 15 units of AMV Reverse Transcriptase (Amersham Biosciences, Uppsala, Sweden) and 1 µM oligo-dT in 50 µl, incubated for 1 h

Table 3. Percent recombination between molecular markers and *Lettuce mosaic virus* (LMV) resistance genes in Col-5 x Nd-1 recombinant inbred lines

Chromosome, marker	Position (cM)[a]	Col-5/Nd-1[b]	Ratio[c]	Recombinant lines/total lines for *LLM1*[d]	Recombination (%) for *LLM1*[e]
I					
nga59	2.90	42/50	0.84	36/85	36.7
nga63	11.48	41/52	0.79	39/86	41.5
SO392	46.71	51/42	1.21	36/86	36.0
gapB	61.21	49/32	1.53	23/75	22.1
nga280	83.83	36/50	0.72	16/80	12.5
genea	88.90	30/57	0.53	24/81	21.12
adh	117.52	25/66	0.38	40/84	45.5
II					
m246	11.03	30/48	0.63	40/72	62.5
PhyB	34.45	37/54	0.69	40/85	44.4
gpa1	48.9	32/32	1.00	35/58	76.1
nga361	63.02	39/55	0.71	38/87	38.8
T9J23	92.0	42/49	0.86	35/84	35.7
III					
nga126	16.35	22/72	0.31	45/87	53.6
nga162	20.56	39/55	0.71	43/87	48.9
MMJ24	48.45	51/42	1.21	49/87	64.5
nga707	78.25	53/38	1.39	45/84	57.7
nga112	87.88	37/28	1.32	33/60	61.1
IV					
T18A10	1.0	56/35	1.60	47/84	63.5
nga8	26.56	59/34	1.73	45/86	54.9
g3883	64.15	41/34	1.20	33/69	45.8
nga1107	104.73	43/50	0.86	37/86	37.8
V					
nga225	14.31	42/33	1.27	36/71	51.4
CA72	29.6	49/42	1.17	44/85	53.7
nga139	50.48	43/45	0.96	42/82	52.5
nga76	68.4	43/39	1.10	41/77	56.9
mi61	98.19	31/49	0.63	45/75	75.0
MUD21	131.20	60/31	1.94	37/84	39.4

[a] Position obtained from the Nottingham Arabidopsis Stock Centre website.
[b] Each fraction represents, for a given marker, the number of lines showing the Col-5 phenotype versus the number of lines showing the Nd-1 phenotype.
[c] Calculated ratios of the fractions from the previous column.
[d] Number of lines for which a recombination event was detected between a given marker and the LMV-0 resistance gene *LLM1*.
[e] Percent recombination calculated using the equation $r = R/2(1 - R)$ (Haldane and Waddington 1931), where R is the calculated ratio from the previous column.

at 42°C. PCR amplification was performed using 5 µl of total cDNAs in 50-µl reactions containing 0.5 units of Extra-Pol I Taq ADN polymerase (Eurobio, Les Ulis, France), using 1 µM of oligonucleotide primers. The primers 5′AGAAAGAGAAGCAGTTCGGAGAAACAATG (the nucleotides underlined show the first codon of the coding region) and 5′AATGCAAAGATTTGAGAGGTTTCA (the nucleotides underlined show the stop codon) were used to amplify the complete coding region of eIF4E, and the primers 5′CAGAAGAAAACTCAACTGCGAAGAATATG and 5′TCTGGCTTCACACTCGTTTCTTCA were used to amplify the complete coding region of eIF(iso)4E. The cycling conditions were 20 s of denaturation at 92°C, 30 s of annealing at 54°C, and 1 min of elongation at 72°C after an initial denaturation at 95°C for 2 min using an iCycler thermal cycler (Bio-Rad Laboratories, Hercules, CA, U.S.A.). The pGEM-T Easy vector system (Promega Corp., Madison, WI, U.S.A.) was used to clone PCR products. Automated DNA sequencing of at least three clones of each construct or of noncloned PCR products was performed at Qbiogene (Evry, France).

The nucleotide sequences were compared with other *A. thaliana eIF4E* and *eIF(iso)4E* retrieved in GenBank using the program Blast (Altschul et al. 1990) and aligned using ClustalW (Thompson et al. 1994), which generates and uses a distance dendrogram (Saitou and Nei 1987) to construct multiple sequence alignments.

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AUTHOR-RECOMMENDED INTERNET RESOURCES

Nottingham Arabidopsis Stock Centre (NASC) website: nasc.nott.ac.uk
The Arabidopsis Information Resource (TAIR): www.arabidopsis.org/home.html
University of California-Davis Arabidopsis resistance genes webpage: niblrrs.ucdavis.edu/At_Rgenes

Cette première phase de mon projet de recherche sur la caractérisation des interactions Arabidopsis/LMV a aussi permis d'introduire cette plante modèle dans les études menées au laboratoire et est devenue l'espèce de choix dans de nombreux projets de notre équipe.

Cette nouvelle expertise que j'ai développée au laboratoire concernant l'utilisation d'Arabidopsis en virologie végétale, m'a aussi permis de développer des collaborations avec plusieurs équipes qui voulaient notamment tester des mutants d'Arabidopsis vis à vis de virus de genre différent. Deux de ces collaborations se sont d'ailleurs traduites par des publications (**Publications [10] et [13]**) avec l'équipe d'Yves Marco (INRA Toulouse) d'une part, et les équipes de Christophe Robaglia (Université Aix-Marseille/CEA Cadarache) et Carole Caranta (GAP, INRA Avignon) d'autre part pour montrer dans cette dernière étude qu'un mutant KO d'Arabidopsis du gène codant pour eIFiso4E était résistant à plusieurs potyvirus dont le LMV mais restait sensible comme le sauvage à d'autres virus appartenant à d'autres genres viraux, démontrant le rôle clef de ce facteur dans le cycle des potyvirus.

Mais surtout mon expérience réussie avec le pathosystème Arabidopsis/LMV m'a conduit à m'impliquer dans le développement d'un autre pathosystème Arabidopsis/potyvirus concernant le virus de la Sharka (*Plum pox virus* ou PPV) en collaboration avec V. Decroocq, qui étudiait jusqu'alors les interactions entre PPV et des espèces végétales du genre *Prunus*.

2.2.2. Développement du pathosystème PPV/*Arabidopsis thaliana*

Le criblage d'un petit panel d'accessions d'Arabidopsis avec le PPV a révélé une diversité de réponse de la plante encore plus riche qu'avec le LMV, avec notamment la description de phénotypes de sensibilité au PPV induisant des symptômes, ce qui n'avait pas été observé avec le LMV. Des différences entre isolats de PPV ont aussi été mises en évidence. Même si des phénotypes de résistance similaires à ceux obtenus avec le LMV ont été observés avec le PPV, la plupart l'étaient sur des accessions différentes. Cependant, nous avons pu aussi identifier des résistances aux deux virus sur les mêmes accessions comme par exemple sur Col. Nous pouvions alors nous demander si ces résistances observées chez une même accession étaient contrôlées ou non par le(s) même(s) gène(s) de résistance.

Dans le cas de Col, une résistance à un autre potyvirus, le *tobacco etch virus* (TEV), présentant le même phénotype de résistance que celui observé pour le LMV (LMV-AF199) et le PPV (PPV-EA et PS), avait précédemment été caractérisé par l'équipe de J. Carrington. Il s'agit d'une résistance dominante contrôlée par au moins 3 gènes, nommés *RTM1*, *RTM2* et *RTM3*. Nous avons alors testé les lignées mutantes de ces 3 gènes, sensibles au TEV, avec les isolats de LMV et PPV incapables d'infecter Col en systémie, et montré qu'elles étaient aussi sensibles à nos 2 virus. Cela démontrait que la résistance observée chez Col vis-à-vis du LMV et du PPV, induisant le blocage du mouvement à longue distance de ces virus, était aussi contrôlée par les gènes RTM. Cette découverte nous a poussé à nous investir davantage sur cette résistance que j'évoquerais plus loin.

L'ensemble des données obtenues avec le PPV ainsi que la mise en évidence de l'implication des gènes RTM dans la résistance observée chez Col vis-à-vis du LMV et PPV ont fait l'objet de la **publication [14]** présentée ci-dessous.

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e-Xtra*

Multiple Resistance Traits Control *Plum pox virus* Infection in *Arabidopsis thaliana*

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Twelve *Arabidopsis* accessions were challenged with *Plum pox potyvirus* (PPV) isolates representative of the four PPV strains. Each accession supported local and systemic infection by at least some of the PPV isolates, but high variability was observed in the behavior of the five PPV isolates or the 12 *Arabidopsis* accessions. Resistance to local infection or long-distance movement occurred in about 40% of all the accession-isolate combinations analyzed. Except for Nd-1, all accessions showed resistance to local infection by PPV-SoC; in the Landsberg *erecta* (L*er*) accession, this resistance was compromised by *sgt1* and *rar1* mutations, suggesting that it could be controlled by an *R* gene–mediated resistance pathway. While most of the susceptible accessions were symptomless, PPV induced severe symptoms on inflorescences in C24, L*er*, and Bay-0 as early as 15 days after inoculation. Genetic analyses indicated that these interaction phenotypes are controlled by different genetic systems. The restriction of long-distance movement of PPV-El Amar and of another member of genus *Potyvirus*, *Lettuce mosaic virus*, in Col-0 requires the *RTM* genes, indicating for the first time that the RTM system may provide a broad range, potyvirus-specific protection against systemic infection. The restriction to PPV-PS long-distance movement in Cvi-1 is controlled by a single recessive gene, designated *rpv1*, which was mapped to chromosome 1. The nuclear inclusion polymerase b–capsid protein region of the viral genome appears to be responsible for the ability of PPV-R to overcome *rpv1*-mediated resistance.

Additional keyword: Sharka.

Sharka disease is the major limiting factor in stone fruit tree production in Europe and North America. It is caused by *Plum pox potyvirus* (PPV) and is one of the most serious viral diseases in peach, apricot, and plum orchards. Losses from Sharka are due primarily to precocious fruit drop and decreases in fruit quality. The potential of PPV to severely affect the fruit tree industry has prompted the European Union to classify it as a quarantine pathogen (European Union council directive 2000/29/EEC, annex II), while the United States federal government has classified it as one of the top ten significant threats to U.S. agriculture (Public Health Security and Bioterrorism Act of 2002, PPV belongs to the family *Potyviridae*. Apart from the atypical El Amar (EA) isolate (Wetzel et al. 1991a) and the sweet and sour cherry–infecting isolates (Crescenzi et al. 1997; Nemchinov and Hadidi 1996), most PPV isolates are classified into two major strains, M (from the isolate Marcus) and D (from the isolate Dideron) (Candresse et al. 1998). Recently, a new atypical strain, W3174, was discovered, but its origin is still unclear (James and Varga 2005).

Studies of the molecular mechanisms underlying the interactions of PPV with its woody hosts have been hampered by the difficulties inherent to the molecular genetic analysis of stone-fruit tree species, in particular the extended generation times and the length and space requirements of phenotypic tests. Four years of monitoring after inoculation are needed to assess the level of resistance or susceptibility of a given *Prunus* cultivar. Standardization of the resistance tests has similarly proved difficult because of delayed responses to inoculation, variability of the virus, physiological state of the host plant, and inoculation method.

In the past few years, the genetic and molecular advantages of model plant *Arabidopsis thaliana* were exploited for the identification of host factors contributing to virus infection. For example, two genes, *Tobamovirus multiplication 1* and *Tobamovirus multiplication 3* (*Tom1, Tom3*), were identified from a screen for *A. thaliana* mutants that show defective infection by *Tobacco mosaic virus* (TMV) (Yamanaka et al. 2000, 2002). Both *Tom1* and *Tom3* are putative transmembrane proteins that may serve as membrane anchors for the TMV replication complex. Similarly, movement-defective mutants and the corresponding RTM loci, which cooperate to restrict *Tobacco etch virus* (TEV) long-distance movement, were described (Mahajan et al. 1998; Whitham et al. 1999). *RTM1* encodes a jacalin-like protein, *RTM2* shows homology to the plant small heat-shock proteins with a predicted transmembrane domain (Chisholm et al. 2000; Whitham et al. 2000), and an additional RTM loci has been identified but not yet fully characterized. The mechanism whereby the three RTM loci cooperate to restrict long-distance movement is still unclear.

Through studies of the interaction of eukaryotic translation initiation factors with viral proteins and genetic and reverse-genetic analysis, eIF4E and eIF(iso)4E were linked to the potyvirus infectious cycle in various plant species (Duprat et al. 2002; Gao et al. 2004; Kang et al. 2005; Lellis et al. 2002; Nicaise et al. 2003; Ruffel et al. 2002; Stein et al. 2005). Our own analyses demonstrated a cosegregation of the *Prunus eIF(iso)4E* ortholog with a major quantitative trait locus (QTL) of resistance to sharka in peach and apricot (Decroocq et al. 2005). To identify new host factors involved in plant-potyvirus interactions, in particular with a potyvirus infecting perennial

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*The *e*-Xtra logo stands for "electronic extra" and indicates the HTML abstract available on-line contains supplemental material not included in the print edition. A table lists the twelve *Arabidopsis* accessions and the five PPV isolates used in this study and Figure 1 appears in color online.

host plants, *A. thaliana* accessions were evaluated for their susceptibility to a range of PPV isolates representing a large part of the pathogen diversity.

Using this approach, we showed considerable variation in the susceptibility to PPV infection of *A. thaliana* accessions. Phenotypes ranging from complete invasion of the plant (accompanied or not by symptom-induction) to the inability of the virus to mount a productive replication in the initially inoculated leaves were observed and the genetic bases of some of these interaction phenotypes were analyzed.

RESULTS

Twelve *Arabidopsis* accessions of diverse geographical origin were inoculated with five isolates of PPV. Responses of *Arabidopsis* to PPV were classified as susceptible (referred to as S in Table 1) when the virus was detected in the uninoculated inflorescence tissues by enzyme-linked immunosorbent assay (ELISA) or by reverse transcriptase-polymerase chain reaction (RT-PCR). While in most cases, the infected plants remained asymptomatic, there were several accession-isolate combinations in which symptoms were observed, such as reduction of plant growth, chlorotic stems, cauline, and rosette leaves, severe inflorescence stunting, and distortion accompanied or not with curling of the cauline leaves (Fig. 1). In Table 1, the severity of the observed symptoms is indicated by the number of + signs. Plants in which both ELISA and RT-PCR assays were negative for PPV detection in inflorescence tissues were noted as resistant. Based on the results of PPV detection by ELISA or RT-PCR in the inoculated leaves, resistance phenotypes were further separated in resistance to local infection (Ri, no virus detected in inoculated leaves) and resistance to long-distance movement (Rsys, virus detected in inoculated leaves but not on distal uninoculated tissues). In preliminary experiments, the plants classified as Ri were assayed for PPV accumulation at 30 days postinoculation (dpi), but no accumulation was detected, and therefore, sampling at such late stages of *Arabidopsis* development was not routinely continued and plants were sampled at 9 and 21 dpi.

PPV is able to systemically infect *Arabidopsis thaliana*.

Although the response of *Arabidopsis* accessions to the five PPV isolates varied, all 12 accessions studied were infected by at least one PPV isolate, with or without development of visible symptoms (Table 1). However, none of the PPV isolates tested was able to infect the *AteIF(iso)4E-1* transposon-disrupted mutant line (Duprat et al. 2002), suggesting that eIF(iso)4E plays an essential role during PPV infection in *A. thaliana*, as already shown for other potyviruses (Duprat et al. 2002; Lellis et al. 2002). Remarkably, the PPV-SoC isolate was able to systemically infect all tested accessions, while infection of uninoculated tissues was never observed for the PPV-SoC isolate (Table 1). In fact, only the Nd-1 accession was able to support local replication of PPV-NAT isolate, while the virus was never detected in the inoculated leaves of any of the other accessions tested. Although Ri is often associated with a hypersensitive response characterized by localized cell death at the infection site, a local lesion response was not macroscopically observed in any of the experiments reported here (data not shown).

In a number of accession-isolate combinations, PPV was only detected (by ELISA or by RT-PCR) in the inoculated leaves, indicating that the virus was unable to spread in the uninoculated tissues. Such a phenotype was observed in most of the accessions inoculated with the PPV-EA isolate. Absence of any long-distance movement was also observed when Cvi-1 was inoculated with PPV-PS and when Shahdara and Kas-1 were infected with PPV-R (Table 1). In contrast, the C24 accession was highly susceptible to PPV infection and developed severe growth defects when infected with M, D, and EA PPV strains. Severe symptoms were also observed when the Landsberg *erecta* (L*er*) accession was infected with the PPV-R isolate, and slightly less severe ones were observed when Bay-0 was infected either with the PPV-NAT or PS isolates (Table 1). Two to three weeks after inoculation, chlorosis was observed on distal uninoculated tissues, such as cauline leaves and inflorescences. During later stages of infection, the plants exhibited severe growth stunting and occasionally lethal necrosis (Fig. 1).

These results show that a high degree of variation exists in the outcome of *Arabidopsis*-PPV interactions and that, in a significant number of cases (40%, 24 out of 60 accession-isolate combinations), some form of resistance against viral infection was observed. The genetic bases of some of the phenotypes observed were then analyzed using either forward or reverse genetics strategies.

Genetic basis of symptom development in the L*er* accession infected with PPV-R isolate.

Limited information on the genetic bases underlying symptom development in virus-plant pathosystems is available so

Table 1. Phenotypic variation of *Arabidopsis–Plum pox potyvirus* (PPV) interactions

	PPV isolates[a]				
***Arabidopsis* accessions**	**NAT (D)**	**R (D)**	**PS (M)**	**SoC**	**EA**
C24	S+++	S+++	S+++	Ri	S+++
Landsberg *erecta* (L*er*)	S	S+++	S	Ri	S
BayO	S++	S	S++	Ri	Rsys
Col-0, Col-3, Col-5	S	S+	S	Ri	Rsys
Ws, Ws-2	S	S	S	Ri	Rsys
Cvi-1	S	S	Rsys	Ri	S
Kas-1, Shahdara	S	Rsys	S	Ri	Rsys
Nd-1	S	S	S	Rsys	Rsys
Arabidopsis mutants in Col-0 background[b]					
AteIF(iso)4E-1	Ri	Ri	Ri	Ri	Ri
Arabidopsis mutants in L*er* background[c]					
sgt1b, *rar-10*	S	nd	S	Rsys	S
sgt1b / rar-*10*	S	nd	S	Rsys	S

[a] S = susceptible but symptomless; S+ = mild symptoms appeared occasionally (1 or a few plants over 12 infected) and did not affect plant growth; S++ = an intermediate situation between the two extremes; S+++ = all plants infected developed severe growth defects leading, in most cases, to death at 3 to 4 weeks postinoculation; Ri = resistance to PPV inoculation; Rsys = resistance to PPV long-distance movement; nd = not determined.
[b] *AteIF(iso)4E-1* was obtained by insertion of a transposon within the *eIF(iso)4E* gene (Duprat et al. 2002).
[c] Provided by J. Parker (Max Planck Institute, Cologne, Germany) and coworkers.

far. Whereas severe symptoms have been observed in L*er* in response to infection by PPV-R isolate, only very mild symptoms were observed in the Col-0 accession (Table 2 and Fig. 1). To determine the number of loci controlling severe symptom development in the L*er* accession infected by the PPV-R isolate, we used a recombinant F2 population between L*er* and Col-0. Parental and F2 plants were scored for development of symptoms and virus accumulation, as determined by ELISA. The results showed that the ratio of symptomatic versus asymptomatic plants did not fit with simple genetic models, suggesting that several loci are involved in the control of this trait. A total of 62 individuals out of 115 developed symptoms, which is consistent with a 9:7 segregation ratio, indicating that this trait is controlled by at least two loci (P = 0.6134 to 0.5311; Table 2). Some F2 individuals developed more severe symptoms than the L*er* parental line; these plants showed severe stunting and died within 21 dpi. This observation suggests that the Col-0 parent might contribute with one or more supplementary genetic factors enhancing or accelerating the severity of the symptoms.

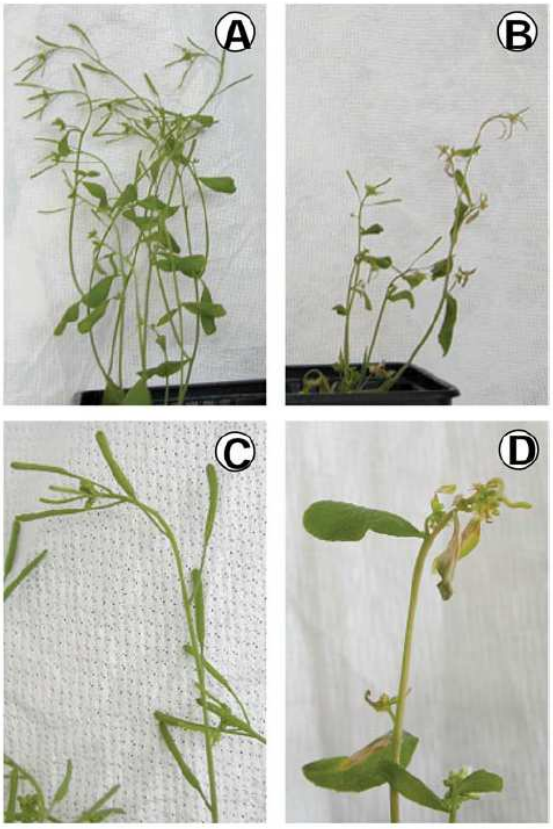

Fig. 1. Symptoms exhibited by *Arabidopsis thaliana* accession L*er* when infected with *Plum pox potyvirus* (PPV)-R. **A,** Healthy plant 3 weeks after inoculation with PPV-R. **B,** Infected Landsberg *erecta* (L*er*) plant with severe symptoms and stunting, 3 weeks after inoculation. **C,** Close-up of A showing healthy inflorescences. **D,** Close-up of L*er* inflorescences infected with the PPV-R isolate and showing leaf curling and chlorosis and severe inflorescence distortion.

Analysis of local resistance to inoculation by the PPV-SoC isolate.

The Ri observed during the interaction between PPV-SoC and most ecotypes tested, failed to detect any virus in the inoculated leaves, suggesting either a link with either *R* gene–mediated resistance pathways or a nonhost passive resistance. The first possibility was evaluated in the L*er* accession by analyzing the behavior of available mutant lines affecting two genes identified as essential in *R* gene–triggered disease resistance, *RAR1* and *SGT1* (Austin et al. 2002; Azevedo et al. 2002; Tornero et al. 2002). Table 1 shows that, contrary to the wild-type *Ler* accession, *sgt1b* and *rar1* mutants and the *sgt/rar1* double mutant supported the local accumulation of the PPV-SoC isolate. However, these mutants still failed to support PPV-SoC systemic infection, indicating the existence in these plants of two levels of resistance, one restricting local PPV-SoC infection, compromised by the *rar1* or *sgt1b* mutations, and the other, independent of these factors, acting to block the long-distance spread of the virus.

Analysis of the resistance to PPV-EA long-distance movement in Col-0.

Segregation data obtained for resistance to PPV-EA infection in the Col-0 × L*er* F2 population was consistent with a single dominant gene model (79:17 resistant/susceptible, P = 0.098, for a segregation ratio of 3:1). In *A. thaliana*, three dominant loci have been shown to confer resistance to the long-distance movement of another potyvirus, TEV, and were designated RTM for restricted TEV movement (Mahajan et al. 1998). Mapping of the resistance trait associated with restriction of PPV-EA systemic spread in the recombinant inbred lines (RIL) of Lister and Dean (1993) allowed the identification of a single region, in the upper part of chromosome 1 in the vicinity of the *RTM1* gene (data not shown) (Mahajan et al. 1998). To analyze the possible involvement of the RTM in the resistance to PPV-EA, chemically induced *rtm* mutants (Mahajan et al. 1998; Whitham et al. 1999) were challenged with the PPV-EA isolate (Table 3). In all three mutants, PPV-EA was able to infect uninoculated tissues and was readily detected in cauline leaves and inflorescence tissues. In order to determine whether the *RTM* loci are also required for restriction of the long-distance movement of other potyviruses, the *rtm* mutants were inoculated with the AF199 isolate of *Lettuce mosaic virus* (LMV), which was previously reported to be unable to systemically invade the Col-0 accession (Revers et al. 2003). Again, restric-

Table 2. Statistical inheritance analysis in selected *Arabidopsis–Plum pox potyvirus* (PPV) interactions

Arabidopsis F2 populations	PPV isolate	Number tested	Segregation	Genetic determinism	χ^2 (P value)[a]
Symptom intensity (ratio of symptomatic over asymptomatic plants)					
Col-0 × L*er*[b]	R	115	62:53	9R:7S	0.6134
		118	63: 55	9R:7S	0.5311
Virus accumulation (ratio of resistant over susceptible plants)					
Col-0 × L*er*	EA	96	79:17	3R:1S	0.0989
Cvi × L*er*	PS	96	17:79	1S:3R	0.0989

[a] P values calculated for a χ^2 of 1 degree of freedom resulted from a chi-square test of fit of the data to two dominant genes (9R:7S), a single dominant gene (3R:1S), and a single recessive gene models (1R:3S). When $P > 5\%$, the hypothesis that the observed ratio of segregation is consistent with the expected ratio cannot be rejected.

[b] The Col-0 × Landsberg *erecta* (L*er*) F2 population was tested twice for symptom appearance.

tion to long-distance movement was found to be abolished in all three *rtm* mutants (Table 3). As further confirmation of the involvement of the RTM system, a T-DNA insertion knockout (KO) line for *RTM2* in the Col-0 background was obtained from the SALK collection, and homozygous mutant plants were selected by PCR on genomic and complementary DNAs. The *rtm2* KO mutant showed no obvious morphological defects, and the chemically induced *rtm2* mutant (Whitham et al. 1999) allowed long-distance movement of PPV-EA and LMV-AF199 (Table 3). The results indicated that the resistance of the Col-0 accession to the long-distance movement of several potyvirus isolates, including PPV-EA and LMV-AF199, is conditioned by the *RTM* genes. In contrast, the Col-0 accession was found to be fully susceptible to the PPV M and D strains (Table 1), indicating that some PPV isolates are able to overcome RTM resistance or are RTM independent.

Table 3. Comparison of susceptibility of *rtm* Col-0 mutants to infection by three different potyviruses

	Potyvirus isolate[a]		
***Arabidopsis* accessions**	**TEV-GUS[b]**	**PPV-EA**	**LMV-AF199**
Col-0	Rsys	Rsys	Rsys
rtm1-1, *rtm1-2*[b]	S	S	S
rtm2-1[b]	S	S	S
rtm3-1[b]	nd	S	S
rtm2[c]	nd	S	S

[a] TEV = *Tobacco etch virus*, PPV = *Plum pox potyvirus*, LMV = *Lettuce mosaic virus*; nd = not determined.
[b] Ethylmethylsulfonate mutants and phenotypes when infected with TEV (Whitham et al. 2000).
[c] T-DNA insertion line (SALK_010448).

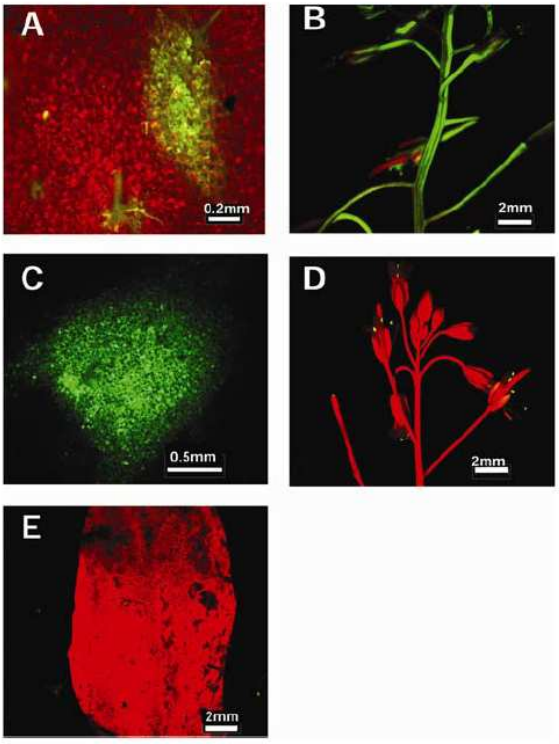

Fig. 2. Green fluorescent protein (GFP)-tagged *Plum pox potyvirus* (PPV)-PS movement is restricted to inoculated leaves of the Cvi-1 accession. Photographs taken under UV light of **A**, and **B**, PPV-R GFP and **C**, and **D**, PPV-PS GFP in Cvi-1-inoculated (A and C) and -uninoculated (B and D) tissues. **E**, Control consisting of *AteIF(iso)4E-1* mutant plants inoculated with PPV-PS GFP. The photographs were taken at 8 (A, C, and E) and 21 (B and D) days postinoculation.

Analysis of the resistance to PPV-PS long-distance movement in Cvi-1.

The resistance that confines PPV-PS infection to inoculated leaves in the Cvi-1 accession was analyzed in further detail. PPV-PS replication does not appear to be inhibited, since the virus was detected by RT-PCR or by ELISA at 9 dpi in inoculated rosette leaves (data not shown). The presence of infectious PPV particles was confirmed by successful back-inoculation from Cvi-1–inoculated rosette leaves to *Nicotiana benthamiana* plants, which developed typical vein clearing and chlorosis PPV symptoms (data not shown). Absence of virus in the upper uninoculated tissues indicated that the PPV-PS long-distance movement was disturbed.

Replication, local movement within the inoculated leaf, and long-distance movement to distal tissues were assessed by inoculation of the Cvi-1 accession with a green fluorescent protein (GFP)-tagged PPV-PS recombinant isolate. Inoculation of

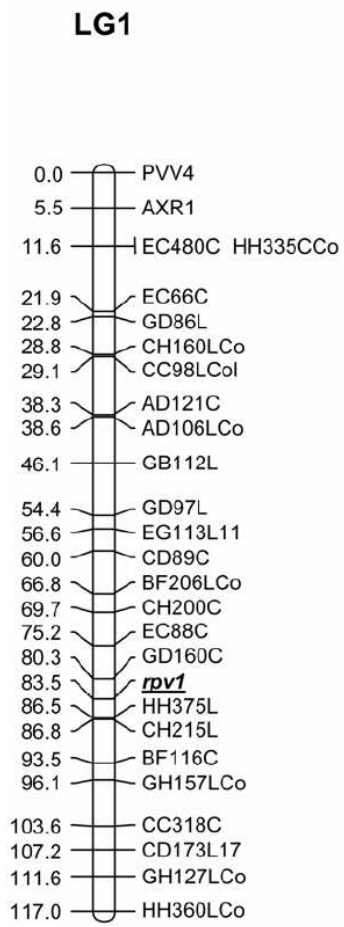

Fig. 3. Map position of *rpv1* on chromosome 1 of *Arabidopsis thaliana* Cvi-1 accession. The *rpv1* (restricted *Plum pox virus*) locus was mapped as a single gene by MAPMAKER analysis and was positioned between GD160C and HH375L on linkage group 1 (LG1). The figure was drawn with MapChart and indicates the genetic distance on the left side (in cM), the markers on the right side, and the target locus *rpv1* in bold and underlined.

Cvi-1 with a GFP-tagged PPV-R recombinant or inoculation of the *AteIF(iso)4E-1* mutant plants served as controls. In the Cvi-1 accession, the GFP-tagged PPV-R isolate replicated within the inoculated leaves (Fig. 2A) and spread systemically into the uninoculated tissues and inflorescences (Fig. 2B). In contrast, the GFP-tagged PPV-PS isolate accumulated only in the Cvi-1 inoculated leaves (Fig. 2C) and GFP fluorescence was never detected in the uninoculated inflorescence tissues (Fig. 2D). In control inoculated leaves of the *AteIF(iso)4E-1* mutant, no GFP fluorescence was observed, irrespective of the recombinant PPV isolate used (Fig. 2E).

Genetic analysis of the F2 progeny derived from a cross made between Cvi-1 and L*er* (Table 2) showed that restriction of PPV-PS infection in Cvi-1 segregated as a single recessive gene (segregation ratio of 1:3; $P = 0.098$). This Cvi-1 locus was designated as *rpv1* for "restricted plum pox virus." Since the *rpv1* resistance is recessive, while that conferred by the RTM genes is dominant, restriction of PPV-PS long-distance movement in Cvi-1 should correspond to a mechanism distinct from the RTM-mediated pathway.

A mapping population of 162 RIL developed from a cross between the Cvi-1 and L*er* accessions (Alonso-Blanco et al. 1998) was used to identify the genomic region encompassing *rpv1*. A single region for the position of *rpv1* in the *A. thaliana* genome was identified with the different statistical procedures assayed. It mapped as a single gene by MAPMAKER analysis or as a single QTL by interval mapping and composite interval mapping (CIM) using MultiQTL analysis. The *rpv1* locus was located on chromosome 1 between the GD160C and HH375L markers (Fig. 3) and was recurrent over two different phenotyping scoring data sets (log of the likelihood ratio [LOD] score of 12 by MAPMAKER analysis). One marker in this region, HH375L, had the strongest correlation seen in the QTL analysis (LOD score of 4.58 by CIM). Interestingly, while the susceptible parental line L*er* was asymptomatic when inoculated with the PPV-PS isolate, the ability to develop symptoms in response to PPV segregated as a single gene in the Cvi-1 × L*er* progeny (F2 population and RIL). This factor of symptomatology should be provided by the Cvi-1 accession but was masked in the parental line by the resistance conferred by the *rpv1* homozygous locus.

Five recombinant viruses constructed between infectious pGPPV-PS and pGPPV-R full-length clones (Sáenz et al. 2000) were used to locate the genetic determinant responsible for breaking the *rpv1* resistance by PPV-R. As shown in Figure 4, the $R/P_{2212\text{-}3628}$ and $R/P_{2212\text{-}7677}$ viruses, carrying the 5′ and 3′ ends from PPV-R, were able to systemically spread on Cvi-1 plants, whereas the complementary recombinant viruses, $P/R_{2212\text{-}3628}$ and $P/R_{2212\text{-}7677}$, in which the 5′ and 3′ sequences come from PPV-PS, did not move outside of the inoculated

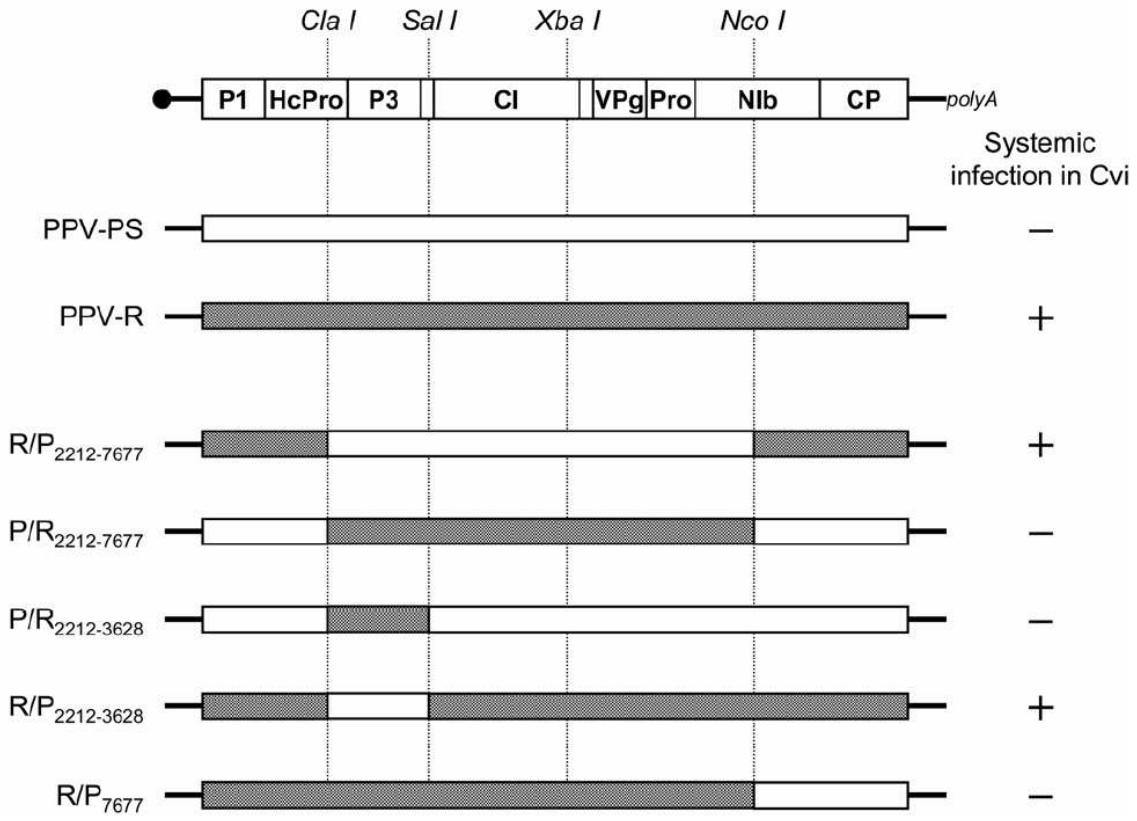

Fig. 4. Schematic representation of the genome of *Plum pox potyvirus* (PPV) and of the R and PS PPV recombinant viruses tested on the Cvi-1 accession. PPV-PS and PPV-R sequences are shown as open and filled boxes, respectively. Restriction sites used in the cloning are indicated. A genetic map of PPV, indicating the positions of the encoded proteins, is shown at the top of the figure. Infectious clones and recombinants are fully described (Sáenz et al. 2000). The names of the virus proteins are indicated in the genome map at the top of the figure. HcPro is the aphid transmission helper component-proteinase with a gene silencing suppression ability; CI is the cylindrical inclusion protein with a role in cell-to-cell movement and an RNA helicase activity; VPg is linked to the 5′ end of the genome (circle); Pro is the major proteinase responsible for polyprotein processing; NIb is the RNA-dependent RNA polymerase; CP is the capsid protein (Urcuqui-Inchima et al. 2001).

leaves, indicating that the ability of PPV-R to break the *rpv1* resistance should either be located in the first 2,212 nt ([5′ noncoding region [NCR], P1, and part of the HcPro coding sequences) or in the 3′ terminal region of the genome (from nt 7,677 to the end, corresponding to the end of the nuclear inclusion polymerase b (Nib) and capsid protein [CP] coding sequences, plus the 3′ NCR) or that both of these regions are involved. R/P_{7677} revealed that the 3′ terminal region from PPV-R is necessary to overcome the *rpv1* resistance. However, we cannot yet rule out the possibility of a role of the 5′ part of the PPV-R genome.

DISCUSSION

The results presented here demonstrate that *A. thaliana* is a suitable host to search for genetic factors involved in controlling susceptibility or resistance to PPV infection. PPV isolates exhibit very different behavior when infecting various *A. thaliana* accessions, providing a wide panel of infection phenotypes ranging from complete local resistance to full susceptibility, with systemic infection accompanied or not by severe symptoms. *A. thaliana* is a small herbaceous plant with a short life cycle that has become an excellent model for genetic studies. It is notably different from the usual PPV host plant *Prunus* spp., but the fact that several PPV strains and isolates infect *Arabidopsis* plants with different efficiency provides an excellent system to study PPV-plant interactions.

The infection of *A. thaliana* by many viruses has been previously reported. Many other viruses systemically infect *Arabidopsis* accessions but show mild or no symptoms. A few of them induce severe symptoms in infected organs, such as *Turnip crinkle virus* (TCV) (Simon et al. 1992) and *Turnip mosaic virus* (TuMV) (Martin-Martin et al. 1999), but they generally display low variation in the severity of these symptoms. Consequently, limited information is currently available on plant genes that may condition symptom development in a susceptible host. In contrast, the results reported here highlight the existence of wide, isolate- or accession-specific variations in symptom severity in the PPV-*Arabidopsis* pathosystem. The development of PPV-PS–induced symptoms in a Cvi-1 background appears to be controlled by a single locus, while symptom severity in L*er* infected with PPV-R is conditioned by at least two genes. The genetic determinants of symptom development in L*er* infected with PPV-R and Bay-0 infected with the PPV-NAT or PS isolates are currently under study, using the Lister and Dean and the Bay-0 × Shahdara RIL progenies, respectively (Loudet et al. 2002).

Variations in symptom severity may correspond to different processes. In particular, they may reflect variations in the timing or intensity of virus accumulation or, alternatively, may result from differential host behavior under comparable virus accumulation conditions (tolerance). Preliminary results tend to indicate that in the Cvi-1 × L*er* progeny, symptomatic and asymptomatic plants accumulate PPV-PS to essentially similar levels. Some single loci controlling symptom development in *A. thaliana* have been identified by genetic screens of *Arabidopsis* mutants or accessions (Fujisaki et al. 2004; Lee et al. 1996; Park et al. 2002; Sheng et al. 1998) and, similar to the Cvi-1 × Ler situation reported here, virus distribution and accumulation were similar in symptomatic and asymptomatic plants; none of these genes has been cloned so far. In contrast, Dardick and associates (2000) showed that, although the Shahdara accession supports rapid accumulation of TMV and develops distinctive disease symptoms, full sensitivity is controlled by at least two genes. One confers rapid virus accumulation, while the other determines the severe symptom phenotype. This second situation could be parallel to the situation observed in L*er* inoculated with PPV-R and in which preliminary results indicate that there could be at least a partial correlation between virus accumulation and symptom severity.

Two L*er* mutants debilitated for *R* gene–mediated resistance, *rar1* and *sgt1b*, were susceptible to PPV-SoC, suggesting that the resistance to inoculation of L*er* to this isolate is possibly controlled by a dominant *R* gene. Dominant genes controlling resistance to other viruses in *A. thaliana* have been described: *HRT* for TCV (Cooley et al. 2000), *RCY1* for *Cucumber mosaic virus* (Takahashi et al. 2001), and *LLM1* for LMV (Revers et al. 2003). Further analysis of other accessions showing Ri to PPV and other mutants affected in the *R* pathway will be required to evaluate the generality of the relationship between an Ri phenotype and one or more *R* genes. Indeed, many components of the *R*-gene signaling pathways are also involved in nonhost resistance independently of *R* genes.

The RTM mechanism restricting virus long-distance movement was initially described as being specific to TEV (Chisholm et al. 2000; Mahajan et al. 1998; Whitham et al. 1999, 2000). This conclusion was based on the observation that *Arabidopsis* accessions RTM-restrictive for TEV, such as Col-0 or Ws-2, were fully susceptible to isolates of several potyviruses such as *Potato virus Y* (PVY), *Tobacco vein mottling virus* (TVMV), or TuMV. In addition, experiments involving three different isolates of TEV failed to reveal variability in their behavior towards the RTM genes (Whitham et al. 2000). By contrast, the results reported here demonstrate that the long-distance movement of both PPV-EA and LMV-AF199 is restricted by the RTM genes in the Col-0 accession. This interpretation is indirectly supported by the observation that these isolates are able to systemically infect *Arabidopsis* accessions known to carry naturally permissive *rtm* alleles such as C24 or L*er*. The RTM-mediated resistance, therefore, appears to have a significantly broader spectrum of action than was initially hypothesized and could even potentially be envisioned as a broad-specificity, *Potyvirus*-specific, long-distance movement restriction system. In such a hypothesis, potyviruses such as PVY, TVMV, and TuMV, which are able to systemically invade accessions carrying restrictive alleles, such as Col-0, would be interpreted as being able to overcome the RTM-mediated resistance. A similar situation applies to the NAT, PS, and R isolates of PPV, which are able to systemically spread on RTM-restrictive accessions such as Col-0 and Wassilewskija (Ws and Ws-2). Despite the cloning of the *RTM1* and *RTM2* genes (Chisholm et al. 2000; Whitham et al. 1999, 2000), the one or more mechanisms by which the *RTM* genes exert their restrictive effect on the potyvirus movement are still a matter of speculation. The discovery that some potyviruses such as PPV are able to overcome this resistance in an isolate-specific fashion opens the way to the identification, through reverse genetics approaches, of one or more viral resistance-breaking determinants. This information should, in turn, help us to understand the functioning of this original resistance system.

Another question that remains open is whether the block of the long-distance movement of RTM-breaking isolates observed in the Kas-1, Shahdara (PPV-R), and Cvi (PPV-PS) accessions is related to the RTM system or represents yet other genetic systems controlling potyviral invasion of *A. thaliana*. In the case of the Cvi-1 accession, however, the inheritance pattern reported here is consistent with a single recessive gene *(rpv1)*, which was mapped away from the known positions of *RTM1* and *RTM2*. Although *RTM3* has not yet been cloned, preliminary mapping data indicate that it is located in a different genome position than *rpv1* (J. C. Carrington, *personal communication*). Therefore, it appears very unlikely that the *rpv1* resistance could be related to the RTM-mediated resistance. Given the recessive nature of the *rpv1* resistance, the

most likely hypothesis is that, similar to the eIF4E-mediated resistance, the *RPV* gene encodes a plant factor strictly required by the virus, in this case for infection of uninoculated tissues. Such a situation would be similar to that reported by Lartey and associates (1998), who described an *A. thaliana* recessive mutant, *vsm1*, defective for *Turnip vein clearing tobamovirus* long-distance spread. The preliminary investigations of the *rpv1* resistance-breaking determinant carried by the PPV-R isolate indicate that it is located either in the C-terminal end of the NIb or in the CP. The results obtained so far do not rule out, however, the possibility that multiple determinants located in either the small genomic region identified, the 5′ part of the PPV genome, or both might be involved in this resistance-breaking phenomenon. The CP is known to be essential for potyvirus long-distance movement (Carrington et al. 1996; Dolja 1994; Urcuqui-Inchima et al. 2001). Since *RPV1* is likely to encode a host factor involved in PPV long-distance movement and therefore interacting, directly or indirectly, with viral proteins involved in this process, it seems reasonable to hypothesize that CP is the viral resistance-breaking determinant. So far, very little is known about plant factors contributing to viral long-distance movement. The positional cloning of the *rpv1* gene, which is currently underway in our laboratory, should provide original information on this largely unknown process of the potyvirus infectious cycle.

One key question that will remain once *Arabidopsis* resistance and susceptibility factors to PPV infection are identified is whether this new knowledge will be transferable to other pathosystems and, in particular, to its natural *Prunus* hosts. Because of the limited number of proteins encoded by the pathogen, the virus completely depends on the host factor to complete its life cycle. Studying recessive resistance genes provides a good opportunity to reveal host factors required for susceptibility. Indeed, results obtained with eIF4E and its isoform (Decroocq et al. 2005) suggest that searching for recessive genes that condition critical steps of the potyvirus infectious cycle allow the identification of key factors that are required for PPV infection, both in its herbaceous and perennial hosts.

MATERIALS AND METHODS

Plant and virus materials.

All plants were grown under greenhouse conditions. Initial seed stocks were obtained from the Nottingham Arabidopsis Stock Centre (Loughborough, U.K.) or from the Institut National de la Recherche Agronomique (INRA) Versailles collection. Each accession was grown and self-pollinated for one or two generations before screening. PPV isolates were propagated on *N. benthamiana* prior to *Arabidopsis* inoculation. The LMV isolate LMV-AF199 described by Krause-Sakate and associates (2002) was routinely propagated on the lettuce cultivar Trocadéro.

The *Arabidopsis RTM2* T-DNA insertion line in the Col-0 background (SALK_010448) was obtained from the Nottingham Arabidopsis Stock Centre. Insertion mutant information was obtained from the Salk Institute Genomic Analysis Laboratory website. The T-DNA insertion site was confirmed by PCR, using the T-DNA left border–specific primer LBa1 5′-TGGTTCACGTAGTGGGCCATCG-3′ and *RTM2*-specific primers LP10448 5′-TGATGACCTGAGACAAAAGAAGAG A-3′ and RP10448 5′-TTCTTGAAGCTTCTTTGCCGC-3′.

Construction of pGPPV-R, pGPPV-PS, and the corresponding recombinant clones was described previously (Sáenz et al. 2000). The recombinant GFP-tagged PPV isolates used in this work derived from the PPV-R cDNA clone pICPPV-NK-GFP (Fernandez-Fernandez et al. 2001) and from the PPV-PS cDNA clone pGPPVPS-RnGFPs (B. Salvador, P. Sáenz, J. B. Quiot, C. Simón-Mateo, and J. A. García, *unpublished results*).

PPV inoculation procedure.

Before inoculation, inflorescences of 4- to 6-week-old *Arabidopsis* plants were removed. Four plants of each accession were hand-inoculated twice, two days apart, on the upper side of three rosette leaves. Inoculum was prepared by homogenizing infected *N. benthamiana* leaves with three volumes of cold citrate buffer (Na_3Citrate 0.05 M, EDTA 0.5 mM, diethyldithiocarbamic acid 0.02 M, pH 7.8). After each inoculation, plants were rinsed with water and were maintained in as S3 level containment greenhouse. Samples of inoculated leaves and new inflorescences were collected 9 dpi, and inflorescence tissues were collected again at 21 dpi. Additional sampling was also initially performed at 30 dpi, but since this sampling time did not provide any additional information, this strategy was not continued in later experiments (data not shown). For infection with the PPV-R/PPV-PS chimeras, *Arabidopsis* plants were inoculated with fresh sap from *N. clevelandii* plants infected with progeny virus derived from RNA obtained by in vitro transcription of the corresponding recombinant plasmids (Sáenz et al. 2000).

PPV detection.

PPV replication in inoculated rosette leaves was assayed by RT-PCR using the P1 and P2 primers (Wetzel et al. 1991b). Total RNA was prepared following the method of Bertheau and associates (1998). For RT-PCR reactions, 2.5 µl of total RNA were added to 22.5 µl of a reaction mixture (TrisHCl, pH 9, 10 mM, KCl 50 mM, bovine serum albumin 0.2 mg/ml, Triton X100 0.3%, $MgCl_2$ 1.5 mM, dNTP 0.25 mM, each primer 1 µM, ABgene *Reverse*-iTreverse transcriptase blend 1.5 U, Invitrogen *Taq* polymerase 0.5 U). A reverse transcription of 15 min at 42°C was followed by 40 cycles of denaturation at 92°C for 40 s, annealing at 56°C for 40 s, and extension at 72°C for 40 s. Infection on uninoculated inflorescence tissue at 9 and 21 dpi was assayed by double antibody sandwich-ELISA using PPV capsid antibody (M+D antibody, LCA Laboratory, Blanquefort, France) and, if needed, was confirmed by RT-PCR. Symptoms were scored visually from 15 to 21 dpi.

Three replicate measurements were made for each accession. In each replicate, leaves and inflorescences were collected from four distinct plants of the same accession. F2 populations, RIL, and mutants were tested similarly to the parental lines. The *AteIF(iso)4E-1* transposon-disrupted mutant line (Duprat et al. 2002; Lellis et al. 2002) was used as a negative control and, at the time of sampling 9 days after inoculation, the inoculum was detected neither by ELISA nor RT-PCR on inoculated leaves of this line.

Mapping of the *rpv1* locus.

The mapping population consisted of 162 RIL developed from a cross between the Cape Verde Islands (Cvi-1) and L*er* accessions (Alonso-Blanco et al. 1998). The genetic linkage map used contains 99 amplification and restriction fragment length polymorphism markers. The chromosomal location of the *rpv1* locus was determined using the MAPMAKER program version 3.0 (Lander et al. 1987), and markers were positioned on the linkage map at a minimum LOD = 3. The genetic map was drawn with MapChart software (Voorrips 2002). To confirm the occurrence of one or more genomic regions, a quantitative trait analysis using MultiQTL software was also performed (Korol et al. 2001). Information on the DNA markers was obtained from the *Arabidopsis* Information Resource (TAIR) database and the European "*Natural*" cooperative database.

Fluorescence detection of GFP-tagged PPV.

Cvi-1 plants 4 to 5 weeks old were mechanically inoculated with GFP-tagged PPV isolates on the tip of four to five rosette leaves as described above. Green fluorescence was scored on inoculated leaves 4 to 14 dpi and at 19 to 21 dpi in uninoculated organs. Fluorescence was observed using a fluorescence stereomicroscope MZFL III (Leica Microsystems, Wetzlar, Germany), equipped with a filter with an excitation window at 470/40 nm for GFP3 and B filters, a barrier filter at 525/50 nm for GFP3, and 515 nm for the B filter.

NOTE ADDED IN PROOF

After partial sequencing, the authors noticed that the PS isolate maintained on *N. benthamiana* in Bordeaux and used initially to screen *A. thaliana* accessions (Table 1) does not correspond to the PS sequence used to generate the infectious GFP-tagged PS PPV and PS/R recombinants (Sáenz et al. 2000). However, data presented in this paper are still relevant since both isolates were compared on the Cvi-1 and L*er* accessions and displayed similar behavior (resistance versus susceptibility).

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AUTHOR-RECOMMENDED INTERNET RESOURCES

The European "*Natural*" cooperative database: www.dpw.wau.nl/natural/resources/populations.htm
The INRA Versailles website: dbsgap.versailles.inra.fr/publiclines
MAPMAKER software: www-genome.wi.mit.edu/genome_software
MultiQTL software: esti.haifa.ac.il/~poptheor and www.multiqtl.com
The Nottingham Arabidopsis Stock Center: nasc.life.nott.ac.uk
United States Department of Agriculture public health security and bioterrorism website: www.aphis.usda.gov/ppq/permits/agr_bioterrorism
The Salk Institute Genomic Analysis Laboratory website: signal.salk.edu
TAIR database: www.arabidopsis.org

2.2.3. Diversité phénotypique des interactions Arabidopsis/LMV-PPV

La diversité des phénotypes d'infection identifiés au cours du criblage d'un petit nombre d'accessions avec le LMV et le PPV nous a donné l'idée d'explorer plus largement cet aspect. En effet, cribler un panel d'accessions d'Arabidopsis représentant une diversité génétique la plus large possible devait nous permettre d'identifier une large gamme de phénotypes en particulier des phénotypes de résistance, chacun pouvant être potentiellement contrôlés a priori par des gènes distincts. Cela pouvait donc représenter une base de données dans la quelle pourrait être identifié un ensemble de gènes de résistance, potentiellement exploitable pour de la lutte génétique. Nous avons donc proposé à nos départements INRA de rattachement (Santé des plantes et Environnement d'une part, et Génétique et Améliorations des plantes d'autre part) de financer une thèse pour exploiter au mieux cette diversité génétique d'Arabidopsis au travers du criblage d'une collection d'accessions avec des isolats de LMV et de PPV. C'est ainsi qu'avec V. Decroocq, j'ai assuré l'encadrement scientifique d'Ophélie Sicard (Thèse de doctorat de l'Université V. Ségalen Bordeaux2 ; Directeur de thèse : Olivier Le Gall) de fin 2004 à fin 2007.

Au cours de cette thèse, Ophélie a criblé une collection de 24 accessions représentant une large diversité génétique de l'espèce (près de 96% de diversité génétique après analyse de plusieurs marqueurs sur une collection de 265 accessions effectué à l'INRA de Versailles, McKhann et al., 2004) avec plusieurs isolats de LMV et PPV. Ce criblage a permis de révéler un ensemble important de phénotypes d'infection (Figure 2) qui représente une base de données importante dans l'objectif d'identifier des facteurs de l'hôte impliqués dans les interactions avec les potyvirus. Ainsi par exemple, ces données ont montré que les infections avec symptômes n'apparaissaient qu'avec le PPV et étaient relativement fréquentes au sein de cette collection.

Compte tenu que très peu de déterminants de la symptomatologie avaient été identifiés jusque là, nous avons décidé de focaliser la suite de la thèse d'Ophélie sur cet aspect.

Figure 2 : Diversité phénotypique des interactions entre Arabidopsis et PPV/LMV. Ri : pas de multiplication virale ; Rsys : pas de mouvement systémique ; S : sensible sans symptôme ; S+ : sensible avec symptômes ; R/S : résistance partielle ; Nd : non déterminé

	PPV					LMV		
Accessions	**R**	**NAT**	**EA**	**PS**	**Soc**	**O**	**E**	**AF199**
Pyl-1	Rsys	Rsys	ND	ND	Ri	Rsys	ND	Ri
Jea	Rsys	S	ND	ND	Ri	ND	S	Rsys
Bl-1	S+	S+	S+	S	Rsys	S	S	S
St-0	Rsys	Rsys	Ri	ND	Ri	Rsys	Ri	Ri
Kn-0	S+	S	Rsys	ND	Ri	ND	S	Rsys
Edi-0	S+	S	ND	ND	Ri	ND	ND	S
Tsu-0	S	S	ND	ND	Ri	S	S	S
Stw-0	S	S	S	ND	Ri	Rsys	ND	Rsys
Mt-0	S+	S+	ND	ND	Ri	ND	Ri	Rsys
Ge-0	ND	ND	S	ND	Ri	ND	Ri	Ri
Ita-0	S	S	Rsys	ND	Rsys	ND	ND	R/S
Ct-1	S+	S+	S+	S	Ri	S	S	S
Can-0	S	S	ND	ND	Ri	ND	S	Rsys
Cvi-0	S+	S+	Rsys	ND	Ri	ND	ND	Rsys
Bur-0	S+	S+	S+	S	Rsys	S	S	S
Alc-0	S	ND	ND	ND	Ri	ND	ND	R/S
Blh-1	S	S	S	ND	Ri	ND	S	S
Gre-0	Rsys	S	ND	ND	Ri	ND	Ri	Rsys
Mh-1	S+	S	ND	ND	Ri	S	ND	S
Oy-0	S+	S/S+	S+	ND	Ri	S	S	S
Shahdara	S	S	ND	ND	Ri	S	S	S
Akita	S+	S+	S+	ND	Ri	S	S	S
Sakata	S	S+	Rsys	ND	Ri	S	S	S
N13	S	S+	ND	ND	Ri	ND	S	R/S

L'identification de déterminants de plante contrôlant la symptomatologie a été réalisée par une analyse génétique en utilisant deux populations de RILs produites d'une part entre Col (produisant peu ou pas de symptôme) et Landsberg erecta (Ler, produisant des symptômes sévères), et d'autre part entre Cape verde island (Cvi) (ne produisant pas de symptôme) et Ler. Il a ainsi été montré que le caractère « symptômes » se manifestant sur Ler après infection par le PPV était contrôlé par des loci de caractère quantitatif (QTL) qui ont pu être cartographiés. Ophélie a pu confirmer l'existence de plusieurs de ces QTL en les isolant des autres grâce à des lignées quasi-isogéniques (NIL), produites dans le groupe de M. Koornneef (Université de Wageningen, Pays-Bas) avec qui nous avons collaboré.

Ces travaux ont fait l'objet de la publication suivante dans la revue MPMI (**Publication [15]**). Comme ces données concernées le PPV, la poursuite de la caractérisation de ces QTLs a été prise en charge par V. Decroocq.

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e-Xtra*

Identification of Quantitative Trait Loci Controlling Symptom Development During Viral Infection in *Arabidopsis thaliana*

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In compatible interactions between plants and viruses that result in systemic infection, symptom development is a major phenotypic trait. However, host determinants governing this trait are mostly unknown, and the mechanisms underlying it are still poorly understood. In a previous study on the *Arabidopsis thaliana–Plum pox virus* (PPV) pathosystem, we showed a large degree of variation in symptom development among susceptible accessions. In particular, Cvi-1 (Cape Verde islands) accumulates viral particules but remains symptomless, Col-0 (Columbia) sometimes shows weak symptoms compared with L*er* (Landsberg *erecta*), which always shows severe symptoms. Genetic analyses of Col × L*er* and Cvi × L*er* F2 and recombinant inbred line (RIL) populations suggested that symptom development as well as viral accumulation traits are polygenic and quantitative. Three of the symptom quantitative trait loci (QTL) identified could be confirmed in near-isogenic lines, including *PSI1* (PPV symptom induction 1), which was identified on the distal part of chromosome 1 in both RIL populations. With respect to viral accumulation, several factors have been detected and, interestingly, in the Col × L*er* population, two out of three viral accumulation QTL colocalized with loci controlling symptom development, although correlation analysis showed weak linearity between symptom severity and virus accumulation. In addition, in the Cvi × L*er* RIL population, a digenic recessive determinant controlling PPV infection was identified.

Two basic types of interaction exist between a virus and its host plant: i) a compatible interaction associated with plant invasion and viral multiplication, followed in most cases by the induction of symptoms, and ii) an incompatible interaction with no or only limited viral multiplication or movement in the host plant. Plant-virus interactions are relatively well documented, but efforts have so far focused on resistance (or resistance-breaking) mechanisms and on the identification of their genetic determinants. Conversely, the one or more mechanisms controlling symptom development in plants are still largely unknown, so that only a very patchy picture of the interaction between a plant and a virus can be presented.

Genetic factors controlling symptoms in compatible plant-virus interactions have been studied both on the host and on the pathogen side. In the host, symptom development has been reported to be controlled by either monogenic or polygenic genetic determinants, dominant or recessive. For instance, the symptom determinants in the pathosystems of *A. thaliana* and *Tobacco ringspot nepovirus*, *Spring beauty latent bromovirus*, or *Turnip mosaic virus* (TuMV) were shown to be monogenic and semidominant traits (Lee et al., 1996; Fujisaki et al. 2004; Kaneko et al. 2004). Similar monogenic controls, however recessive, were reported in the case of the symptom development during infection of *A. thaliana* by *Beet curly top virus* and *Tobacco mosaic virus* (Dardick et al. 2000; Park et al. 2002). On *Nicotiana tabacum* infected with *Cucumber mosaic virus* (CMV), the genetic determinism of symptoms was shown to be digenic and recessive (Takahashi and Ehara 1993). Finally, even more complex mechanisms controlling symptom development have been observed, with multiple contributing genes. Such polygenic control is the case with the *A. thaliana–Cauliflower mosaic virus* pathosystem, for which three distinct quantitative trait loci (QTL) have been identified (Callaway et al. 2000). Similarly, quantitative symptom determinants have also been identified in several other pathosystems such as pepper-CMV (Ben Chaim et al. 2001), tomato–*Tomato yellow leaf curl virus* (Zamir et al. 1994), and *Prunus davidiana–Plum pox virus* (PPV) (Decroocq et al. 2005). However, none of these genes have been cloned yet.

The viral determinants involved in symptom development or controlling symptom severity have frequently been observed to be inhibitors of the plant RNA silencing antiviral defense system (Dunoyer and Voinnet 2005; Vance and Vaucheret 2001; Voinnet 2001). Such is, for example, the case for P1/HC-Pro, a silencing suppressor of the potyvirus TuMV, which appears to exert its effect through interference with the miRNA-controlled developmental pathway, thus inducing developmental abnormalities and symptom development in the host plant (Dunoyer et al. 2004; Kasschau et al. 2003).

As detailed above, the model plant *A. thaliana* has often been used for the identification of plant genetic factors involved in plant-pathogen interactions. Recently, we have shown that PPV, the causal agent of sharka disease in stone fruit trees, is able to infect *A. thaliana* (Decroocq et al. 2006). A wide range of interaction outcomes were observed, depending on the host genotype

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or on the particular PPV isolate being used: resistance at the inoculation point (with no local virus multiplication detectable), resistance to systemic viral invasion (with viral accumulation in the inoculated leaves but not distantly from the inoculation point), and full systemic susceptibility. Depending on the particular combination between a susceptible *Arabidopsis* accession and the viral isolate, a broad range of reactions were observed, from the absence of symptoms to severe symptom development. Initial efforts toward the identification of one or more genetic factors underlying this variability in symptom development indicated that, when confronted by the PPV-R (Rankovic) isolate, the difference in response between the Col-0 (Columbia) accession (mild symptoms) and the L*er* (Landsberg *erecta*) accession (severe symptoms) is controlled by at least two loci and segregates as a quantitative trait (Decroocq et al. 2006).

The focus of the present study was the further characterization of plant determinants controlling symptom development as well as PPV accumulation in *A. thaliana*. For that, a quantitative genetic analysis was performed within the Col × L*er* F2 and recombinant inbred line (RIL) populations. A similar approach was followed using populations derived from the L*er* parent and Cvi-1 (Cape Verde islands), an accession that shows only extremely limited symptom development. The use of near-isogenic lines (NILs) allowed the confirmation of QTL identified during the quantitative analysis. Finally, a digenic and recessive resistance has been identified following the analysis of the Cvi × L*er* population, in which each parent contributes one resistance genetic determinant.

RESULTS

Symptom occurrence in *Arabidopsis thaliana* accessions infected with PPV-R.

To identify the genetic determinants controlling symptom development and viral accumulation during PPV infection in *A. thaliana*, a detailed phenotypic characterization of the most promising *Arabidopsis* accession and viral isolate combinations identified by Decroocq and associates (2006) was performed. Two parameters were scored, i.e., the virus accumulation in infected plants and symptom development. Approximately 2 weeks after inoculation up to 21 days postinoculation (dpi), each inoculated plant was rated visually for symptoms (SYMP). A score was given to the obtained phenotype following a notation scale between 0 (no symptom but viral accumulation) and 4 (Fig. 1). At three weeks postinoculation, virus accumulation (ACC) was determined by optical density and a 1:100 dilution was performed in order to have a better value distribution. The PPV-R isolate was selected because it results in a wide range of infection phenotypes in different accessions and because an infectious cDNA clone of this isolate is available, thus eliminating potential problems associated with genetic drift of the isolate between experiments.

Upon inoculation with PPV-R, the three *A. thaliana* accessions L*er*, Col-0, and Cvi-1 differed substantially in symptom development and virus accumulation. The L*er* accession showed the most consistent response. All of the 99 inoculated plants developed severe symptoms (SYMP = 3.0 ± 0) and heavily accumulated the virus in noninoculated inflorescence tissues (ACC = 40.7 ± 8.6% of the positive *Nicotiana benthamiana* control). Plants of the Col-0 accession showed a more variable phenotype but, overall, expressed much milder symptoms than L*er* (SYMP = 1.29 ± 1.25). Irrespective of the presence of symptoms, all inoculated Col plants accumulated PPV-R but with substantial variation in the accumulation level (ACC of 40.0 ± 35% of positive control). There was only a weak correlation between symptom severity and viral accumulation (R^2 of 0.42). The Cvi accession showed an extremely variable response. Of 96 inoculated Cvi plants, 69 did not accumulate the virus in the noninoculated tissues and, therefore, were considered to be resistant. In all other experiments performed, a similar fraction of the inoculated Cvi plants were found to fail to accumulate the virus (discussed below). This observation was in contrast with our previous data (Decroocq et al. 2006), in which we reported Cvi to be a susceptible, symptomless host of PPV-R. This discrepancy may be explained by the fact that the noninfected plants observed by Decroocq and associates (2006) were considered as inoculation escapes and were therefore discarded from further analyses.

Of the remaining 27 Cvi plants in which viral accumulation was detected, only four developed weak symptoms (SYMP = 1.6 ± 0.75), all other plants remained symptomless. In all experiments performed in this study, these four Cvi plants were the only ones that developed symptoms upon PPV-R inoculation. The 27 PPV-R-accumulating plants had a mean ACC value of 42.4 ± 44.6% of the positive control and could be separated in two groups: 12 plants accumulating the virus at high levels (ACC = 86.0 ± 30% of control) and 15 accumulating the virus at a low levels (ACC = 7.5 ± 3%). The four plants that showed symptoms belong to the high-accumulating group.

It should be noted that the SYMP scores are relatively stable from one experiment to another but that the ACC values can vary substantially. This variation may be a consequence of

Fig. 1. Various degrees of symptom severity in the Columbia × Landsberg *erecta* recombinant inbred line population. Similar phenotypes were observed in the Cape Verde islands × L*er* population. Healthy plants are on the left of each picture and inoculated plants, on the right. **A,** Plants exhibiting symptoms scoring 1, one floral hamp with top incurvation, **B,** plants showing symptoms scoring 2, a majority of the floral hamps are curved but no or very low reduction size, **C,** plants showings a phenotype scored 3 with all hamps presenting incurvation and a strong reduction of their size, and **D,** plant corresponding to a symptom score of 4, with heavily reduced hamp development and stunted hamp. **E,** Details of a symptomatic floral hamp.

changes in environmental conditions, which may influence the virus accumulation in the *Arabidopsis* host plants or even in the *N. benthamiana* positive control. However, when evaluated simultaneously, the difference in response of the various accessions was always consistent and reproducible in various experiments. PPV-R accumulation was always found to be higher in L*er* than in Col (discussed below), while comparable average accumulation levels were reached in L*er* and in Cvi (though with a larger standard deviation for Cvi). However, the high-accumulating subpopulation of Cvi clearly reached higher viral titers than any other evaluated plant, despite the fact that these plants displayed no or only mild symptoms.

Phenotypic variation in F2 populations.

In order to determine the genetic basis controlling the SYMP and ACC traits, F2 populations derived from the Col × L*er* and L*er* × Cvi crosses were inoculated with PPV-R, and SYMP and ACC were scored on individual F2 plants. Both traits showed a normal distribution (Supplemental Fig. S1) in the F2 Col × L*er* population. In the Cvi × L*er* F2 population, ACC values were also normally distributed, but this was not the case for the SYMP trait. In addition, no virus could be detected in the inflorescence tissues of 12 of the 110 inoculated F2 plants. Given the 100% infection rate observed in the plants of the Col × L*er* population and the resistance phenotype observed in part of the Cvi PPV-R–inoculated plants, these 12 plants are unlikely to be inoculation escapes but should rather be considered as resistant to PPV-R. The analysis of this resistance is described below.

For both traits, transgressive segregation was observed in all populations. In particular, a substantial number of plants (between one-quarter and one-third) of the F2 populations developed severe symptoms (Fig. 1, score 4 on the symptom scale), whereas such symptom intensity was never observed in any of the parents. These data suggest that SYMP and ACC are quantitative traits controlled by multiple genes.

Correlation, genetic effect, and heritability.

RIL populations derived from the Col × L*er* (Lister and Dean 1993) and L*er* × Cvi (Alonso-Blanco et al. 1998b) crosses were inoculated with PPV-R, and SYMP and ACC were scored. For each RIL, the values obtained were averaged over five (101 Col × L*er* RILs) and four (156 Cvi × L*er* RILs) individual plants, respectively. For the Col × L*er* population, 100% of the inoculated RILs showed viral accumulation. For the Cvi × L*er* population, 24 of the 156 RILs did not show detectable virus accumulation and were classified as resistant to PPV-R infection. Resistance is defined in this study by the absence of the virus in noninoculated leaves and, therefore, the absence of symptoms. These RILs were used for the analysis of the resistance (discussed below) but were excluded from further analysis of the SYMP and ACC traits, since the effect of the virus on its host was biased. The correlation between SYMP and ACC scores was evaluated for each RIL population. In both crosses, the symptom severity showed only weak correlation with viral accumulation (Supplemental Fig. S2). Coefficients calculated with the statistical software R showed average (0.48, $P = 9.71 \times 10^{-7}$) to weak (0.23, $P = 9.2 \times 10^{-3}$) correlation in the Col × L*er* and L*er* × Cvi populations, respectively. For example, in both populations, RILs could be identified that have severe symptoms (SYMP score = 3) with ACC scores of between 25 and 90% of the positive control. Conversely, other RILs presented weak or no symptoms but a high virus titer.

Fischer's F-values and heritabilities, obtained from the analysis of variance (ANOVA) on RIL population data, are presented in Table 1. The genetic effect is highly significant for both traits ($P(f) < 0.0001$) in the two populations. Heritabilities of 43 and 54% were obtained in the Col × L*er* population for the ACC and SYMP traits, respectively, indicating that approximately half of the variation observed in the phenotype can be explained by genotypic differences. For the L*er* × Cvi cross, heritabilities were even higher for both traits (75 and 65% for ACC and SYMP, respectively).

QTL analysis.

The QTL analyses were performed on the Col × L*er* and Cvi × L*er* RIL populations and using the quantitative values for the SYMP and ACC traits described above as phenotypic data.

Concerning the Col × L*er* population, three QTL were detected for the ACC trait on chromosomes 1 (*ACC1*), 3 (*ACC3*), and 5 (*ACC5*), respectively (Table 2, Fig. 2). The L*er* alleles of the *ACC1* and *ACC3* QTL increased ACC values (positive additive effect), while the L*er* allele of the *ACC5* QTL decreased ACC values (negative additive effect). The occurrence of multiple QTL with opposite additive effect segregating independently is expected to cause transgression above the parental L*er* phenotypic value (Table 2). The percentage of phenotypic variance explained by each QTL ranged from 9 to 17%. Two QTL were found to contribute to the SYMP trait explaining respectively 13 (*PSI1.1*, PPV symptom induction, located on chromosome 1) and 17% (*PSI3,* located on chromosome 3) of the phenotypic variance. A weak epistatic interaction, significant at $\alpha = 0.05$, was detected between these two QTL. *PSI1.1* maps on top of chromosome 1, near the nga59 marker. This position is very close to that identified for the *ACC1* QTL (marker ve006), being only 12 cM distant. The other SYMP QTL, *PSI3*, colocalizes with the *ACC3* QTL on chromosome 3, since it is linked to the same marker, m424.

In the Cvi × L*er* population, only one QTL for the ACC trait could be identified, *ACC4* on chromosome 4, with a negative additive effect (ACC values of Cvi are greater than those of L*er*). For the SYMP trait, because of the nonnormal distribution of the data in the Cvi × L*er* F2 and RIL populations, a Kruskal-Wallis rank-sum test was performed. Results identified three contributing loci on chromosomes 1 (*PSI1.2*), 2 (*PSI2*), and 5 (*PSI5*) (Table 2 and Fig. 2), with explained variances of 13.5, 5.6, and 9.2%, respectively. *PSI1.2* and *PSI2* had positive additive effect (SYMP values of L*er* are greater than that of Cvi) while *PSI5* had a negative additive effect (SYMP values

Table 1. Phenotypic variation for viral accumulation (ACC) and symptom development (SYMP) in recombinant inbred line (RIL) populations

Trait	Columbia (Col) mean	Landsberg *erecta* (L*er*) mean	RIL mean	RIL range (min to max)	Fischer's F[a]	Heritability (%)
ACC	40.34 (± 35.8)	75.16 (± 19.7)	58.01	13.01 to 98.8	3.42***	43
SYMP	1.29 (± 1.25)	3.4 (± 0.84)	2.94	0.25 to 4	4.73***	54
Trait	**Cape verde islands (Cvi) mean**	**L*er* mean**	**RIL mean**	**RIL range (min to max)**	**Fischer's F**	**Heritability (%)**
ACC	53.56 (± 11.54)[b]	37.73 (± 6.9)	34.31	14.37 to 86.52	9.48***	75
SYMP	0	3	1.11	0.25 to 4	6.24***	65

[a] *** = significant at the 0.01% level.

[b] Mean calculated with infected plant (with virus accumulation).

Table 2. Quantitative trait loci (QTL) controlling viral accumulation (ACC) and symptom development (SYMP) in the Columbia (Col) × Landsberg *erecta* (L*er*) and in the Cape verde islands (Cvi) × L*er* recombinant inbred line populations

Trait	Chr[a]	QTL name	Nearest marker	LOD score	Additive[b]	PEV[c]	
QTL controlling symptom and viral accumulation in the Col × L*er* population after infection with the *Plum pox virus* Rankovic (PPV-R) isolate							
SYMP	1	PSI1.1	nga59	3.7797	–0.3514	13.15	
	3	PSI3	m424	4.4367	–0.439	17.4	
			nga59 × m424	0.0339[d]	4.65		
ACC	1	ACC1	ve006	2.8513	–5.7044	9.66	
	3	ACC3	m424	4.2848	–8.3012	15.07	
	5	ACC5	nga139	3.287	7. 5449	16.59	
Trait	**CHR**	**QTL name**	**Nearest marker**	**LOD score**	**Additive[b]**	**PEV[c]**	
QTL controlling viral accumulation in the Cvi × L*er* population after PPV-R infection							
ACC	4	ACC4	EC.306L	3.41	–0.0583	11.84	
Trait	**CHR**	**QTL name**	**Nearest marker**	**Mean rank L*er***	**Mean rank Cvi**	**PEV[c]**	**K[e]**
QTL controlling symptom development in the Cvi × L*er* population after PPV-R infection							
SYMP	1	PSI1.2	PVV4	1.65495	0.773522	13.5	******
	2	PSI2	FD.150C	1.49876	0.894489	5.6	***
	5	PSI5	GH.117C	0.876126	1.6423	9.2	****

[a] CHR = chromosome; LOD = logarithm of the odds ratio.
[b] In the Col × L*er* population, a negative value indicates the contribution of the L*er* parent and in the Cvi × L*er* population, this information is given by a positive value.
[c] Percentage of variance (PEV) explained by the QTL.
[d] Value given is the *P* value instead of the LOD score, at the 5% level.
[e] *P* value associated with the Kruskal-Wallis test: *** = 0.01 **** = 0.005 and ****** = 0.005.

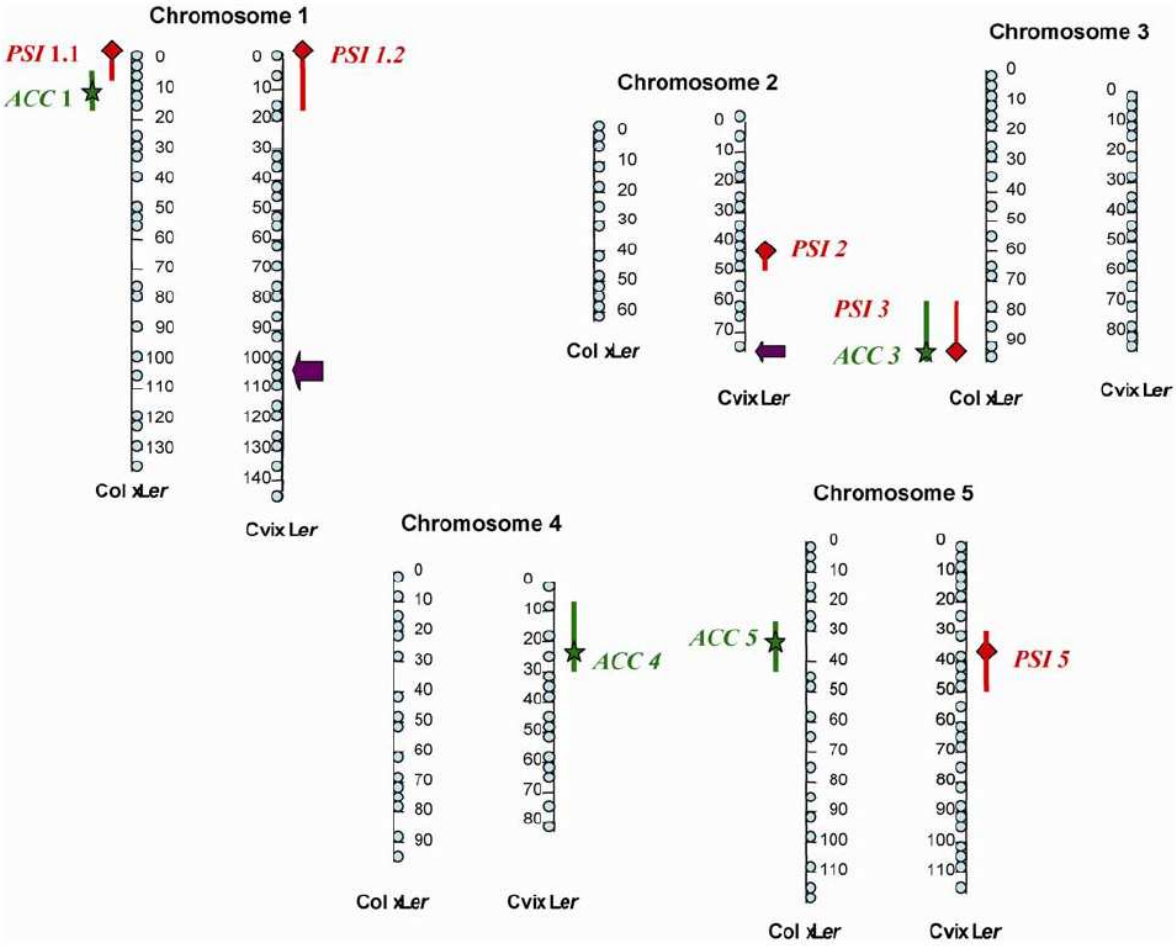

Fig. 2. Columbia (Col) × Landsberg *erecta* (L*er*) (left chromosome) and Cape Verde islands (Cvi) × L*er* (right chromosome) genetic maps showing quantitative trait loci (QTL) for symptom severity (red square) and virus accumulation (green star). Vertical lines associated with each QTL denote the 2-LOD (logarithm of the odds ratio) support interval. Approximate positions of resistance genes are indicated with a purple arrow. Units shown are in centimorgans, and markers used to construct the genetic maps are depicted as blue closed circles.

of Cvi are greater than those of L*er*). Interestingly, *PSI1.2*, cosegregating with the PVV4 marker, colocalizes with the *PSI1.1* QTL detected in the Col × L*er* RIL population. However, despite colocalization and similarity in additive effect, we cannot rule out, at this stage, that *PSI1.1* and *PSI1.2* are two distinct loci.

QTL confirmation and fine mapping using NILs.

To confirm and fine-map the *PSI1* QTL located on top of chromosome 1, seven NILs (Keurentjes et al. 2007), each containing a different Cvi introgression in a L*er* background, were used (Fig. 3). As *PSI1* is genetically linked to the PVV4 marker, LCN1-2.3, LCN1-2.4, and LCN1-2.5 should not contain the L*er* allele of *PSI1*, whereas LCN1-2.10, LCN1-2.11, LCN1-2.12, and LCN1-2.16 should. Indeed, after inoculation of this set of NILs by PPV-R, LCN1-2.3, LCN1-2.4, and LCN1-2.5 did not develop any symptoms, despite the fact that they accumulated the virus in their inflorescence tissues to concentrations similar to those observed in the other four NILs that developed severe symptoms (mean scores between 2.4 and 2.8) (Fig. 3). These results confirm the *PSI1.2* QTL and demonstrate a major effect of this QTL on the development of symptoms in response to infection by PPV-R. The distinct phenotypes of the NILs LCN1-2.3 and LCN1-2.10 locate *PSI1.2* between 0 and 1,471 kbp at the long distal part of chromosome 1.

PSI1.1 was also confirmed by using a Col × L*er* NIL called SRL1-52-10/1, which contains a L*er* introgression between 0 and 24 cM on chromosome 1 in a Col background (Fig. 4). A SYMP score of 2.8 ± 0.3 was recorded for this NIL, significantly higher than the symptom score of the Col parent (1.4 ± 1.1), while there was no significant difference in viral accumulation (42.0 ± 9% compared with 52.0 ± 24%).

The *PSI3* QTL detected in the Col × L*er* population, having a positive additive effect, could be confirmed using the SRL3 NIL, which contains a 10-cM L*er* introgression in a Col background (Fig. 4). The PPV-R–infected SRL3 NIL showed a significantly higher SYMP score than that of the Col parent (3.2 ± 0.4 compared with 1.4 ± 1.1), despite the fact that virus accumulation, with an ACC score of 45.0 ± 10% for SRL3, was not significantly different from that observed in Col (52.0 ± 24%).

To confirm *PSI5,* the SYMP QTL identified on chromosome 5 with Cvi alleles increasing symptom severity (Table 2), two NILs with a Cvi introgression in a L*er* background, LCN5-4 and LCN5-7, were inoculated with PPV-R. LCN5-7, which carries a large Cvi introgression covering the QTL region, displayed extremely severe symptoms (mean SYMP score of 4.0, with 7 of the 11 tested plants ultimately dying from infection, which, in this study, was never observed in the L*er* parent). This score is significantly different ($P(f) < 0.0001$) from that for LCN5-4, which carries a smaller, nonoverlapping Cvi

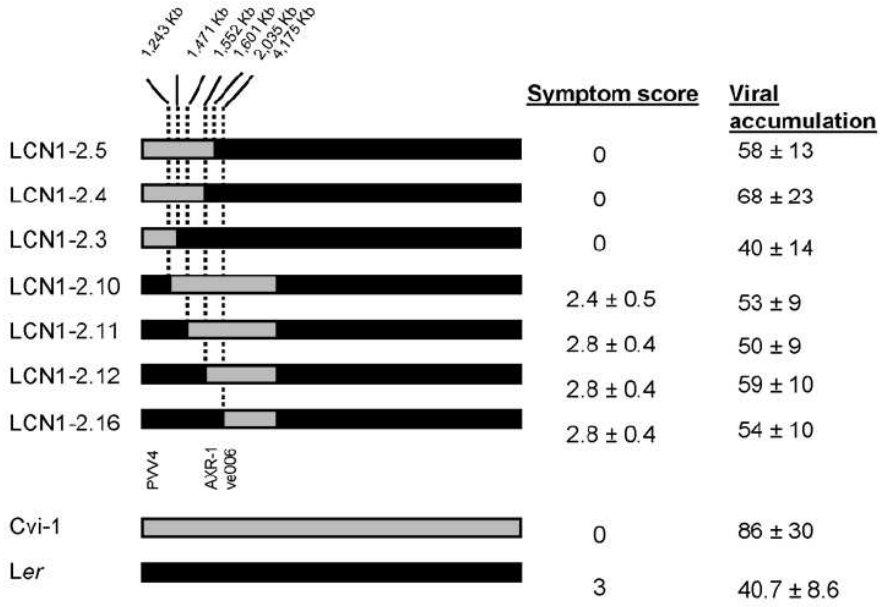

Fig. 3. Schematic representation of near-isogenic lines (NILs) showing chromosome 1 Cape Verde islands (Cvi) introgressions (in gray) in a Landsberg *erecta* (L*er*) background (in black) and their responses to *Plum pox virus* Rankovic (PPV-R) infection (symptom score and viral accumulation).

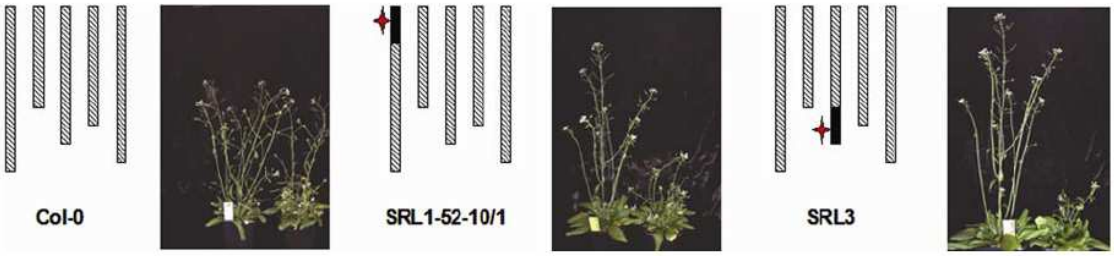

Fig. 4. Representation of two near-isogenic lines (SRL 1-52-10/1 and SRL3) showing chromosome 1 and 3 Landsberg *erecta* introgressions (in black) in a Col-0 (Columbia) background (in hatched black) and their phenotypic responses to *Plum pox virus* Rankovic infection, compared with the Col-0 parent. Healthy plants are on the left and infected plants on the right. The red star indicates the approximate quantitative trait loci positions on chromosomes 1 and 3.

introgression, or for the L*er* parent (3 ± 0 and 3 ± 0.4, respectively) (Fig. 5). The direct comparison of symptom development in the two NILs thus confirms the *PSI5* symptom QTL and demonstrates the additive effect of the Cvi *PSI5* allele in a L*er* background (Fig. 5), namely a dramatic increase in symptom severity. The opposite effects for the various SYMP QTL explain, at least partly, the transgression observed for the SYMP phenotypic trait values in the Cvi × L*er* F2 and RIL populations.

Since the 2-LOD (logarithm of the odds ratio) *ACC1* and *ACC3* intervals overlap with the *PSI1.1* and *PSI3* SYMP QTL, the phenotypic analyses of the SRL1-52-10/1 and SRL3 NILs reported above were also used to try to confirm the two *ACC* QTL. Since, in both cases, viral accumulation in the NILs was not significantly different from that observed in the recurrent Col-0 parent, we were not able to confirm the effect of any of these two QTL. Similarly, the *ACC4* QTL was not confirmed in the PPV-R–infected LCN4-3 NIL (Keurentjes et al. 2007).

Resistance determinants in the Cvi × L*er* population.

In the Cvi × L*er* F2 population, 12 of the 110 inoculated plants failed to accumulate PPV-R to detectable levels in their inflorescence tissue. In the corresponding RIL population, 24 of the 156 RILs behaved similarly (discussed above), indicating that one or more resistance factors segregate in these populations. A first resistance determinant originating from the Cvi parent was mapped on chromosome 1 between the CH.200C and EC.88C markers, with a LOD score of 4.89 (Fig. 2). Interestingly, it colocalizes with the *rpv1* locus, conferring resistance to the PPV-PS isolate, identified by Decroocq and associates (2006) in Cvi. As expected from a recessive resistance model, the L*er* (susceptibility) allele at this position is strongly associated with susceptibility (of 88 RILs that have the L*er* allele at this position, 85 are susceptible to PPV-R as demonstrated by detectable viral accumulation in the inflorescence tissue). On the other hand, the Cvi allele is not correlated with resistance. Of 46 RILs with the Cvi allele, 25 are susceptible and 21 are resistant, indicating that a second genetic determinant may be needed for resistance. Those data were confirmed by using the frequency of resistant plants in each of the 156 RILs (data not shown). Due to nonnormal distribution of the data, a nonparametric test was performed and showed the closest linkage of *rpv1* with the EC.88C marker (significance of 5×10^{-4}).

Using the subpopulation of the 46 RILs carrying the Cvi allele, a second determinant, named *rpv3*, originating from the L*er* parent, was mapped close to the EC-235L genetic marker on chromosome 2, with a LOD score of 5.09. Again, the predictive value was high for the susceptibility allele at this locus (of 56 RILs with the Cvi susceptibility allele, 50 are susceptible) but not for the resistance L*er* allele (75 susceptible and 18 resistant). Analysis of the four genotypic classes for the two loci (Supplemental Fig. S3) suggests that the resistance is conferred by the simultaneous action of at least two recessive genes on linkage groups 1 and 2.

To determine if this resistance is PPV-specific, two resistant and two susceptible RILs were inoculated with two other potyviruses, LMV-AF199 (for which Cvi is resistant and L*er* symptomless susceptible) (Revers et al. 2003) and TuMV-UK1 (Cvi and L*er* are both susceptible and exhibit symptoms) (Martin Martin et al. 1999). The two resistant RILs carried resistance alleles at both loci controlling PPV resistance but lacked the gene controlling resistance to LMV-AF199 infection described by Revers and associates (2003). The four RILs displayed symptoms upon TuMV-UK1 inoculation, and analysis of viral accumulation indicated that they were symptomless susceptible to LMV-AF199, demonstrating that this recessive, digenic resistance does not confer broad-spectrum protection against potyviruses (data not shown).

DISCUSSION

In this work, we have analyzed the genetic basis of symptom development during viral infection in *A. thaliana*. F2 and RIL populations originating from two different crosses were used to evaluate the variability of symptom severity as well as of virus accumulation in *Arabidopsis* inflorescences infected with the PPV-R isolate. For both traits, the quantitative variations observed were shown to be complex regulated. Quantitative trait analysis allowed the mapping of a number of genetic determinants for both traits in two RIL populations, Col × L*er* and Cvi × L*er*.

With respect to symptom severity, two to three QTL were reported, depending on the segregating population, while for virus accumulation, one to three QTL were observed. The effect of each QTL was moderate to medium (explained variance ranging from 5.6 to 17.4%), but a high percentage of explained phenotypic variance was observed when considering all the covariants (total explained variance in the Col × L*er* population is 45% for SYMP and 38% for ACC). These relatively low explained-variances values are in agreement with the significant but relatively low heritability values observed for both traits, in particular in the Col × L*er* population. These observations can be explained by several reasons that are not mutually exclusive.

A first one is that the phenotyping strategy used did not allow a sufficiently precise and reproducible evaluation of the quantitative traits under study. In this context, it is noteworthy that the large-scale evaluation of the Col and Cvi parents showed significant plant-to-plant variability, in particular for the ACC trait, as indicated by the large standard deviations obtained. Moreover, from one experiment to another, substantial variations in ACC values were observed for the parental lines.

Fig. 5. Representation of two near-isogenic lines (NILs 46 and 55) showing chromosome 5 Cape Verde islands-1 introgressions (in gray) in a Landsberg *erecta* background (in black) and their phenotypic responses to *Plum pox virus* Rankovic infection. For each NIL, healthy plants are on the left and infected plants on the right. The red star indicates the approximate quantitative trait loci position on chromosome 5.

It is thus conceivable that a phenotyping performed under more stable conditions than those available in the BL3-level containment greenhouse required for PPV manipulation could have allowed a more powerful QTL identification.

A second reason that could account for intermediate level heritability values and low impact of the identified QTL is that these traits are controlled by a larger number of genetic determinants, each contributing only little to the overall trait. The confirmation of some QTL by NILs does not support this hypothesis. However, we cannot rule out the occurrence of many more QTL that we failed to detect because of their small effect.

Whatever the reason or reasons for the moderate to low explained variances of the QTL detected, the involvement of these genetic determinants in the traits studied was confirmed by independent detection in the two different recombinant populations in the case of the *PSI1* locus and the use of NILs for at least two other QTL. Besides *PSI1*, no other QTL were identified in both RIL populations. The simplest explanation is that this could be due to environmental effect, since the populations were tested separately. Three types of QTL-environment interactions have been proposed to explain differential QTL detection while using at least one similar parent (Li et al. 2003): i) some QTL are expressed in one environment and not in another, ii) QTL can be expressed more in one condition than in an other, or finally, iii) QTL can have opposite effects in two different environmental conditions. However, another explanation is that different genetic factors are polymorphic in the two crosses or that effects are dependent on genetic background. Similarly, at least two other studies showed contrasting effects of L*er* alleles in distinct crosses (El-Lithy et al. 2006; Symonds et al. 2005).

The use of connected crosses has been proposed to increase the genetic variability addressed and to test for epistatic interactions between QTL and the genetic background. Such an approach allows increasing the power of QTL detection, owing to a higher probability that a QTL will be polymorphic in at least one cross. Despite those potentialities, we detected only weak epistatic effect between the above QTL and in one single population, Col × L*er*. Similarly to what was observed in our study, others reported poor QTL congruency when analyzing six connected maize populations (Blanc et al. 2006).

A direct validation of the phenotypic effect of the three symptom QTL, *PSI1*, *PSI3*, and *PSI5* was obtained using NILs.

The location of *PSI1* was narrowed in Cvi-introgression lines to a small segment of approximately 1,500 kb at the distal part of chromosome 1. Usually a QTL will account for only a small fraction of the genetic variation as revealed by quantitative analysis in the recombinant populations. This was confirmed in NILs, as shown for *PSI3* and *PSI5*. However, surprisingly, analysis of the introgression lines covering the *PSI1* interval revealed qualitative variation, since *PSI1* alone leads to symptom development upon PPV infection. *PSI1* accounts, therefore, for a major part of the symptom trait and appears to act independently from other loci. However, the low explained variance suggests that symptom development can be diminished by other Cvi or Col loci.

On the contrary, we showed that the presence of the Cvi *PSI5* allele in NILs produced more severe symptoms than in the parent L*er*. Since Cvi is asymptomatic when infected with PPV-R, we hypothesize that the presence of symptoms requires at least the L*er PSI1* allele and the Cvi *PSI5* allele acts synergistically with *PSI1*, causing the recombinant lines to exceed the parental phenotypes.

Similarly, the quantitative effect of the *PSI3* QTL on symptom severity was demonstrated through the use of lines carrying L*er* introgressions in a Col-0 background. Based on the results obtained in both Cvi and L*er* introgression lines, we postulate that *PSI1* and the *PSI3* or *PSI5* are functionally integrated components determining symptom development and severity.

This work also points out another interesting element that concerns the relationship between the two traits studied, symptom development and virus accumulation. It is commonly accepted that a positive correlation exists between fitness and virulence that corresponds to the basic trade-off model (Anderson and May 1982; Ewald 1983; Frank 1992; Nowak and May 1994). In contrast, in our experimental populations, we found little evidence of correlation between virus accumulation and symptom severity, though at least two accumulation QTL, *ACC1* and *ACC3*, colocalize with *PSI1* and *PSI3*, respectively. In the Cvi × L*er* population, no colocalization between SYMP and ACC QTL was detected. Noteworthy, some Cvi × L*er* RILS and NILs consistently presented a high virus titer but no symptoms at all. Three models have been proposed to explain possible association between two traits mapping to the same genetic locus (Lebreton et al. 1995): i) two closely linked QTL control the two traits independently, ii) one single QTL controls both trait, iii) or one QTL controls one trait that is causally associated to the other one. However, we could not rule out one or the other model concerning the QTL controlling symptom severity and virus accumulation on chromosomes 1 and 3.

Although multiple determinants for symptom development and severity were detected, transgressive segregation resulted in several RILs of the Cvi × L*er* population that were fully resistant upon PPV infection. The data presented here indicate that both parents possess genetic determinants that affect PPV infection. Cvi shows intermediate susceptibility to PPV-R infection, while L*er* is fully susceptible. Therefore, the genetic basis for the PPV-R resistance in the Cvi × L*er* RILs is more complex than can be explained by a single-gene model. Our data points out to a recessive resistance conditioned by two unlinked loci, one coming from Cvi and the other from L*er*. The nature of this resistance is still unknown. Since initially PPV-R was detected in inoculated Cvi leaves (Decroocq et al. 2006), we hypothesized that this partial resistance was related to an inhibition of PPV long-distance movement. However, in a *rpv1/rpv3* background, the mechanism leading to the digenic resistance has not yet been described. It should be noted that in two other model plants, tobacco and soybean, a similar digenic, recessive mechanism that restricts virus long-distance movement was described (Goodrick et al. 1991; Schaad and Carrington 1996). For example, *Tobacco etch virus* is able to establish infection and move from cell to cell in inoculated leaves of tobacco line V20 carrying two unlinked recessive genes, but systemic infection in distal tissues is impaired (Schaad and Carrington 1996). Recently, Ruffel and associates (2006) also identified digenic, recessive determinants controlling the resistance to *Pepper veinal mottle virus* (PVMV) infection in pepper. But in this case, PVMV is restricted early during infection in the inoculated leaves, and the resistance is linked to the eIF4E and eIFiso4E eukaryotic initiation factors.

Since recessive genes that control these restriction phenotypes are important to dissect the host requirements for virus infection, positional cloning of *Arabidopsis* genes is currently going on. One of them is mapping in the same region as the previously mapped resistance gene *rpv1* (Decroocq et al. 2006). *rpv1* is controlling the resistance to PPV-PS infection, and we hypothesize that it is also partially controlling infection by PPV-R. The second recessive gene mapped at the bottom of chromosome 2 and is holding from L*er*. Neither the *rpv1* nor *rpv3* locus is colocalizing with the *Arabidopsis eIF4E* and isoform copies.

In this study, QTL mapping provided a means of identifying specific host factors associated with symptom development during virus infection. These results will be further coupled with fine-scale mapping, followed by QTL positional cloning. *PSI1* will be our first candidate. It is also noteworthy that we failed initially to detect *PSI1* dominance over other QTL, despite the significant major effect of *PSI1* in NILs. This result illustrates the limit of resolution typical of QTL studies and is even more accurate when detecting loci explaining only a small part of the trait variation.

MATERIALS AND METHODS

Plant materials.

F2 and RIL populations derived from the crosses between Col-0 and L*er* (Lister and Dean 1993) and between Cvi-1 and L*er* (Alonso-Blanco et al. 1998b) were obtained from the Nottingham *Arabidopsis* Stock Center (NASC) or from the Institute for Agronomy Research (INRA)-Versailles collection. Plants were grown in a BL-3 containment greenhouse under temperature- and humidity-controlled conditions (20°C and relative humidity of 60%).

A total of 102 F2 individuals of the Col × L*er* population and six replicates (one mock-inoculated control and five inoculated plants) for each of the 101 RILs derived from this cross were phenotyped following PPV-R inoculation.

Similarly, 110 F2 individuals of the Cvi × L*er* population and five replicates (one mock-inoculated control and four inoculated plants) for each of 156 RILs derived from this cross were also phenotyped consequently to PPV-R inoculation. This experiment was performed using a complete random-block design.

NILs derived from the Col × L*er* cross and in which L*er* genomic regions have been introgressed into a Col-0 background were provided by M. Kearsey (University of Birmingham, U.K.) or were obtained from the NASC (Koumproglou et al. 2002). NILs originating from the L*er* × Cvi cross and in which Cvi-1 genomic regions were introgressed into a L*er* background have been developed at the Wageningen Agricultural University (Alonso-Blanco et al. 1998a; Keurentjes et al. 2007). A total of 12 to 24 replicates of each NIL were tested by inoculation with PPV-R.

Virus material.

The PPV-R isolate is available as an infectious cDNA clone called pICPPV (Saenz et al. 2000). It was initially inoculated by particle-gun bombardment and was then further propagated in *N. benthamiana*. The inoculation of *Arabidopsis* plants with PPV-R was performed as previously described (Decroocq et al. 2006).

Two other potyviruses were also used: TuMV, available as an infectious clone of the UK1 isolate (Jenner et al. 2000) provided by F. Ponz (Centro de Investigacion y tecnologica del Instituto Nacional de Investigaciones Agraria, Madrid) and propagated on turnip, and LMV-AF199, available in our laboratory (Krause-Sakate et al. 2002) and propagated in lettuce (cv. Trocadéro). Both viruses were inoculated as for PPV.

Phenotyping.

RIL and F2 plants were monitored for the appearance of symptoms and were scored 15 to 21 dpi. PPV symptoms consist of inflorescence distortions with more or less severe stunting. In severe cases, yellowing or browning of the inflorescences is also observed. Scoring of symptom severity was performed using a notation scale ranging from 0 to 4: 0 corresponds to plants in which the virus is present but that show no morphological differences as compared with the healthy plant: 1 to plants with no stunting but showing top incurvation on a single floral hamp, 2 to plants presenting distortions on a majority of hamps but with only a slight reduction of their size, 3 to plants with distortions on all floral hamps and showing a strong growth reduction, and finally, 4 to plants with complete inhibition of floral hamp development and severe stunting of the whole plant (Fig. 1). The scoring of F2 populations and RILs was performed at least twice by two independent evaluators. The final score used is the mean of these independent scorings.

At 21 days after inoculation, virus accumulation in inflorescence tissues was evaluated for each individual plant, using a semiquantitative double antibody sandwich-enzyme-linked immunosorbent assay (ELISA) assay with anti-PPV commercial reagents (D+M polyclonal antibody, LCA Laboratory, Blanquefort, France). Optical densities were normalized using a PPV-R infected, *N. benthamiana* positive control deposited on every ELISA plate of an assay. In the case of RILs, the final ACC value is the average of normalized measurements from all PPV-infected replicates of each RIL. In order to obtain more reproducible results and to eliminate potential interference from plant substances, all assays were performed on a 1:100 dilution of crude plant extracts prepared by grinding the plant tissues (1:4 wt/vol) in a phosphate buffered saline-Tween polyvinylpyrrolidone (PVP) buffer (136.9 mM NaCl, 1.47 mM KH_2PO_4, 2.68 mM KCl, 8.1 mM de Na_2HPO_4, 0.05% [vol/vol] Tween 20, 21% [wt/vol] PVP 25K). Independently of the evaluation of the viral concentration, this test allowed the confirmation of the presence of the virus in symptomlessly infected plants (0 in SYMP scale), thus excluding potential inoculation escapes.

Statistical analysis.

Data analysis was performed using the generalized linear model (PROC GLM) of the SAS package (SAS institute, Cary, NC, U.S.A.). ANOVA allowed the determination of the specific effect of 'genotype' and broad-sense heritability, which is the ratio between the genetic variance and the total phenotypic variance and is calculated using the formula:

$h^2 = \sigma^2_g/[\sigma^2_g + (\sigma^2_e/n)]$, where σ^2_g is the genetic variance, σ^2_e is the environmental variance, and n the number of replicates. Frequency distribution and Pearson's correlation coefficients were calculated using the statistical and graphical functions of EXCEL and the statistical software R.

Genetic determinants mapping.

A set of 91 markers was previously scored in the Col × L*er* RIL population as displayed on the NASC website. They were selected following two criteria. i.e., acceptable Chi square and few missing data. For the Cvi × L*er* linkage map, 99 markers were chosen and extracted from the European *Natural* cooperative database. The RIL-derived genetic maps were constucted using MAPMAKER 3.0 software and the Kosambi map unit function (Lander and Green 1987). MAPMAKER 3.0 was also used to map monogenic determinants in the Cvi × L*er* RIL population.

Both Col × L*er* and Cvi × L*er* linkage maps served as the basis for QTL analysis in QTL Cartographer for MS Windows version 2.5 (Basten et al. 1994; North Carolina QTL Cartographer website). Interval mapping (Lander and Botstein 1989) was first performed to determine putative QTL, and then, composite interval mapping (Zeng 1994) was performed, scanning the genome at 2-cM intervals and using a window size of 10 cM. Five markers were selected as cofactors under the forward regression method. A 1,000-permutations test allowed estimation of the LOD significance threshold for each trait, as described by Churchill and Doerge (1994). A conservative LOD

score of 2.7 was used as the threshold for detecting QTL significance. The percentage of the phenotypic variation explained by the QTL corresponds to the regression value R^2 taken at the peak LOD score of the QTL.

In addition, in cases of nonnormal trait distribution, a nonparametric Kruskal-Wallis rank-sum test was performed (Lehmann 1975). An association between trait and molecular markers was considered significant below the 1% level.

The program PROC GLM was used to perform a two-way interaction ANOVA to detect significant QTL × QTL epistasis via the corresponding marker × marker interactions and epistasis.

ACKNOWLEDGMENTS

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AUTHOR-RECOMMENDED INTERNET RESOURCES

European *Natural* database:
www.dpw.wau.nl/natural/resources/populations.htm
NASC website: arabidopsis.info/RI_data/full_markers.may2001.xls
INRA Versailles collection: dbsgap.versailles.inra.fr/vnat/
North Carolina State University QTL Cartographer website:
statgen.ncsu.edu/qtlcart/WQTLCart.htm

2.2.4. Bases génétiques, cartographie génétique et clonage positionnel de gènes de résistance au LMV

A partir du moment où nous avions identifié lors du criblage d'accessions d'Arabidopsis des phénotypes de résistance au LMV, notre intention était bien entendu d'identifier les gènes contrôlant ces résistances. Les résistances qui nous intéressent en premier lieu sont les résistances récessives dont l'allèle de sensibilité code pour un facteur nécessaire au cycle viral. En effet suite aux études qui ont conduit à identifier eIF4E comme non seulement produit de plusieurs gènes de résistance récessive chez plusieurs espèces végétales mais aussi comme facteur de sensibilité nécessaire au cycle viral (Robaglia et Caranta, 2006), ce type de résistance est non seulement une source potentielle de nouvelles résistances pouvant être transférée sur des espèces végétales d'intérêt agronomique mais offrent également de par leur nature un accès à une meilleure compréhension des interactions moléculaires plante/virus. L'autre type de résistance qui nous intéresse est constitué des résistances dominantes bloquant le mouvement à longue distance du virus. En dehors de la résistance RTM, ces résistances sont très mal connues. Nous avons écarté d'emblée les résistances dominantes pour lesquelles aucune multiplication virale n'est détectée. En effet il est très probable que ces résistances soient contrôlées par des gènes de type R, ce qui est beaucoup moins original, très compétitif et dont le transfert vers des espèces d'intérêt agronomique est plus délicat. Bien avant la thèse d'Ophélie et le criblage qu'elle a réalisé sur la core 24, j'ai analysé plus précisément 2 résistances, celle chez Cvi qui était active vis-à-vis des 3 isolats de LMV testés, et celle sur Col que j'ai introduit dans le paragraphe de 2.2.

2.2.4.1. Cartographie génétique du gène *RLM1* chez Cvi

Lorsque j'ai démarré l'analyse génétique de la résistance chez Cvi, nous n'avions encore pas de généticien dans l'équipe ni l'expertise en manipulation des marqueurs moléculaires. J'ai à ce stade contacté un certain nombre de groupe de généticiens d'Arabidopsis pour obtenir des populations (F1, F2, Ril, etc.) mais aussi des conseils pour la culture d'Arabidopsis et sur les sources d'informations disponibles.

Nous avons été très excités lorsque j'ai analysé une population F2 issue d'une population F1 fournie par nos collègues de Wageningen entre Cvi (résistant au LMV) et Ler (sensible au LMV) puisque l'analyse indiquait que cette résistance était contrôlée par un gène de résistance récessif. Cependant en reproduisant l'analyse sur un croisement réalisé au laboratoire, j'ai finalement constaté que la résistance était bien monogénique mais dominante (n'ayant pas contrôlé la F2 de Wageningen, je pense que les plantes F1 utilisées n'en étaient probablement pas mais plutôt du parent sensible). Au niveau phénotypique, j'ai pu montrer que cette résistance bloquait le mouvement du virus dans la plante puisque je ne pouvais détecter le virus qu'au niveau des feuilles inoculées et seulement par RT-PCR. J'ai tout de même entrepris la cartographie de ce gène (nommé RLM1 pour Resistance to Lettuce mosaic virus 1) à l'aide de RILS et NILS et ai localisé ce gène dans le haut du chromosome 1. Cependant faute de forces humaines suffisantes, le clonage positionnel de ce gène n'a pas encore abouti puisque j'ai choisi de porter notre effort sur la caractérisation de la résistance RTM avec en particulier le clonage positionnel de *RTM3*.

2.2.4.2. Clonage de *RTM3*

La résistance RTM étant active pour au moins trois potyvirus (TEV, LMV et PPV) et l'équipe de J. Carrington ne travaillant plus sur ce sujet, nous avons décidé de nous investir dans une étude plus approfondie de cette résistance.

L'équipe de Carrington a montré que cette résistance était sous le contrôle d'au moins trois gènes nommés *RTM1*, *RTM2* et *RTM3* (pour restricted TEV movement, Mahajan et al., 1998; Whitham et al., 1999). Seuls *RTM1* et *RTM2* ont été clonés et codent respectivement pour une protéine « jacalin-like » et une protéine ayant un domaine de « small heat shock protein » mais pour lesquelles aucune fonction n'avait été attribuée (Chisholm et al., 2000, Whitham et al., 2000). Il a été cependant montré que ces deux gènes étaient exprimés uniquement dans les tissus du phloème (Chisholm et al., 2001). Manquait donc le clonage de *RTM3*.

J'ai donc développé une collaboration avec J. Carrington qui était favorable à ce que ce projet se poursuive dans notre équipe et qui m'a fourni d'une part ses données préliminaires de cartographie de *RTM3* (cartographié dans un intervalle de 6 cM sur le chromosome 3) et d'autre part la population F2 utilisée pour la cartographie, produite entre le mutant *rtm3* (sensible au virus) et WS-2, porteur comme Col de la résistance RTM.

En 2004, j'ai obtenu un financement pour recruter un postdoc (Hien Le, bourse Haigneré de 18 mois de décembre 2004 à mai 2006)) afin de démarrer le clonage positionnel de *RTM3*. Après le départ de Hien qui n'avait pu affiner la cartographie du gène que dans un intervalle de 2 cM, Patrick Cosson, ingénieur d'étude, recruté dans notre laboratoire en février 2006 pour ces compétences en génétique moléculaire, a poursuivi sous mon encadrement ce clonage. *RTM3* a été cartographié dans un intervalle comprenant 22 gènes. Pour son identification, nous avons réalisé le séquençage de ces 22 gènes chez le mutant *rtm3* où une mutation non synonyme a été identifiée dans le gène At3g58350 (ceci fin 2007).

Pour valider que ce gène était bien *RTM3*, nous avons utilisé 2 lignées knock-out (KO) qui existaient pour ce gène (que nous avons génotypées et séquencées pour être sur de l'homozygotie de ces lignées ainsi que de la bonne insertion du T-DNA dans le gène) et qui se sont bien avérées sensibles au LMV. De plus, nous avons croisé ces lignées KO avec le mutant *rtm3* (test d'allélisme) et les F1 obtenues se sont aussi révélées sensibles au LMV.

At3g58350 code pour une protéine de 301 acides aminés de type « Meprin and Traf homology (MATH) domain-containing protein ». Cette famille de protéines est surtout connue chez les mammifères, les protéines Traf étant associées aux récepteurs du facteur de nécrose tumorale (Tumor Necrosis Factor ou TNF) et les méprines étant des métalloprotéases (Sunnerhagen et al., 2002). Ces protéines partagent un domaine conservé d'environ 180 acides aminés présentant un repliement de 7-8 brins β antiparallèles et sont trouvés chez de nombreux eucaryotes (Zapata et al., 2007). Ce domaine est situé entre les acides aminés 13 à 136 de RTM3. Un alignement du domaine MATH de RTM3 avec ceux de protéines TRAF humaines met en effet en évidence des homologies de séquences au niveau des brins β des domaines MATH entre RTM3 et les protéines TRAF (Figure 3A). En revanche, aucune information de structure secondaire n'était indiquée au niveau de la région C-terminale de RTM3 dans laquelle une longue hélice α est prédite (Figure 3B). En analysant cette région avec plusieurs programmes de prédiction de structures protéiques, j'ai pu identifier un domaine coiled coil (CC) entre les positions 216 et 293. Il est à noter que la mutation présente dans le mutant *rtm3* est située dans ce domaine, ce qui signifierait que ce dernier est nécessaire à la fonction de résistance de cette protéine (Figure 3B).

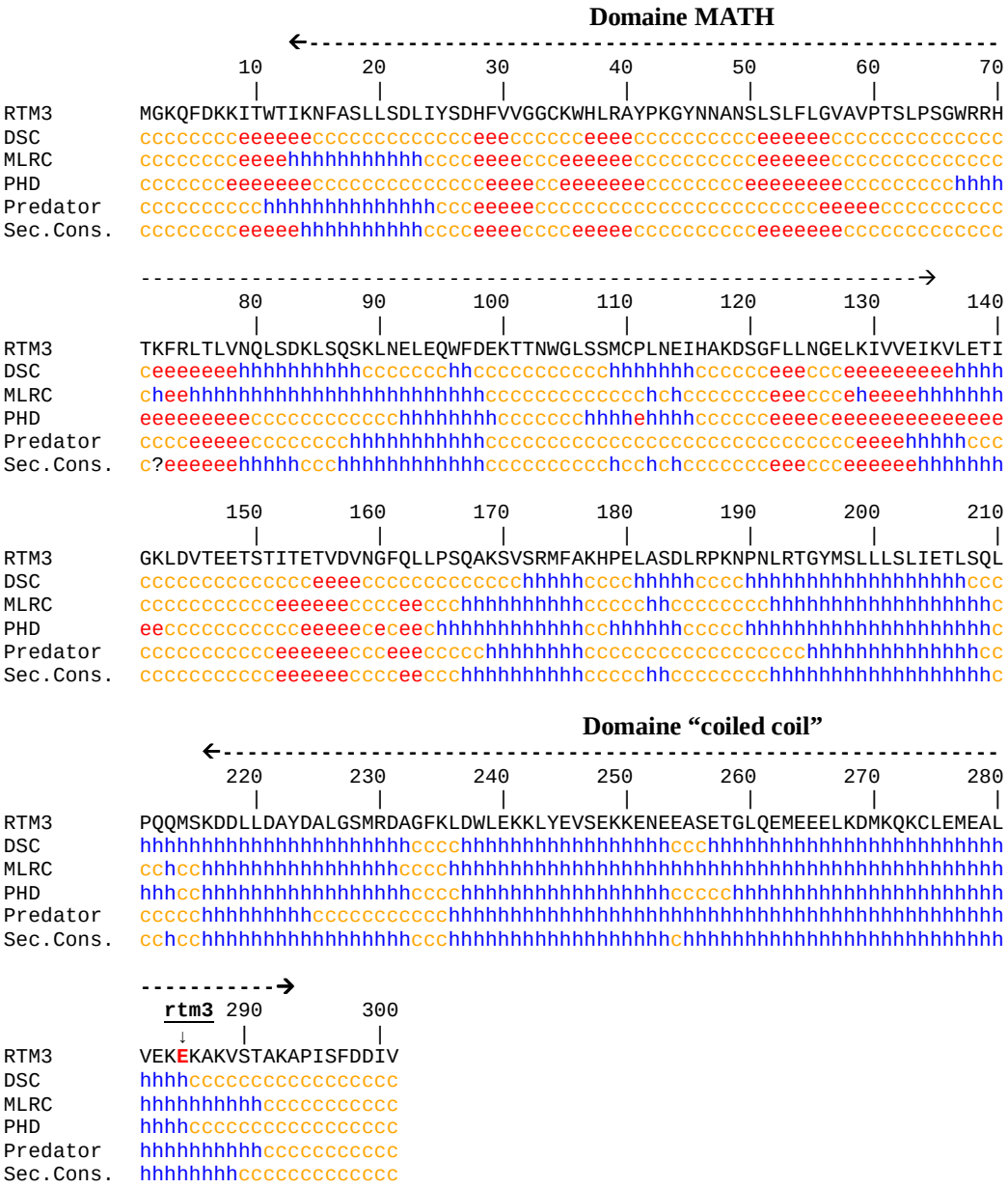
Figure 3: **Prédiction de la structure secondaire de la protéine RTM3**.

(A) Alignement de la séquence en acide amine du domaine MATH de RTM3 avec 4 protéines TRAF humaines. Les 8 feuillets β identifiés dans TRAF2 (Park et al., 1999) sont indiqués par des flèches. Les nombres indiqués entre parenthèse indiquent la position des acides aminés de début et de fin des domaines MATH de chacune des protéines. Les acides aminés conservés entre RTM3 et au moins une protéine TRAF sont grisés.
(B) Prédiction en utilisant 4 programmes différents à partir du site NPS@ Web server (Network Protein Sequence @nalysis) du pôle bioinformatique lyonnais. Sec. Cons correspond à la prédiction consensus.. “h” indique une hélice, “e” indique un feuillet β et “c” indique un « random coil ». Les nombres au dessus de la séquence indiquent la position des acides aminés dans la séquence de la protéine RTM3. Le domaine « coiled coil » prédit entre les positions 216 et 293 est indiqué par la double flèche. La position de la mutation chez le mutant *rtm3* est indiquée à la position 284 (en rouge).

A

Figure 3 (suite): **Prédiction de la structure secondaire de la protéine RTM3**.

B



J'ai pu identifier 71 gènes pour lesquels la protéine correspondante contient un ou plusieurs domaines MATH (Figure 4). Parmi ces gènes, 29 codent pour une protéine qui possède également un domaine CC (domaine plus large d'ailleurs que la structure CC proprement dite). De façon intéressante, j'ai pu également identifier une dizaine de protéines homologues à RTM3 qui ne contenaient qu'un domaine CC, sans domaine MATH, pour lesquelles les gènes correspondant sont situés dans des clusters comprenant des gènes avec domaine MATH et CC. Ainsi *RTM3* est situé dans un cluster de gènes comptant 17 gènes avec domaines MATH et CC, 5 gènes avec un domaine CC uniquement et un gène avec un domaine MATH uniquement, suggérant que ces gènes ont subi des événements de duplication associés à des événements de délétion ou d'insertion. Il n'est donc d'ailleurs pas surprenant de constater que les gènes ayant des pourcentages d'identité les plus élevés avec *RTM3* font

partie de ce cluster. Un schéma présentant la structure des protéines homologues à RTM3 est présenté sur la figure 5.

A ce jour, aucune fonction n'a été attribuée à ces gènes (avec domaine MATH et/ou CC) chez *Arabidopsis thaliana*. L'implication de *RTM3* dans le blocage du mouvement à longue distance des potyvirus est donc la première fonction connue de ce type de gène.

Un papier décrivant le clonage de *RTM3* et incluant des aspects fonctionnels présentés dans le paragraphe 2.4.3 devrait être soumis à PNAS dans les semaines à venir.

Figure 4: **Localisation des gènes homologues à *RTM3* sur le génome d'Arabidopsis**. Carte réalisée avec le « the chromosome map tool" du TAIR. Les flèches noires indiquent les gènes codant des protéines avec des domaines MATH et CC. Les flèches grises indiquent les gènes codant pour des protéines ne contenant que le domaine CC. Les chiffres en haut des chromosomes indiquent le numéro des chromosomes d'Arabidopsis

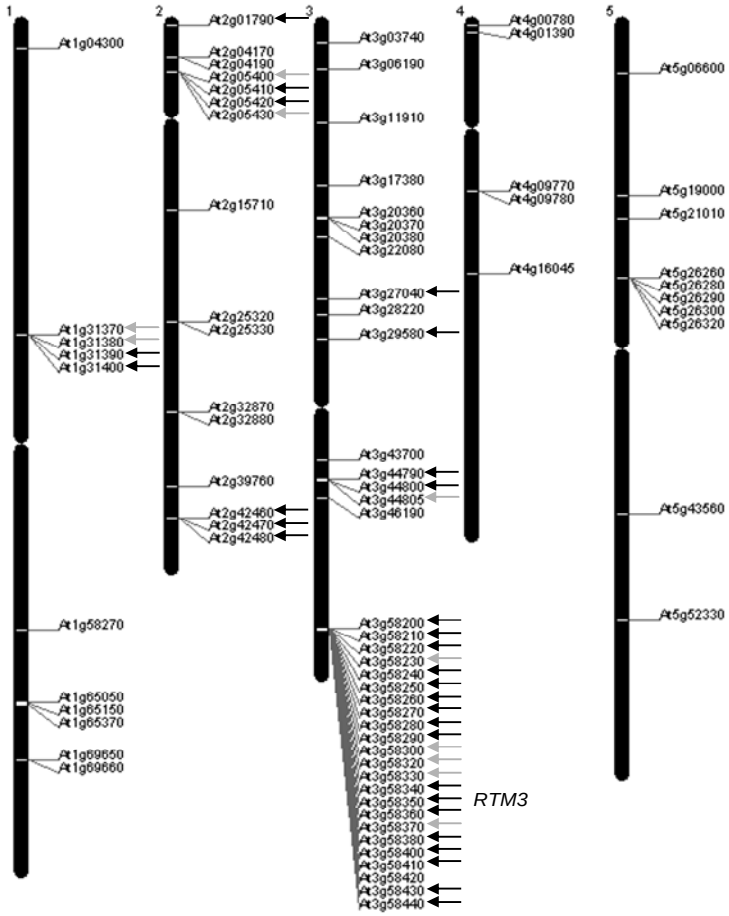
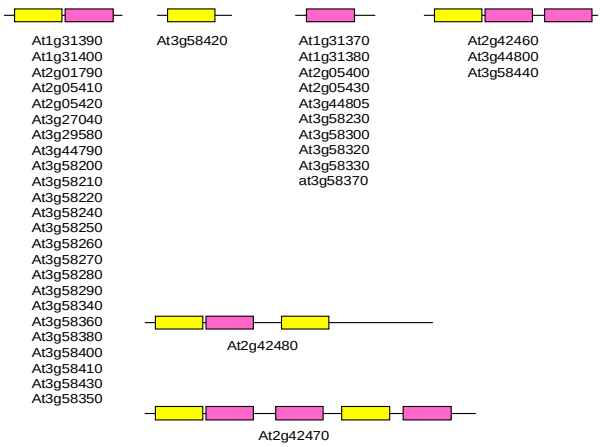


Figure 5: **Organisation schématique des protéines homologues de RTM3**. En jaune : les domaines MATH ; en rose : les domaines CC



2.2.4.3. Caractérisation des bases génétiques d'autres résistances au LMV

Suite à mes travaux de phénotypage de quelques accessions d'Arabidopsis avec le LMV publiés en 2003 et à ceux de la thèse d'O. Sicard réalisés sur la collection des 24 accessions de Versailles, j'ai initié, en plus des travaux de cartographie et de clonage de *RLM1* et *RTM3*, la caractérisation génétique d'autres résistances obtenues. L'objectif de ces analyses préliminaires était d'établir la base génétique de ces résistances, c'est-à-dire déterminer le caractère récessif ou dominant de chacune des résistances par l'analyse de populations F1 ainsi que d'établir si possible le nombre de gènes impliqués à l'aide de populations F2 pour nous permettre ensuite d'identifier les projets de clonage positionnel.

Pour cela, nous avons produit des populations F1 et F2 entre ces accessions résistantes et l'accession Ler sensible à tous les isolats de LMV, que j'ai inoculées avec l'isolat AF-199 de ce virus. Ces données montrent qu'au moins deux résistances seraient de nature récessive. La confirmation de ces données ainsi qu'une cartographie génétique grossière de ces deux résistances est actuellement menée sous ma responsabilité par Valérie Schurdi-Levraud (MC Université Bordeaux2), assistée de Patrick Cosson (IE) et Mélodie Bousquet (TR, recrutée dans notre groupe en septembre 2008). Si nous confirmons le caractère récessif de ces résistances, l'une d'entre elles (ou les deux si différentes et si force humaine suffisante !) fera l'objet d'un projet de clonage positionnel.

Pour nous aider dans ces analyses de cartographie de gènes, nous sommes de plus en train de développer un panel de marqueurs moléculaires basés sur des microsatellites. Nous les choisissons de telle façon qu'ils soient répartis à intervalle régulier sur l'ensemble du génome d'Arabidopsis, qui soient le plus polymorphes que possible afin de pouvoir les utiliser dans des analyses de cartographie génétique entre le plus grand nombre d'accessions d'Arabidopsis que possible, en particulier au sein de la core 24 de la collection de l'INRA de Versailles que nous avons testée avec LMV et PPV. Ces marqueurs ont l'avantage d'être facilement exploitables en laboratoire (révélation sur gel d'agarose la plupart du temps) et de permettre de réaliser des cartographies génétiques grossières préalablement aux cartographies fines

conduisant au clonage positionnel des gènes d'intérêt. **Ce travail est conduit sous ma responsabilité par P. Cosson et devrait être valorisé sous forme de publication début 2010**.

2.2.5. Analyse fonctionnelle de la résistance RTM

2.2.5.1. Identification d'allèles RTM non fonctionnels pour la résistance au LMV

Parallèlement au clonage de *RTM3*, nous avons démarré des études visant à comprendre comment ces gènes fonctionnaient. Dans un premier temps, nous avons voulu savoir pourquoi certaines accessions d'Arabidopsis n'étaient pas résistantes aux isolats viraux bloqués par les gènes RTM chez Col. Comme une mutation dans un des gènes RTM semble suffisante pour rendre inactive cette résistance, nous avons fait l'hypothèse que dans les accessions sensibles aux virus, au moins un allèle d'un des trois gènes RTM était muté et donc plus fonctionnel pour cette résistance. Nous avons donc entrepris le séquençage des gènes *RTM1*, *RTM2* et *RTM3* de la collection d'accessions de Versailles (Figure 6). Si RTM1 ne présente pas un grand nombre d'allèles distinct (4 allèles identifiés), RTM2 et RTM3 sont beaucoup plus variables (12 allèles pour RTM2 et 11 pour RTM3).

Nous avons ensuite réalisé des tests de complémentation (ou test d'allélisme) en complémentant les mutants rtm avec l'allèle correspondant (lorsque cet allèle était bien entendu différent de l'allèle de Col) provenant d'une accession sensible. Pour cela, nous avons donc produit des F1 entre mutant rtm et accessions sensibles que nous avons inoculées avec LMV-AF199. Si la F1 se révélait sensible, cela signifiait que l'allèle rtm de l'accession sensible n'avait pas complémenté le mutant rtm correspondant et était donc un allèle ayant perdu sa fonction de résistance. En revanche si la F1 s'avérait résistante alors l'allèle rtm n'avait pas perdu sa fonction de résistance. Cette analyse nous a permis de montrer que 2 allèles RTM1, 4 allèles RTM2 et 6 allèles RTM3 étaient des allèles ayant perdu leur fonction de résistance (Figure 6). De plus en fonction de la position des mutations dans ces allèles, nous avons pu pour certains cas déduire l'implication de certains domaines des protéines RTM dans la fonction de résistance. Ainsi le domaine jacaline ainsi que l'extrémité C-terminale de RTM1, le domaine correspondant à une longue structure en hélice alpha ainsi que le domaine entre l'hélice et le domaine transmembranaire de RTM2 et les 2 domaines de RTM3 semblent impliqués dans la résistance.

Pour confirmer que la perte de la fonction de résistance était bien liée aux mutations dans la protéine et non liée à un problème d'expression, des mesures de l'expression des 3 gènes RTM chez les accessions sensibles au LMV-AF199 sont en cours pour compléter ce travail.

Un manuscrit décrivant l'ensemble de ces données est en cours de préparation (soumission envisagée fin 2009).

Figure 6: Séquences des protéines RTM1, RTM2 et RTM3 d'un ensemble d'accessions d'Arabidopsis. Seuls les acides aminés qui différent de la séquence de Col et leur position dans les protéines RTM de Col sont indiqués. La colonne « statut » indique si les allèles RTM sont des allèles de sensibilité (Sens) ou de résistance (Res) suite aux tests d'allélisme. nd= non déterminé (quand accessions résistantes au LMV) ; del= délétion. La position des différents domaines des protéines RTM est indiquée au dessus de la position des acides aminés. Les accessions en gras sont résistantes au LMV.

RTM1:

										←----------**Domaine jacaline**----------→									
Accessions										11	29	56	62	65	93	132	139	169	statut
Col-0, Ws-2, Edi-0, Tsu-0, **Ita-0**, **Can-0**, Bur-0, **Alc-0**, Blh-1, Mh-1, Oy-0, Sha, Akita, Sakata, **N13**, **Jea**, **Stw-0**, **Mt-0**, **Cvi-0**, **Gre-0**, **Pyl-1**, **St-0**, **Ge-0**										A	S	S	F	N	N	G	D	S	Res
Bl-1, Ct-1, C24											Y		C	D	I				Sens
Kn-0										T									Nd
Ler																		Stop	Sens

RTM2:

	←----------**HSP**----------→				←----------**helice α**----------→														
Accessions	11	28	71	94	127	135	176	188	225	232	233	243	287	288	317	350	351	355	statut
Col-0, Jea, St-0, Edi-0, **Mt-0, Ge-0, Can-0**, Bur-0, Mh-1, Oy-0, **N13**	G	Q	S	N	A	L	A	K	K	L	K	E	S	A	S	S	S	S	Res
Ler												K							Res
Stw-0					T														Nd
Ita-0						F				Q				T					Res
Bl-1, Sakata									N							del	del		Sens
Sha, Blh-1, **Pyl-1**							V												Sens
Akita											R								Sens
Cvi-0															N				nd
Ws-2, Gre-0, Tsu-0	L		N					N											Res
Alc-0, C24			N																Res
Ct-1			N					N					L						Sens
Kn-0			N					N										F	Nd

Figure 6 (suite): Séquences des protéines RTM1, RTM2 et RTM3 d'un ensemble d'accessions d'Arabidopsis

RTM3:

	←----------Domaine MATH----------→						←--insertion--→									←----------Domaine CC----------→											
	3	5	12	20	40	50					70	77	78	91	101	107	138	139	146	159	176	183	216	261	274	293	statut
Col-0, Ler, **Ws-2, Jea, Kn-0, Stw-0, Ita-0, N13**	K	F	T	L	A	S					H	L	V	N	T	S	E	T	T	N	A	S	K	Q	C	A	Res
Pyl-1, St-0		Y		P			G	V	G	V			I	T	A		Q				E			K			Nd
Bl-1, **Gre-0**, Sha, Akita, **Mt-0**, Ct-1		Y		P	V											P					E	T		K		S	Sens
Sakata	N			P	V											P					E	T		K		S	Sens
Tsu-0, **Alc-0**	N			P	V											P		I			E	T				S	Sens
Edi-0, Mh-1, Oy-0	N			P	V						R					P		I			E	T			Y		Sens
Ge-0				P																K			N				Nd
Can-0				P															I								Nd
Cvi-0				P																							Nd
Bur-0			K	P								P															Sens
Blh-1				P		stop																					Sens

2.2.5.2. Identification du déterminant viral impliqué dans le contournement de la résistance RTM

Identifier le facteur viral impliqué dans le contournement de résistance est un élément déterminant pour mieux comprendre le mécanisme moléculaire contrôlant cette résistance. Dans le cas de la résistance RTM, nous avons pu développer deux approches, l'une avec le PPV et l'autre avec le LMV, qui nous ont conduits à identifier ce déterminant dans la région N-terminale de la capside (CP). La stratégie développée avec le PPV par V. Decroocq a consisté à travailler avec des virus chimères obtenus entre un isolat contournant et un autre ne contournant pas la résistance RTM. De mon coté avec le LMV, assisté de L. Svanella (AI) et P. Cosson (IE), j'ai exploité le fait qu'occasionnellement lors d'inoculation de Col, porteur de la résistance RTM, des plantes apparaissaient sensibles à ce virus. Après une à deux phases d'inoculation sur Col à partir de ces plantes infectées, j'arrivais à obtenir 100% de plantes sensibles, mettant en évidence la sélection de variants de LMV. Après séquençage de la région N-terminale de la CP (et séquençage complet d'un des variants pour s'assurer que d'autres mutations n'étaient pas en cause), nous avons identifié une mutation ponctuelle chez chacun des variants analysés engendrant un changement en acide aminé (Figure 7). Cette position était très souvent variable entre variants mais toujours située dans une même région d'une dizaine d'acides aminés à proximité du triplet DAG, motif conservé dans la région N-terminale des CP de potyvirus. Conjointement aux expériences menées sur le PPV, ces données mettaient ainsi en évidence le rôle clef de cette région de la CP dans le contournement de la résistance RTM. Il est intéressant de noter ici que la CP, et en particulier le domaine N-terminal, avait d'ailleurs été impliquée dans le mouvement à longue distance des potyvirus (Dolja et al., 1995).

Figure 7. **Mutations identifiées dans différents variants de LMV capables de contourner la résistance RTM**. Seule la séquence en acide aminé de l'extrémité N-terminal de la CP est représentée. « / » correspond à la jonction Nib/CP. - indique les acides aminés identiques à la séquence de LMV-AF199.

```
LMV-AF199   Q/VDAKLDAGQGSKTDDKQKNSADPKDNIITEKGSGSGQVKKDDDINAGLHGKHTIPRTKA
LMV-AFVAR1  -/---------D-----------------------------------------------
LMV-AFVAR2  -/----------N-----------------------------------------------
LMV-AFVAR3  -/-------------G-------------------------------------------
```

L'ensemble de ces données a fait l'objet d'une publication actuellement « sous presse » dans MPMI.

2.2.5.3. Etude des interactions protéine-protéine impliquées dans la résistance RTM

Après avoir cloné *RTM3* et identifié la CP comme facteur viral du contournement de la résistance RTM, nous avons récemment engagé des expériences pour tenter de mettre en évidence des interactions entre les différents facteurs de plante et viraux impliqués dans ce mécanisme de résistance. L'approche la plus simple à mettre en œuvre pour ce type d'étude est l'utilisation du système double-hybride chez la levure. Nous avons donc testé par cette technique les interactions potentielles entre les 3 protéines RTM et les CP du LMV et PPV.

Les résultats obtenus (Figure 8A) montrent que :

- RTM1 interagit faiblement sur elle-même, confirmant ainsi les résultats publiés par l'équipe de Carrington (Chisholm et al. 2001)
- RTM3 interagit fortement avec elle-même
- RTM3 interagit fortement avec RTM1
- Le domaine CC de RTM3 interagit fortement sur lui-même
- RTM2 n'interagit ni avec elle-même ni avec les autres protéines RTM ou les CP virales
- Aucune des protéines RTM n'interagit avec les CP du LMV ou PPV (CP issue d'isolats contournant ou non contournant).

Ces premiers résultats d'interaction semblent indiquer que les protéines RTM pourraient former un complexe, composé au minimum de RTM1 et RTM3. Ces données d'interaction produites par le système double-hybride ont aussi été confirmées *in planta* en utilisant la technique de « Bimolecular Fluorescence Complementation » (BiFC) sur cellules d'épidermes d'oignons (Figure 9).

Afin dévaluer l'importance de ces interactions dans le mécanisme de la résistance, nous avons également testé les interactions entre la protéine RTM3 issue du mutant *rtm3* qui du fait de la mutation est sensible au LMV, TEV et PPV, ainsi que l'allèle RTM1 de l'accession Ler, identifié lors des tests d'allélisme décrits ci-dessus comme étant un allèle ayant aussi perdu sa fonction de résistance.

De façon intéressante, la forme RTM3 mutée n'est plus capable d'interagir avec RTM1 mais reste capable d'interagir sur elle-même (Figure 8B) alors que la forme RTM1 de Ler reste capable d'interagir aussi bien avec RTM3 qu'avec elle-même. Ces résultats indiquent donc que l'interaction RTM1-RTM3 est nécessaire pour la résistance mais indiquent aussi que la région C-terminale de RTM1 tout en étant non impliqué dans l'interaction avec RTM3, intervient dans le mécanisme de la résistance. Identifier le rôle de cette extrémité C-terminale de RTM1 est évidemment une perspective fort excitante.

Ces dernières expériences d'interaction avec RTM3 du mutant *rtm3* ont été pour l'instant uniquement réalisées en double-hybride et sont en cours de réalisation *in planta* par l'approche BiFC.

L'ensemble des données d'interactions présentées ici seront incluses dans le papier « RTM3 ».

Figure 8 : **Expériences de double-hybride avec les protéines RTM**

A. Expériences d'interaction entre les protéines RTM sauvages. 6 répliquâts de chaque combinaison de co-transformation ont été repiqués sur milieu –LT (contrôle de co-transformation), -LTH et –LTHA (milieux de sélection des interactions). C- indique les contrôles négatifs et C+ indiquent les contrôles positifs. Les clones dans le vecteur pGAD-T7 sont indiqués à l'horizontale et les clones dans le vecteur pGBK-T7 sont indiqués à la verticale

B. Expériences d'interaction avec la protéine RTM3 mutée issues du mutant *rtm3*. Pour chaque combinaison le premier clone indiqué est dans le vecteur pGAD-T7 et le second dans pGBK-T7. pGAD-T et pGBK-53 sont les contrôles fournis par Clontech utilisés comme contrôle positif. pGAD et pGBK sont respectivement les plasmides vides pGAD-T7 et pGBK-T7 utilisés comme contrôle négatif d'interaction.

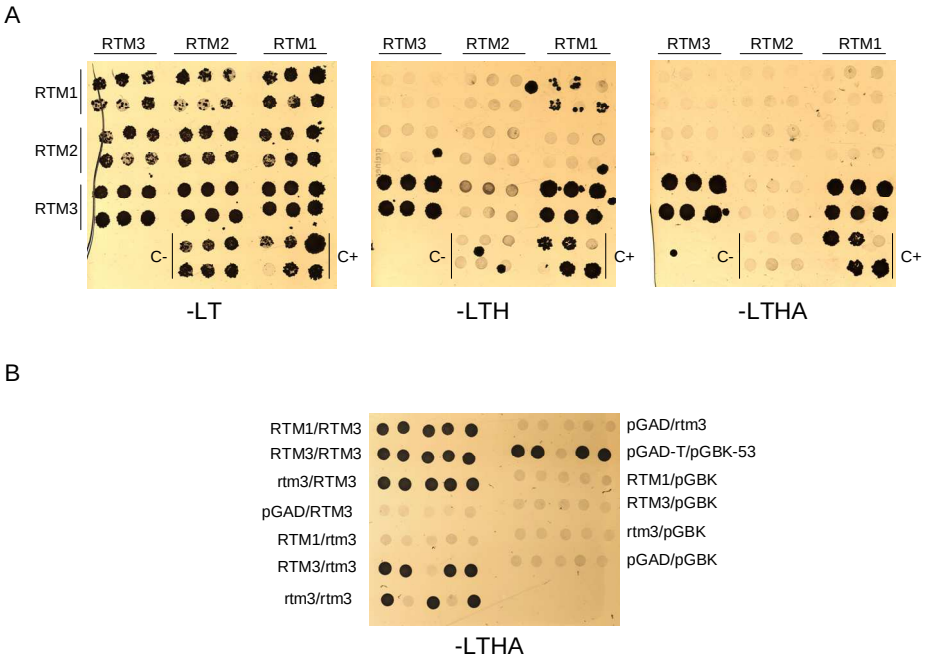
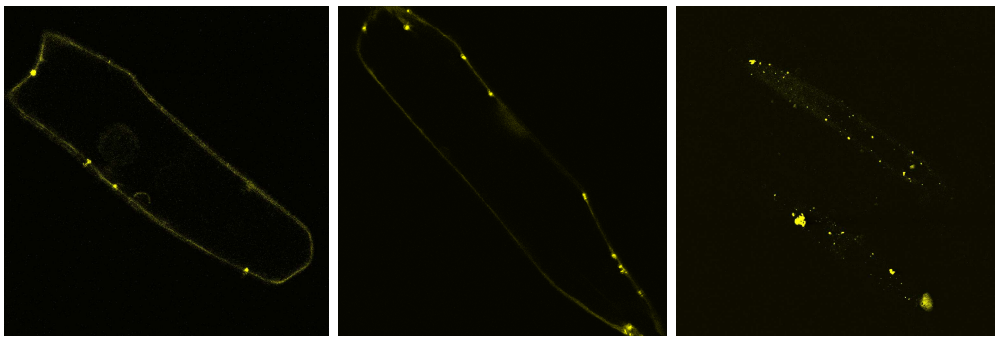


Figure 9 : **Expériences d'interaction entre les protéines RTM1 et RTM3 en BiFC**. Images réalisées en microscopie confocale (avec un Leica TCS SP2) avec l'objectif x40 de cellules d'épiderme d'oignon co-transformées par biolistique et excitées à 514 nm afin d'observer la YFP.



RTM3/RTM3 RTM1/RTM1 RTM1/RTM3

2.2.6. Analyse du transcriptôme de plantes infectées par des potyvirus

En parallèle de l'approche génétique qui consiste à aller du phénotype au gène, j'ai aussi développé au laboratoire une approche d'analyse du transcriptôme de plantes infectées par des potyvirus. Ce projet a été développé fin 2002 en collaboration avec Carole Caranta (GAP INRA Avignon) afin d'analyser et comparer les transcriptomes de la tomate et d'Arabidopsis infectées par un même potyvirus, le virus de la marbrure du tabac (TEV). Les principaux objectifs de ce projet étaient :

- L'identification de gènes dérégulés d'une part par l'infection virale et d'autre part en relation avec la symptomatologie;
- La caractérisation de la cinétique de régulation de ces deux types de gènes;
- L'identification de régulations transcriptionnelles communes à deux dicotylédones différentes, ou au contraire propres à l'une d'entre elles;
- La cartographie et étude de conservation de synténie des gènes régulés par l'infection virale chez *Arabidopsis thaliana* et chez la tomate ;
- Evaluer une corrélation entre la cartographie de ces gènes et celle de gènes et QTL connus en relation avec l'infection par un potyvirus.

Nous avons pu bénéficier pour ce projet Aratom (pour ARAbidopsis et TOMate) d'un financement postdoctoral de décembre 2002 à novembre 2003 par le Ministère de la recherche (recrutement de Xiaoyan Feng, postdoc chinoise, qui a partagé son séjour entre Avignon et Bordeaux), et d'une aide financière de quelques k€ provenant de l'Action Transversale Structurante Tomate de l'INRA. Après avoir identifié au moins une souche de TEV infectant les deux espèces végétales et avoir réalisé la cinétique d'infection de cette souche virale dans ces plantes, nous avions décidé de mener successivement 2 approches pour l'identification de gènes dérégulés au cours de l'infection, la technique de cDNA-AFLP, réalisée à Avignon et l'hybridation de microarays, réalisée à Bordeaux.. Les hybridations des microarrays de tomate ont été réalisées au niveau de la plateforme de génomique fonctionelle à l'IBVM et celles d'Arabidopsis à l'INRA d'Evry. L'analyse a été effectuée à la fois sur feuilles inoculées et sur feuilles supérieures. Environ 600 gènes dont l'expression était modifiée ont été détectés chez la tomate et plus de 1600 chez Arabidopsis dont 90% sur feuilles inoculées et seulement 10% au niveau des feuilles supérieures, ceci chez les deux espèces étudiées. Un petit nombre seulement de gènes communs aux deux espèces ont été identifiés, en partie lié à la proportion beaucoup plus faible des gènes totaux de tomate représentée sur les microarrays utilisées. Les modifications d'expression de quelques gènes ont été validées chez la tomate par RT-PCR semi-quantitative lors du stage de DESS de Maud Rivard en 2004.

Suite au bref séjour de X. Feng, ce projet n'a pas été immédiatement poursuivi faute de moyen humain et il n'a été repris qu'à partir de 2006 par V. Schurdi-Levraud (MC Bordeaux2), à un moment où elle a du réaliser un changement thématique.

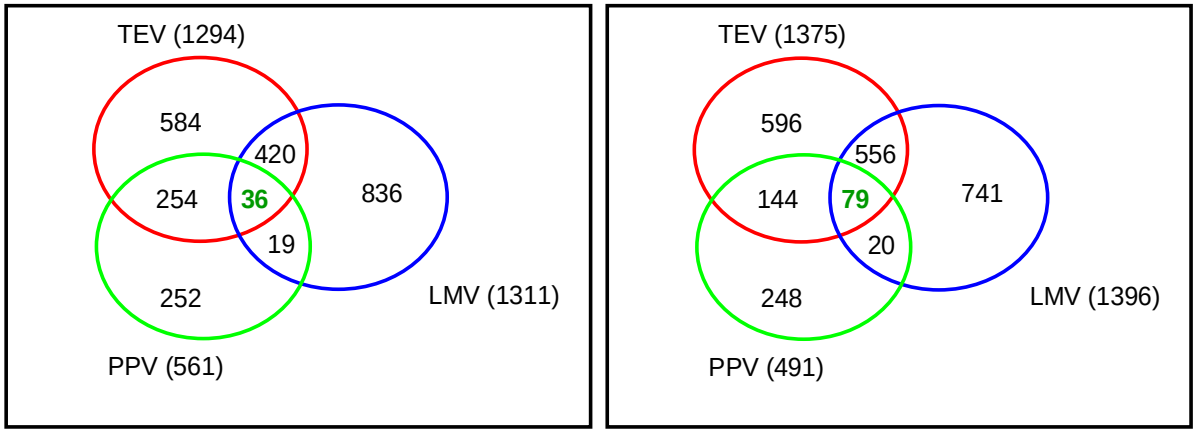
Nous avons décidé, non pas de réaliser une validation des données de microarrays par une approche classique de qRT-PCR sur quelques gènes, mais de valider ces données en répétant des analyses de microarrays sur plusieurs pathosystèmes potyvirus/Arabidopsis. Par cette approche nous pouvions ainsi valider un panel de gènes retrouvés en commun dans plusieurs pathosystèmes lors d'infection avec des potyvirus. Pour cela, nous avons de nouveau hybridé des lames CATMA d'Arabidopsis en collaboration avec l'URGV d'Evry à partir d'ARN totaux issus de feuilles inoculées avec le TEV (virus utilisé lors de la première expérience), le LMV et le PPV sur la même accession d'Arabidopsis, Ler. L'analyse a révélé

d’une part qu’une grande proportion des gènes dérégulés lors de la première expérience avec le TEV l’était aussi dans cette deuxième expérience, et d’autre part que plusieurs dizaines de gènes dérégulés étaient identifiés en commun dans les 3 pathosystèmes analysés (Figure 10).

Afin de distinguer parmi ces gènes dérégulés ceux dont la dérégulation est une conséquence de l’infection virale de ceux qui ont un rôle actif lors de l’infection, nous avons opté pour analyser plusieurs dizaines de lignées KO. Ces lignées correspondent principalement à des gènes dérégulés identifiés dans les 3 pathosystèmes décrits et aussi identifiés dans des travaux publiés. Près de 70 lignées ont été récupérées et génotypées. Nous nous heurtons malheureusement depuis 1 an à un problème de phénotypage de ces lignées. En effet, nous avons choisi pour une lecture simple du phénotype sur feuille inoculée d’utiliser un virus étiqueté avec le gène GUS permettant de révéler et quantifier les foyers d’infection. Des essais avec le TEV-GUS ou le PPV-GUS montrent que sur Columbia (parent dont sont issues les lignées KO), le nombre de foyers d’infection varie de 0 à 2 en moyenne ce qui est faible pour une comparaison significative avec des lignées qui seraient devenues résistantes (indiquant que le gène muté est essentiel pour l’infection virale). Nous sommes en train de modifier les paramètres d’inoculation en concentrant notamment l’inoculum viral pour espérer avoir un phénotype d’infection efficace sur Col. Ces données seraient en effet particulièrement utiles pour pouvoir raisonnablement bien valoriser ce travail, sachant que peu d’études de transcriptomique publiées ont présenté des données d’analyse de lignées KO de gènes dérégulés.

Si l’analyse de ces lignées KO ne montre aucune implication directe d’un ou plusieurs gènes dérégulés lors de l’infection virale, nous pourrons clore ce projet. En revanche, si nous identifions au moins un gène montrant, suite à l’analyse du KO correspondant, son implication potentielle dans l’infection virale (KO résistant au virus) ou dans les réactions de défense (KO avec une sensibilité accrue), nous développerons une analyse approfondie de ce(s) gène(s).

Figure 10 : Répartition des gènes dont l'expression est modifiée lors d'infection virale par le TEV, LMV et PPV. Le nombre entre parenthèses qui suit le nom des virus correspond au nombre total de gènes dérégulés pour un virus donné.



Gènes induits **Gènes réprimés**

Projet :

Etudes des interactions moléculaires *Arabidopsis thaliana*/potyvirus : étude du mouvement à longue distance

Suite au criblage d'accessions d'Arabidopsis avec le LMV et le PPV, nous avons identifié un panel de résistance dont le phénotype se caractérise par une absence de mouvement à longue distance des virus bien qu'une multiplication virale soit détectée dans les feuilles inoculées. Mise à part la résistance RTM sur laquelle j'ai investi une partie des forces de mon groupe, aucune autre résistance de ce type n'a pour l'instant été caractérisée par d'autres équipes (Scholthof, 2005 ; Gomez et al., 2009). L'étape même du mouvement à longue distance des potyvirus est actuellement très mal connue sans qu'aucun facteur de plante impliqué dans ce processus biologique n'ait été identifié jusqu'à présent (Gomez et al., 2009).

Le projet que je me propose de développer visera ainsi à poursuivre et à développer un ensemble d'approches (génétique, biologie moléculaire et biochimique) dans l'objectif d'identifier des facteurs de l'hôte impliqués dans le mouvement à longue distance des potyvirus et de déterminer la fonction de ces facteurs afin d'élucider le ou les mécanismes moléculaires qui gouvernent cette phase du mouvement à longue distance à la fois lors d'interaction compatible (infection virale) que lors d'interaction incompatible (blocage de ce mouvement). Cette thématique comme celles développées dans le reste de l'équipe s'inscrit dans le champ thématique 1 **«** Génomique fonctionnelle et biologie intégrative des interactions biotiques autour de la plante cultivée », OP3 « Mécanismes du pouvoir pathogène des bioagresseurs et réactions de défense des hôtes (plantes, insectes) » du département SPE de l'INRA.

1. Caractérisation génétique de résistance bloquant le mouvement à longue distance du LMV

Identifier par clonage positionnel d'autres facteurs de résistance sera un axe majeur du projet. Comme présenté dans la partie 2.3.3., nous ciblerons les résistances qui bloquent le mouvement systémique du LMV, qu'elles soient dominantes ou récessives.

Nous avons déjà établi la base génétique d'une partie importante des résistances identifiées lors du criblage de la core24 avec LMV-AF199 avec en particulier l'identification de 2 résistances récessives. Nous poursuivrons cette caractérisation génétique, en particulier pour savoir si ces résistances sont gouvernées par les mêmes gènes ou des gènes distincts. Dans le cas des résistances dominantes, nous établirons si ces résistances sont dépendantes ou indépendante des gènes RTM en analysant des populations F2 à l'aide de marqueurs moléculaires à proximité de ces gènes.

Nous envisageons également de caractériser des résistances sur une deuxième collection d'accessions d'Arabidopsis pour laquelle des milliers de SNP ont été développés pour des analyses de cartographie (Clark et al., 2007).

Il est évident que même si potentiellement ces analyses ouvrent un nombre important de projet de clonage positionnel, nous ne ciblerons que quelques gènes. En effet, à la fois la taille modeste de l'équipe ainsi que la possibilité de ne réaliser des analyses qu'à moyen débit, ne permet pas d'envisager plusieurs clonages simultanément. De ce fait, nous nous attacherons dans un premier temps à l'une des 2 résistances récessives identifiées dans la core 24.

Nous continuerons également à analyser la base génétique de la résistance RTM sur Col-0. En effet, le criblage d'une banque de mutant de Col-0 par l'équipe de Carrington avait révélé 3 gènes RTM. En analysant une population F2 puis des RILs entre Col-0 et l'accession Nd, sensible à LMV-AF199, une cartographie préliminaire met en évidence l'implication de trois gènes. Si un de ces gènes a pu être cartographié à proximité de *RTM3*, les 2 autres ne

sont absolument pas liés à *RTM1* et *RTM2*. Cela laisse donc supposer qu'au moins 2 autres gènes différents des 3 gènes RTM identifiés seraient aussi impliqués dans la résistance RTM. De ce fait, nous réaliserons une analyse de différentes populations F2 produites entre Col-0 et des accessions sensibles au LMV afin de voir si nous pouvons révéler par cette approche d'autres gènes RTM.

Pour cette partie, sont impliqués V. Schurdi-Levraud, MC Université Bordeaux2, généticienne de formation, P. Cosson, IE, expert en génétique moléculaire et M. Bousquet, TR, impliquée dans le phénotypage des populations d'Arabidopsis.

2. Identification de facteurs de plante impliqués dans le mouvement à longue distance des potyvirus : le projet « Viromouv »

En France, très peu de groupe travaillent sur le mouvement à longue distance des virus de plante. Deux équipes travaillent sur des virus qui sont restreint aux tissus vasculaires et qui appartiennent au genre *Polerovirus*, l'équipe de Véronique Brault, travaillant dans l'équipe « virologie-vection » de l'UMR « santé de la vigne et qualité du vin » à l'INRA de Colmar et l'équipe de Véronique Ziegler-Graff travaillant dans l'équipe « virologie intégrative » à l'IBMP à Strasbourg. Je leur ai proposé une collaboration afin de proposer un projet au programme de génomique de l'ANR en 2008. Ce projet intitulé « Identification de facteurs de l'hôte impliqués dans le mouvement à longue distance des virus de plante » a été retenu et j'en assure la coordination. Ce projet a démarré début 2009 et se terminera fin 2011. L'objectif est d'identifier des facteurs des tissus phloémiens impliqués dans l'étape du mouvement à longue distance de nos 2 modèles viraux, le LMV pour les potyvirus et le Beet western yellow virus (BWYV) pour les polérovirus en utilisant des approches biochimiques et moléculaires.

Ce projet est structuré en 5 phases :

Phase 1 : Production d'une banque ADNc de cellules compagnes d'Arabidopsis

Il s'agit ici en utilisant une technique de microdissection laser d'isoler des cellules compagnes (cc) marquées à la GFP (exprimé à l'aide d'un promoteur spécifique des ces cellules dans l'accession Col-0, Ivashikina et al., 2003), d'en extraire les ARN totaux et de synthétiser une banque d'ADNc dans un vecteur double-hybride. Cette phase est réalisée à Strasbourg dans le cadre d'une thèse ayant démarré en mars 2009.

Nous avons également prévu de réaliser un séquençage massif de l'ordre de 15 000 clones de cette banque afin d'avoir une connaissance plus précise de l'identité des gènes exprimés dans les tissus phloémiens.

Phase 2 : criblage avec le système double-hybride de la banque d'ADNc de cc à l'aide d'appâts viraux

En utilisant le système double-hybride chez la levure, nous criblerons cette banque ADNc en utilisant pour chacun des deux virus les protéines virales pour lesquelles l'implication dans le mouvement à longue distance a été démontrée. Dans le cas du LMV qui me concerne, ce criblage sera réalisé avec la CP, HC-Pro et la VPg (Revers et al., 1999).

Une fois une première liste de candidats établie suite à ce criblage (après confirmation des interactions en double-hybride et test de spécificité de l'interaction), nous développerons une ou plusieurs approches visant à confirmer l'interaction *in planta* (par co-immunoprécipitation, BiFC, etc.).

Phase 3 : Identification de gènes phloémiens dérégulés lors de l'infection virale

Les ARN totaux de cc seront extraits à partir de plantes saines, infectées par le LMV et infectées par le BWYV et seront utilisés pour hybrider des microrrays d'Arabidopsis (lames CATMA, collaboration avec l'équipe de JP Renou à l'INRA d'Evry). A l'issue de ces analyses, nous dresserons une liste de gènes dérégulés au cours de l'infection avec chacun des deux virus, en dressant éventuellement une liste de gènes communs aux deux virus testés. Ensuite, une sélection de quelques dizaines de gènes sera effectuée pour une validation de la dérégulation par qRT-PCR. Nous prendrons en charge à Bordeaux les gènes dérégulés lors de l'infection avec le LMV.

Phase 4 : Identification de facteurs de l'hôte interagissant avec les virions

Nous envisageons de développer plusieurs approches pour tenter d'identifier des facteurs d'Arabidopsis qui interagissent directement avec les virions.

Une première approche consistera à révéler des protéines qui co-purifient avec le virus. Des résultats préliminaires semblent montrer que des protéines de plante restent accrocher au virus durant la purification. Si c'est le cas, nous montrerons par divers approches s'il y a bien interaction spécifique entre particules virales et ces protéines de plante

Une deuxième approche consistera à fractionner par différentes méthodes des extraits de protéines totales d'Arabidopsis produits à partir d'inflorescences dans lesquelles les tissus phloémiens sont relativement abondants et de réaliser des far-westerns avec du virus purifié.

Enfin une troisième approche que nous développerons est de réaliser une chromatographie d'affinité en utilisant du virus purifié sur lequel nous ferons passer de l'extrait de plante.

Pour l'ensemble de ces approches, si des facteurs de plante interagissant avec les virions sont identifiés, nous tenterons de révéler de la colocalisation de ces protéines avec le virus en combinant des marquages avec des fluorophores et de l'immunolocalisation.

Phase 5 : Analyse fonctionnelle des candidats identifiés

La phase finale du projet consistera à démontrer ou non que ces différents facteurs interagissant avec les virions ou les facteurs viraux impliqués dans le mouvement à longue distance ont un rôle actif dans cette étape du cycle viral.

Pour cela, nous travaillerons soit avec des lignées KO correspondant aux gènes qui codent pour ces facteurs si elles sont disponibles soit avec des lignées obtenues par RNAi. Ces lignées seront donc inoculées avec LMV et BWYV et nous suivrons l'infection virale pour savoir si dans ces lignées le mouvement à longue distance de ces virus est bloqué ou non.

Si nous arrivons à identifier de tels facteurs, nous examinerons de plus près leur localisation cellulaire et subcellulaire, leur expression en condition saine et infectée, éléments qui permettront de déboucher sur de nouvelles études sur ces facteurs afin d'apporter des éléments de compréhension sur leur mode d'action dans le mouvement à longue distance de nos virus modèles. Ces études seront développées postérieurement au projet Viromouv.

Pour réaliser ce projet, je suis assisté de L. Sofer, TR à 60% sur ce projet, en particulier sur la phase 2, de B. Doublet, TR et T. Michon, CR1, tout deux sur la phase 4 et de V. Schurdi-Levraud sur la phase 3. Deux M2 et une thèse sont également prévus.

3. Etude fonctionnelle des facteurs de l'hôte impliqués dans le mouvement à longue distance des potyvirus

A ce jour, seuls les facteurs de résistance RTM ont été clonés et nous poursuivrons naturellement l'analyse fonctionnelle de ces facteurs afin de mieux comprendre le mécanisme moléculaire du blocage du mouvement à longue distance des potyvirus qui peut ouvrir des pistes pour la compréhension même du mouvement à longue distance de ces virus.

Parmi les questions auxquelles nous aimerions répondre, au moins trois me semblent prioritaires :

1. Dans quel type cellulaire et au niveau de quel compartiment cellulaire, le blocage du mouvement à longue distance des virus se met en place

2. Avec quel(s) facteur(s) de plante les particules virales (ou complexe ribonucléoprotéique) dont le mouvement est bloqué interagissent-elles ? (Facteurs/complexe RTM ? autre facteur ?)

3. Les protéines RTM forment-elles véritablement un complexe *in planta* et dans quel compartiment cellulaire sont-elles localisées ?

L'hypothèse de travail sur laquelle nous nous appuierons est celle d'un complexe RTM, composé d'au moins RTM1 et RTM3 d'après nos données d'interaction protéine-protéine en double-hybride et en BiFC, qui pourrait séquestrer les particules virales. Cette interaction pourrait s'établir via la région N-terminale de la CP, puisque nous l'avons identifiée comme la région impliquée dans le contournement de la résistance RTM, empêchant ainsi le virus d'atteindre les tubes criblés du phloème et donc de mouvoir à longue distance. On peut facilement imaginer que ce complexe RTM soit associé à une membrane puisque RTM2 possède un domaine transmembranaire à son extrémité C-terminale. Mais comme pour l'instant aucune interaction n'a été révélée entre protéine RTM et CP (uniquement testé pour l'instant en double-hybride), on peut également imaginer que d'autres facteurs cellulaires assurent le lien entre virus et complexe RTM.

Pour répondre à ces questions, différentes expériences sont envisagées :

- Production des protéines RTM en fusion avec différents fluorophores qui vont permettre de réaliser soit en système épiderme d'oignon ou agroinfiltration de feuilles de tabac:
    - De la localisation et co-localisation des 3 protéines RTM (à l'aide notamment de marqueurs moléculaires ciblant les différents compartiments cellulaires). Ces expériences devraient nous indiquer en particulier si RTM2 est associé à des membranes et si les 3 protéines RTM co-localisent
    - Des études d'interaction via des approches de type Fret/Flim (éventuellement couplé à du BiFC pour révéler une interaction entre les 3 protéines)
- Test d'interaction entre CP du LMV et protéines RTM par BiFC sur cellules d'épidermes d'oignon (en émettant l'hypothèse qu'en système levure, toutes les modifications post-traductionnelles apportées sur les protéines testées ne sont pas équivalentes à celles se produisant *in planta*, expliquant l'absence d'interaction CP-RTM en double-hybride)
- Révélation d'interaction entre virus et protéines RTM par far-western à partir de protéines RTM purifiées en système bactérien et de virus purifié.
- Immunomarquage du LMV pour identifier le site du blocage du virus dans les tissus phloémiens d'Arabidopsis
- Co-localisation du LMV avec les protéines RTM à partir d'Arabidopsis produisant les protéines RTM fusionnées à la GFP et dont les gènes sont exprimés sous

contrôle de leur propre promoteur (lignées disponibles pour RTM1 et RTM2, en cours d'obtention pour RTM3).

Nous possédons également une banque d'ADNc dans le vecteur double-hybride pGAD-T7, produite à partir de protoplastes de cellules compagnes d'Arabidopsis (ce qui ne sera pas le cas de la nouvelle banque produite dans le cadre du projet Viromouv qui évitera de passer par ce stade stressant d'isolement des cellules végétales), fournie par V. Brault. Nous avons initié un criblage de cette banque avec RTM1 dans l'optique d'identifier des facteurs cellulaires impliqués dans le mécanisme de la résistance RTM, voire directement dans le processus du mouvement à longue distance si on émet l'hypothèse que le complexe RTM pourrait aussi priver le virus de facteurs nécessaires à ce mouvement, en les piégeant et de ce fait les empêchant d'interagir avec les particules virales. Parmi les protéines péchées par cette approche, nous avons identifié RTM1. Cette donnée confirme l'interaction de cette protéine sur elle-même et d'autre part démontre que nous ciblons bien des protéines des tissus phloémiens.

Nous étendrons le criblage de cette banque ou de celle produite dans le cadre du projet Viromouv, dès que celle-ci sera disponible, avec RTM2 et RTM3 mais aussi avec la CP puisque cette protéine est à la fois un facteur viral nécessaire au mouvement à longue distance des potyvirus mais également le déterminant de contournement de la résistance RTM. Le criblage avec la CP étant programmé dans le projet Viromouv à partir d'une CP d'isolat de LMV infectant Col-0 (donc contournant la résistance RTM), nous réaliserons également un criblage avec une CP de LMV ne contournant pas la résistance RTM (Ces CP ne différant que par un seul acide aminé dans la région N-terminale). Nous espérons ainsi observer un différentiel d'interaction entre ces deux CP qui pourront déboucher sur l'identification de facteurs clefs impliqués soit dans le mécanisme de la résistance soit dans le mécanisme du mouvement proprement dit.

En fonction du nombre de candidats identifiés lors de ces criblages par double-hybride, de la spécificité des interactions obtenues et de la fonction connue ou putative des protéines, une sélection de candidats nous paraissant les plus intéressants sera réalisée. Nous testerons pour ces candidats si les interactions observées en double-hybride se produisent aussi *in planta* par des approches évoquées plus haut (BiFC, Co-IP, Flim). Si c'est le cas, des lignées KO ou silencées correspondant à ces candidats seront inoculées avec le LMV pour voir si une infection systémique se met en place. Si des candidats s'avèrent être directement impliqués dans le processus de la résistance RTM ou dans le mouvement à longue distance, cela ouvrira de nouvelles perspectives d'études fonctionnelles de ces facteurs.

Pour accomplir cette partie, j'ai déposé un sujet de thèse auprès de l'école doctorale « sciences de la vie » de Bordeaux2 pour la rentrée 2009/2010 mais cela va dépendre du classement des étudiants ayant choisi ce sujet lors du concours de l'école doctorale, dans la mesure où je n'ai pu avoir de financement (cette partie, hormis le criblage de la banque de cc par la CP de LMV, n'est pas intégré à Viromouv). L. Sofer est aussi largement investi sur cette partie.

L'ensemble de ces données devrait apporter des éléments significatifs pour la compréhension de ce mécanisme de résistance et du mécanisme du mouvement à longue distance des potyvirus. L'identification de tels facteurs impliqués directement dans ces processus biologiques est non seulement importante d'un point de vue fondamental puisque cela apportera des éléments majeurs pour une meilleure compréhension des interactions

moléculaires plante/virus mais revêt également un intérêt plus appliqués. En effet ces facteurs de plante seront des cibles privilégiées pour leur utilisation en lutte génétique chez des espèces d'intérêt agronomique. Cela passera par l'identification des orthologues chez ces espèces d'intérêt puis par la génération d'allèles de résistance soit en exploitant la biodiversité de l'espèce soit par criblage de collection de mutants obtenus par TILLING.

Enfin il est concevable de penser que l'identification de tels facteurs puisse apporter des éléments de connaissance sur leur rôle putative dans la physiologie normale de la plante notamment dans le transport de macromolécules dans les tissus vasculaires.

BILAN DE MON ACTIVITE DE RECHERCHE

L'analyse de pathosystèmes Arabidopsis/potyvirus dans laquelle je me suis engagé depuis 2000 a ouvert la voie vers des projets d'identification d'un nombre important de gènes impliqués dans les interactions plante/potyvirus. En effet depuis que j'ai introduit Arabidopsis dans nos études, plusieurs chercheurs du laboratoire ont développé des projets en utilisant le couple Arabidopsis/LMV comme modèle d'étude au travers de diverses approches qu'elles soient moléculaires, biochimiques ou génétiques, toutes dans l'objectif de décortiquer les mécanismes moléculaires qui gouvernent les interactions plante/virus.

De mon coté en étant virologiste moléculaire à la base, j'ai pu, grâce à des collaborations multiples, notamment avec des généticiens, développer au laboratoire des projets de caractérisation génétique de gènes de plante. Cela s'est traduit par l'aboutissement d'un premier clonage positionnel qui est celui du gène *RTM3*. Ce premier clonage nous a permis de mettre en place une méthodologie et de mettre au point un dispositif expérimental pour mener à bien ce type de projet. J'ai de plus bénéficié d'un renforcement, bien que tardif, en ITA (1 IE et 1 TR en 2006 et 1 TR fin 2008) qui permet forcément d'être beaucoup plus efficace en termes de rendement.

La disponibilité de moyen humain suffisant fut certainement un frein important à un développement plus rapide de ces projets et par conséquent un frein à une production scientifique plus importante. En effet jusqu'en 2006, aucune aide technique n'a pu venir en soutien sur mes projets car tout simplement au sein de notre groupe composée de 6 chercheurs, nous n'avions qu'une IE et un TR (et 1 TR dédié aux purifications de protéines). Le message de renfort du dispositif humain est enfin passé au sein de notre hiérarchie qui s'est traduit par des recrutement en 2006 et 2008.

Il a aussi été relativement difficile d'attirer des étudiants sur cette thématique de génétique. Le problème de recrutement d'étudiants ne s'arrange d'ailleurs pas malgré la proposition de sujets en lien avec des études fonctionnelles que ce soit en thèse ou en M2.

Devenir très attractif semble être la clef de la réussite à la fois pour attirer du monde et pour faire financer sa recherche. Quel challenge !! En espérant que cela suffise ! Et je suis bien conscient que cela passera par une production scientifique de qualité, en publiant en particulier dans des journaux à IF plus élevés que MPMI qui est une revue où j'ai publié la plupart de mes derniers papiers. J'espère être sur le bon chemin avec la publication prochaine du clonage de RTM3 et les résultats qui sortiront des projets en cours

Collaborer plus intensément sera aussi cruciale. Une partie du succès du projet Viromouv auprès de l'ANR est pour moi liée au partenariat affiché avec les équipes de Colmar et de Strasbourg puisque la soumission les années précédentes de projets sur des thématiques proches mais sans partenaire n'avait pas été couronnée de succès. Les collaborations locales en particulier avec les autres équipes de l'IBVM et les plateformes technologiques (génotypage, imagerie, etc.) seront aussi un atout important. Il faudra aussi s'ouvrir au niveau européen en tentant autour de ces projets les quelques équipes travaillant sur les interactions plantes/virus. A condition bien entendu que les virus de plantes soient parmi les priorités de l'Europe !

Dans ce contexte, il est clair que dans le cadre du projet que je présente il faudra aussi faire des choix sur les gènes et facteurs végétaux à étudier, chacun ouvrant potentiellement sur autant de nouveaux projets (clonage, études fonctionnelles, etc.). Il me faudra tenir compte pour cela à la fois des moyens humains et financiers disponibles mais aussi du contexte et de

la politique scientifique conduite à la fois localement (équipe de virologie, IBVM, région) et nationale (département SPE, INRA, ministère de tutelle).

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Communications présentées à des congrès

Communications orales :

(Le nom de la personne ayant présenté la communication est souligné)

(1) **Macquaire, G., Candresse, T., Wetzel, T., Revers, F., Lanneau, M. & Dunez, J.** L'hybridation moléculaire et l'amplification de séquences géniques appliquées à la détection des viroïdes et des virus phytopathogènes. *Colloque Franco-Polonais de Virologie Végétale, Rennes, France, 12-18 Octobre 1992.*

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(11) **Yang, S.J., Revers, F., Le Gall, O., Candresse, T., Lot, H., Souche, S. & Dunez, J.** Analysis of resistance-breaking determinants using an infectious complementary DNA of lettuce mosaic potyvirus. *EMBO Workshop "Molecular Mechanisms in the Replicative Cycle of Viruses in Plants", Las Navas del Marques, Espagne, 15-19 Juin 1997.*

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(32) **Sicard, O., Loudet, O., Candresse, T., Le Gall, O., Revers, F. and Decroocq, V.** Recherche des déterminants génétiques de la plante hôte et du virus contrôlant l'apparition des symptômes dans le pathosystème *Arabidopsis thaliana / Plum pox virus. XIèmes Rencontres de Virologie Végétale.* Aussois, France, 28 janvier-1er février 2007.

(33) **Cosson, P., Le, H., Leger, V., Decroocq, V., Le Gall, O., Candresse, T., C. Carrington, J. and Revers, F.**.. Genetic and functional characterization of the RTM-mediated resistance. *Resistvir International congress "Genetic control of plant pathogenic viruses and their vectors: Towards new resistance strategies"*, Puerto de Santa Maria, Spain, 23-27 november 2008.

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(35) **Cosson, P., Le, H., Leger, V., Decroocq, V., Le Gall, O., Candresse, T., C. Carrington, J. and Revers, F..** Caractérisation génétique et fonctionnelle de la résistance RTM bloquant le mouvement à longue distance de virus de plante. *7ème Colloque National de la Société Française de Phytopathologie*, Lyon, France - Du 8 au 11 Juin 2009.

Poster :

(Le nom de la personne ayant présenté la communication est souligné)

(1) **Revers, F., Lanneau, M., Candresse, T. & Dunez, J.** Détection et caractérisation de souches du virus des taches foliaires chlorotiques du pommier (ACLSV) par PCR. *4èmes Rencontres de Virologie Végétale CNRS-INRA, Aussois, France, 25-29 Janvier 1993.*

(2) **Revers, F., Cao, T.L. & Cazenave, C.** Radiomarquage par photoaffinité des protéines de l'ovocyte de xénope se fixant spécifiquement sur les hybrides ADN-ARN. *12èmes Colloque Annuel de la Société Française de Biophysique- L'ARN... de la structure à la fonction- Figeac, France, 18-22 Septembre 1993.*

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(6) **Revers, F., Candresse, T., Le Gall, O., Lot, H. & Dunez, J.** Variabilité moléculaire et biologique du virus de la mosaïque de la laitue (LMV). *IV^{èmes} Forum des Jeunes Chercheurs en Physiologie Végétale, Clermont-Ferrand, France, 6-8 Septembre 1995.*

(7) **Revers, F., Yang, S. J., Walter, J., Souche, S., Le Gall, O., Candresse, T. & Lot, H.** Molecular basis of the biological diversity of lettuce mosaic virus. *8th International Congress on Molecular Plant-Microbe Interactions, Knoxville, E.U., 14-19 Juillet 1996.*

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(9) **Yang, S. J., Revers, F., Walter, J., Le Gall, O., Lot, H., Dunez, J. & Candresse, T.** Complete sequence of two lettuce mosaic virus isolates differing in their seed-transmissibilty and in their resistance-breaking properties. *Xth International Congress of Virology, Jerusalem, Israël, 11-16 Août 1996.*

(10) **Revers, F., Candresse, T., Le Gall, O., Souche, S., Lot, H. & Dunez, J.** Comparaison des propriétés biologiques et de la variabilité moléculaire du virus de la mosaïque de la laitue (LMV). *6èmes Rencontres de Virologie Végétale CNRS-INRA, Aussois, France, 9-13 Mars 1997.*

(11) **Yang, S.J., Revers, F., Le Gall, O., Candresse, T., Lot, H. & Dunez, J.** Analysis of resistance-breaking determinants using an infectious complementary DNA of lettuce mosaic potyvirus. *5th International Congress of Plant Molecular Biology, Singapour, 21-27 Septembre 1997.*

(12) **Candresse, T., Yang, S.J., German-Retana, S., Redondo, E., Le Gall, O., Revers, F., Lot, H., Souche, S. & Dunez, J.** Use of infectious complementary DNA, recombinant or tagged with the GFP to study the pathogenicity of lettuce mosaic potyvirus (LMV). *7th International Congress of Plant Pathology, Edinburgh, Ecosse, 9-16 Août 1998.*

(13) **Revers, F., Harrison, S., Thomas, C. & Maule, A.** Vers la caractérisation du gène récessif *sbm-1* conférant une résistance au virus de la mosaïque du pois transmise par la graine (PSbMV) chez le pois. *7èmes Rencontres de Virologie Végétale CNRS-INRA, Aussois, France, 14-18 Mars 1999.*

(14) **Harrison, S., Revers, F., Thomas, C. & Maule, A.** Towards the characterisation of the *sbm-1* resistance locus in pea. *The 13th John Innes Symposium, Attack and Defence in Plant Disease, Norwich, Angleterre, 20-23 Juillet 1999.*

(15) **Redondo, E., Yang, S.J., Revers, F., German-Retana, S., Souche, S., Lot, H., Le Gall, O. & Candresse, T.** Mapping of aggressiveness and resistance-breaking determinants of lettuce mosaic virus. *9th International Congress on Molecular Plant-Microbe Interactions, Amsterdam, Pays-Bas, 25-30 Juillet 1999.*

(16) **Otzen Petersen, B., Redondo, E., Revers, F. & Albrechtsen, M**. Inhibition of post-transcriptional gene silencing by HC-Pro from various potyviruses. *6th International Congress of Plant Molecular Biology, Quebec, 18-24 Juin 2000.*

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(20) **Harrison, S., Revers, F., Thomas, C. & Maule A**. VIP1, a putative transcriptional regulator, interacts with the VPg from several potyviruses. *10th International Congress MPMI, Madison, USA*, 10-14 juillet 2001.

(21) **Revers, F., Guiraud, T., Le Gall, O. & Candresse, T.** Viral and plant factors involved in the LMV-Arabidopsis interaction. In *XIIth IUMS Virology meeting*, Paris, France, 27th July-12th August 2002.

(22) **Ruffel S., Dussault M.H., Duprat A., Palloix A., Moury B., Revers F., Bendahmane A., Robaglia C., Caranta C**. The eukaryotic translation initiation factor 4E (eIF4E) : a target for plant resistance against potyviruses. *In 7th international congress of Plant Molecular Biology, Barcelone, Espagne, 23-28 juin 2003.*

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(24) **Guiraud, T.; Peypelut, M.; Redondo, E.; Houvenaghel, M.C.; Mauduit, T., Revers, F., Candresse, T., Le Gall, O**. Mapping of the viral determinants responsible for the ability of some isolates of *Lettuce mosaic virus* to infect lettuce plants with a defective eIF4E. *In AAB conference "Advances in Plant Virology", Montpellier, France, 29 September - 01 October 2003.*

(25) **German-Retana, S., Revers, F., Houvenaghel, M. C., Guiraud, T., Le Gall, O., and Candresse, T**. Restriction of *Lettuce mosaic virus* long-distance movement in resistant lettuce and *Arabidopsis thaliana. Le phloème dans tous ses états, Poitiers, 1-2 Avril 2004.*

(26) **Decroocq, V., Eyquard, J. P., Le Gall, O., and Revers, F**. Preliminary exploration of the genetic variability of Arabidopsis/Plum pox virus interactions. *European Meeting '04 on Plum pox virus, Rogow, Poland, 1-4 September 2004.*

(27) **Feng, X., Rivard, M., Petit, J., Rothan, C., Renou, J. P., Schurdi-Levraud, V., Le Gall, O., Caranta, C., and Revers, F**. Modifications de la régulation du génome de plante consécutives à l'infection par un virus: étude comparative entre la tomate et *Arabidopsis thaliana. 10èmes Rencontres de Virologie Végétale, Aussois, France, 6-10 Mars 2005.*

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(29) **Decroocq, V., Lansac, M., Le, H., Sicard, O., Candresse, T., Le Gall, O., and Revers, F. *Arabidopsis thaliana*: a naive species to study plant/virus interactions.** *17th International Conference on Arabidopsis Research, Madison, WI, USA, 28 June - 2 July 2006.*

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(32) **Schurdi-Levraud, V., Feng, X., Sourrigues, Y., Rivard, M., Petit, J., Taconnat, L., Rothan, C., Renou, J.P., Le Gall, O., Caranta, C. & Revers, F. (2006).** Approche transcriptomique pour l'identification de gènes impliqués dans les interactions *Arabidopsis thaliana* /potyvirus. In *Rencontres Scientifiques du DGAP, Batz sur mer, 25-27 septembre 2006.*

(33) **Sicard, O., Candresse, T., Le Gall, O., Revers, F. and Decroocq, V.** Genetic determinants of symptom development in *Arabidopsis thaliana*/Potyvirus interactions. XV Meeting on Plant and Animal Genome, San Diego, USA, 13-18 January 2007.

(34) **Cosson, P., Le, H., Sicard, O., Lansac, M., Decroocq, V., Candresse, T., Le Gall, O. and Revers, F.** Exploitation of the genetic diversity of *Arabidopsis thaliana* to identify host resistance factors which restrict long distance movement of potyviruses. *XIèmes Rencontres de Virologie Végétale.* Aussois, France, 28 janvier-1er février 2007.

(35) **Sicard, O., Candresse, T., Le Gall, O., Revers, F. and Decroocq, V.** Identification of plant genetic determinants for symptoms development after a Potyvirus (PPV - *Plum pox virus*) infection in *Arabidopsis thaliana*. *7ème journée scientifique de l'Ecole doctorale "Sciences de la vie et de la santé".* Arcachon, France, 5 April 2007.

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2. Documents à vocation de transfert ou relatifs à l'animation de la recherche

2.2. Travaux encadrés ou coordonnés par l'auteur

2.2.2. Mémoires de stages

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