ETIOLOGY AND MOLECULAR EPIDEMIOLOGY OF A SEVERE RHIZOMANIA DISEASE OCCURING IN CONFINED LOCATIONS IN EUROPE: HYPOTHESIS FOR THE IMPLICATION OF THE RNA-3 AND/OR -5 OF *BEET NECROTIC YELLOW VEIN VIRUS* (BNYVV)-P PATHOTYPE

OLIVIER LEMAIRE[1], MONIQUE BEUVE[1], CHRISTELLE WEBER[1], AUDREY SCHIRMER[1], DIDIER LINK[2], ALEXANDRE MEUNIER[3], CLAUDE BRAGARD[3], AND DAVID GILMER[2]

1 : INRA, UR-BIVV , 28 rue de Herrlisheim, 68021 COLMAR, FRANCE.
2 : IBMP, 12 rue du Général Zimmer, 67084, STRASBOURG, FRANCE
3 : UCL-FYMY, Croix du Sud, 2 bte 3 1348 LOUVAIN-LA-NEUVE, BELGIUM

ABSTRACT

A fifth *Beet necrotic yellow vein virus* (BNYVV) RNA (RNA-5) component has been identified in sugar beets grown in the Pithiviers area, where more severe symptoms of rhizomania including foliar systemic symptoms were observed. Molecular and epidemiological studies have been carried out to verify if this BNYVV "P" type or variants of this strain could have led to such symptomatology and yield losses. To monitor the spread of the P pathotype, an European survey has been conducted in 2002. Immunocapture followed by multiplex RT-PCR was performed to detect BNYVV P type. PCR products from RNA-2 (coat protein), RNA-3 (P25, involved in symptom expression) and RNA-5 (P26, putatively involved in symptom severity) were obtained from susceptible (Roberta) and partially resistant (Parade) sugar beet genotypes. Sequence alignments revealed only minor amino acid changes compared to the previously RNA-3-encoded P25 and RNA-5-encoded P26 sequences from the Pithiviers isolates. Phylogeny analysis of P25 protein, reveals that pathotype P is grouped in a distinct cluster compared to those of A and B P25 proteins. Moreover, P-type RNA-3 seems to be present around the Pithiviers area as well as in some particular beet growing areas in Belgium but without any detection of RNA 5. Preliminary molecular epidemiological data shows that BNYVV P type is restricted to the French Pithiviers area and in a confined location of East Anglia (UK), with a very slow extension in space and time. RNA-5 from France and UK resembles to the formerly described Kazakhstan RNA-5 and exhibits nucleotides substitutions insertions or deletions when compared to the Chinese-japanese RNA5 sequences. RNA-3 and 5 sequences appear highly stable over the time, suggesting a very slow evolution of BNYVV.

ABRÉGÉ - ÉTIOLOGIE ET ÉPIDEMIOLOGIE MOLECULAIRE D'UNE FORME SÉVÈRE DE RHIZOMANIE EN EUROPE : HYPOTHÈSE DE L'IMPLICATION DES ARN-3 ET/OU -5 DU BNYVV PATHOTYPE P

Un cinquième ARN a été identifié chez le virus des nervures jaunes et nécrotiques de la betterave (BNYVV) dans des betteraves sucrières infectées dans la zone cultivée proche de Pithiviers. Dans cette zone, les symptômes de la rhizomanie apparaissent plus sévères et sont accompagnés par d'éventuels contournements de gènes de résistance ces 4 dernières années. Des études moléculaires et épidémiologiques ont été menées pour vérifier si le type P du BNYVV ou des variants de ce pathotype ont pu induire cette symptomatologie et ces pertes de rendement. Nous avons mené des expériences de RT-PCR après immunocapture pour analyser les isolats de BNYVV afin de détecter la présence de l'isolat P dans et hors zone Pithiviers ainsi que dans de nombreux pays européens en 2002. Nous avons comparé les séquences nucléotidiques du BNYVV dans l'ARN-3 (P25, implication dans l'expression des symptômes) et l'ARN-5 (P26, implication probable dans la sévérité des symptômes) provenant de betteraves sucrières de variétés sensibles (Roberta) et partiellement résistantes (Parade). Les études phylogéniques menées indiquent que les séquences des protéines P25 de l'isolat P sont regroupées dans des clusters distincts de ceux obtenus pour les types A et B. Par ailleurs, nous avons identifié autour de Pithiviers des isolats contenant de l'ARN-3 ayant de fortes homologies avec celui du pathotype P, mais avec absence d'ARN-5. La même situation a été décrite en Belgique. L'épidémiologie moléculaire de ce syndrome à pathotype P, montre qu'il est restreint à la région d'origine de Pithiviers et à quelques parcelles d'East Anglia (UK) et que son extension est très réduite. Les alignements de séquences effectués montrent uniquement des variations mineures dans la composition des protéines par rapport aux précédents séquençages des isolats de type P de la zone Pithiviers. Les ARN-5 français et anglais appartiennent au même cluster que celui des ARN-5 du Kazakhstan qui diffèrent de ceux originaires de la Chine et du Japon. Enfin ces ARN présentent une très forte stabilité dans le temps, le BNYVV n'évoluant probablement que très lentement.

INTRODUCTION

Beet necrotic yellow vein virus (BNYVV), the causal agent of sugar beet rhizomania transmitted by the soil-borne fungus *Polymyxa betae* (TAMADA & BABA, 1973), is world-wide distributed in most sugar beet growing areas. BNYVV is composed of 4 genomic messenger–like RNAs with sometimes an additional fifth RNA (TAMADA *et al.*, 1989). Molecular analyses (RFLP and SSCP) have shown the existence of three major pathotypes in Europe, called A, B and P type (KRUSE *et al.*, 1994), (KOENIG *et al.*, 1995). BNYVV RNA-5 containing isolates have been identified in the French Pithiviers area (KOENIG *et al.*, 1997), Kazakhstan, Japan and China (KOENIG & LENNEFORS, 2000). In sugar beets grown in the Pithiviers area severe symptoms of rhizomania including more foliar systemic symptoms were observed. A pathogenicity study

initiated in 1997 by IIRB and performed by the Institut fur Zuckerrubenforschung (IFZ, Gottingen) had already pointed out differences in aggressiveness of the above-described BNYVV pathotypes toward sugar beet genotypes linked to their sources of resistance. BNYVV isolates harboring RNA-5 seem to be more pathogenic toward sugar beet and *Chenopodium quinoa* than conventional isolates A and B (MIYANISHI *et al.*, 1999). These authors showed that the French P type was closely related to A type, according to the RNA-2 sequence. This finding suggests that A type may have evolved from P type by loss of RNA-5, or that ancestral RNA-5 was originally associated to an A-like type common ancestor with the BNYVV A type. Moreover, the same authors found mixed infections between the 2 types of viruses, leading potentially to pseudo-recombinations. JUPIN *et al.* (1992), have shown that the RNA-3 encoded protein (P25) was responsible for the yellow local and severe lesion phenotypes, thus leading us to study beside P26, the variability of P25 between the BNYVV isolates sampled within and out of the severe area syndrome.

In 2001, even more severe symptoms have been detected in the Pithiviers area, and particularly on tolerant genotypes harboring the "Holly" resistance gene, compared to the previous years. *Beet soil-borne mosaic virus* (BSBMV), another *P. betae* transmitted soil-borne virus endemic in USA (HEIDEL *et al.*, 1997), was not found in France and especially in the Pithiviers area. Thus, we postulated for an hypothetic evolution of the BNYVV genome which could be related to the increased pathogenicity of the BNYVV P-type isolate within the last years. Molecular and epidemiological studies have been carried out to verify if this BNYVV "P" type or variants of this strain could have led to such symptomatology and yield losses. To monitor the spread of the P pathotype, epidemiological and phylogenetic studies have been conducted in 2002 as well as fundamental research to find hypotheses about the origin of this disorder and to assess the potential spread of the new syndrome. The distribution of the BNYVV P-type in Europe was assessed in 1997 by R. Koenig but on a limited number of samples. The occurrence of BNYVV isolates containing RNA-5 was studied in England in an area of Norfolk, west of Norwich, on 2 isolates (HARJU *et al.*, 2002).

This European survey has been initiated to get additional information about the potential evolution of BNYVV and the molecular epidemiology of RNA-5, and has been conducted at INRA Colmar in close cooperation with IBMP Strasbourg. An accurate sampling in the neighborhood of Pithiviers was performed by ITB collaborators as well as an extensive survey by IIRB in severe rhizomania spots from 8 countries, on about 140 symptomatic field sugar beet samples. The main objectives to address the questions about the origin, spread and potential threat of the « P » disease on the fundamental and applied levels, were:

- Methodology of BNYVV P-type detection by setting up molecular tools for detecting and genotyping these variants.
- Sampling and analysis of BNYVV isolates in the Pithiviers area where the severe rhizomania occurs.
- RT-PCR on RNA-3 and -5 putatively involved in the aggressiveness. Sequence determination and comparison with the published sequences (Japan, Kazakhstan and Europe) of RNA-3 (P25) and RNA-5 (P26) sequences.

- Comparison of RNA-3 and -5 content, between susceptible (Roberta) and tolerant (Parade) sugar beet genotypes.
- Characterization of proteins variability and phylogenetic studies including the P25 sequences obtained in Belgium.
- Accurate assessment of the potential spread of this virulent type around Pithiviers and molecular epidemiology of this severe rhizomania in France and Europe where abnormal rhizomania was observed, with susceptible (Roberta) and resistant (Parade) genotypes in micro-plots.

MATERIAL AND METHODS

1. SUGAR BEET SAMPLING

Two sugar beet genotypes were used in this survey, Parade and Roberta respectively resistant and susceptible to BNYVV. Sugar beets were sown in plots where a severe rhizomania was supposed to occur in France, Germany, Sweden, Belgium, Italy, Spain, Hungaria, England and The Netherlands, for a total sampling of 140 beets. Soils from these locations were sent in order to back up the isolates (BNYVV and *P. betae*). Additional sampling was performed in a larger perimeter (30 km) around Pithiviers to find the border between the A or B BNYVV types and the P type, mainly to the west and south-east of Pithiviers.

2. IMMUNOCAPTURE FOLLOWED BY MULTIPLEX RT-PCR (IC-RT-PCR)

This single-tube procedure was set up by combining the works of KOENIG *et al.*, 1995, and those from HAUSER *et al.*, 2000 and used in routine tests to detect BNYVV types, by amplifying a 350 bp fragment for RNA-2 (within the CP gene) and a 885 bp fragment for RNA-5 (within the P26 encoded gene). Presence of P type was checked by the amplification of both fragments whereas identification of A or B types was assessed by amplification of the single 350 bp band (RNA-2) (Figure 1). Our sheep polyclonal antiserum raised against BNYVV F3 isolate (B type) was used as coating. Sequences of primers are given in Table 1. This methodology does not required RNA extraction. Additionally, it eliminates plant RNAs, thus reducing non specific signals.

3. RNA EXTRACTION, HIGH FIDELITY RT-PCR, CLONING AND SEQUENCING

RNA extractions were done using PUREscript kit (Gentra system, Inc USA) and Rneasy (Qiagen SA, France), depending on the starting material, tissue or plant species. To check the RNA extraction prior to the following analyses, we used a fast and reliable single tube RT-PCR system (RTG RT-PCR Beads, Amersham, France).

Determination of RNA-3 and RNA-5 sequences was performed after a high-fidelity RT-PCR (Expand High Fidelity, Roche, Meylan, France) followed by PCR-product cleaning step (Qiagen). When the concentration or the quality of the RT-PCR products was not satisfying, PCR products were cloned and sequenced (TOPO TA cloning kit for Sequencing, Invitrogen, Carlsbad, USA). Sequence alignments were performed by the algorythm ClustalW and the cladograms were drawn using the Neighbor Joining method with 1000 bootstrap replicates.

RESULTS

1. METHODOLOGY

IC-RT-PCR multiplex was preferred to standard single tube RT-PCR multiplex. Immuno-trapping the virions on the microplate enabled to get rid of most of plant contaminants (data not shown) giving specific signals (Figure 1). However in a few samples, molecular analyses did not permit to detect BNYVV from rhizomania-infested area. ELISA was carried out to confirm such negative results. In very few cases, BNYVV was detected by ELISA but not by RT-PCR, probably due to RNA degradation in senescent beets (data not shown).

2. LOCALIZATION OF P-TYPE ISOLATES IN FRANCE AND EUROPE IN 2002

Molecular epidemiological data collected from 140 samples analyses revealed that BNYVV P type was strictly restricted to the French Pithiviers area and in a confined location of East Anglia, close to Norwich (UK), with a very slow extension in space and time compared to the situation of 1997 (Figure 2 and Figure 3). Most of the time, both cultivars, Roberta and Parade were infected by BNYVV, with a slight difference in virus concentration, Parade being less infected by BNYVV (on the basis of PCR bands intensity and ELISA O.D. values). In some cases, viral RNA was not detectable in Parade samples, mainly due to the presence of its resistant gene.

3. P TYPE SPREAD AROUND THE INITIAL SPOT OF PITHIVIERS

The French P type isolate is mainly restricted in the south-east of Pithiviers with an extension to the west area (Figure 3) around the Neuville, Angerville and Châtillon towns. On the basis of the presence or the absence of RNA-5, 24 sites harboured P type isolate, whereas 20 were infested by A or B types and 13 were free of BNYVV (out of the 57 locations tested).

4. P TYPE WITHOUT RNA-5 ? SITUATION OF HANCHES

However, in a plot where the presence of P type has been already shown, we found in very closely localized beet tap-roots, BNYVV with and without the fifth RNA (Figure 4). After sequencing the RNA-3 from these samples, we showed that the encoding P25 sequence was that of P type. Consequently, the question to be addressed was the following: does a P-like type without RNA-5 (PΔ5) exist?

5. PHYLOGENETIC STUDY OF P25

We compared sequences of RNA-3 and RNA-5 obtained from susceptible (Roberta) and partially resistant (Parade) sugar beet genotypes. Sequence alignments showed only minor nucleotides changes with the previously sequenced RNA-5 and RNA-3 from Pithiviers. Compared to A or B type infections, sugar beets from Pithiviers area exhibited a few more deleted forms of RNA-3 in resistant genotype, but not enough to propose a clear cut correlation between P disease and presence of truncated P25.

Phylogeny of RNA-3-encoded P25, reveals that P25 from P type are grouped in distinct clusters compared to the cluster gathering the A and B P25 proteins as shown on Figure 5.

This first cluster grouping the A or B isolates from France and Belgium (18 out of 27) is characterized by the tetra amino acid residue AYHR and brings together most of the French and Belgium (B) isolates, formerly described as A or B type.

The second cluster is organized in three sub-clusters called 2a, 2b and 2c, gathering the P type isolates (Pithiviers, Kazakhstan, UK, Japan...) and others named A1 or A2 types from Belgium. This tetra amino acid stretch could be used to distinguish between the sub-clusters and to make a more accurate typing of the isolates compared to the SSCP procedure on RNA-2 PCR products. The tetra amino acid stretch is composed of the following amino-acids depending on the geographic origin:

- AHHG, AFHR for the 2 Belgium sub-clusters A1 or A2 and ALHG for the Kazakhstan sub-cluster, being grouped with the AHHG Belgium isolates. The Belgium ones (9 out of 27 locations analyzed and called A1 or A2) did not contain detectable RNA-5, conversely to the Kazakhstan isolates.
- SYHG for the Pithiviers and UK isolates from Norwich including the isolate collected at Hanches without detectable RNA-5.
- AYRV for the Japanese and Chinese isolates.

These results indicate that it is most probable to find P-like type isolates carrying only four genomic RNAs. Moreover, according to the symptoms expression, most of those isolates are more pathogenic compared to the A or B type isolates harboring the AYHR stretch.

6. PHYLOGENETIC STUDY OF P26

Phylogeny of P26 enabled to distinguish 3 clusters as shown on Figure 6.

- Cluster #1 contains one French isolate (Mondreville) and the Kazakhstan isolates.
- Cluster #2 contains most of the French (Yèvres, Reigneville...) and the UK isolates including a Yèvres-la-Ville isolate sampled 17 years ago and stored dessicated at 4°C, displaying 100% similitude with the P26 sequence of the Yèvres isolates collected in 2002.
- Cluster #3 contains Japanese and Chinese isolates.

If we combine the phylogenic trees (Figure 7) between P25 and P26, the diverse clusters described previously segregate with the same geographically collected isolates. This interesting segregation suggests that specific interactions may occur between P25 and P26 in the pathogenesis process of the rhizomania disease. Such interactions would putatively explain part of the accentuated rhizomania symptoms.

DISCUSSION

The presence of RNA-5 seems to be correlated with the Pithiviers disorder and is associated to an RNA-3 cluster. To date, no RNA-5 has been found outside of the Pithiviers area of France, but in a single site in Europe (East Anglia, UK), this P type probably originating from the Pithiviers area. The RNA-3 deleted forms are randomly distributed (with the tendancy to be more frequent in the Pithiviers area on resistant genotype). P25 and P26 sequences from the BNYVV isolates sampled in the Pithiviers area as well as in the Norwich site are well conserved when compared to the published European sequences. If the modification of virulence observed these last years in the Pithiviers site is caused by P25 and/or P26 BNYVV variants, it may be induced by point mutations.

P25 and P26 are organized in clusters, which seem to be geographically associated raising the following questions: are P25 and P26 closely interacting in the physio-pathology of the severe disorder? Could it explain the hypothetic co-evolution between RNA-3 and RNA-5? The P25 tetra-amino acid sequence « AYHR » is variable between the types and could be used for typing more accurately the BNYVV isolates, following their pathogenic effect toward sugar beet.

A P-like type RNA-3 can be detected in neighbouring beets around the Pithiviers area and in Belgium, without the presence of RNA-5; is RNA-5 undetectable by conventional RT-PCR or is P-type evolving to a 4 RNAs structure more adapted to tolerant genotypes by loss of RNA-5, which could be named PΔ5 type?

According to this first set of results, and before drawing early conclusions, the Koch postulate has to be applied to validate our hypothesis. The use of point mutated infectious transcripts will reinforce the molecular epidemiology of P25 (RNA-3). In 2003, we will focus our work on the « hot spots » of rhizomania in Europe by RNA-3 typing, and also make an accurate survey around Pithiviers

and Norwich where P types are present. The essential steps in this deepening analysis are the following:

- Real time RT-PCR to quantify RNA-3 and -5 in the tolerant and susceptible genotypes, and to assess the systemic spread of the small RNAs. Use of this highly sensitive detection method (possible detection threshold of 10 RNA copies) to check the absence of RNA-5 in the P\5 types
- Molecular diagnostic tool to identify the P-type RNA-3 in routine tests
- RNA-3 and -5 infectious transcripts and analysis of their infectivity in different RNA environment (A or B type), using point mutations
- Confirmation of the Koch postulate, to check the P25/P26 hypothesis for inducing the « P » disease
- Molecular epidemiology of RNA-3 around Pithiviers and in worldwide locations (i.e. Imperial Valley, USA), where severe rhizomania symptoms occur. Is P\5 type common around places where RNA-5 is found?
- Hypothesis of the loss of RNA-5 in the BNYVV evolution. How does each component of the BNYVV genome evolve at present, with the probable emergence of variants exhibiting various biological properties, including possible overcoming of resistance genes?

ACKNOWLEDGMENTS:

The work was partly supported by the Institut Technique de la Betterave (ITB, France) and the breeding company Florimond-Desprez (Cappelle, France).

REFERENCES

1. HARJU, V. A., MUMFORD, R. A., BOCKLEY, A., BOONHAM, N., CLOVER, G. R. G., WEEKES, R. & HENRY, C. M. (2002). Occurence in the United Kingdom of *Beet necrotic yellow vein virus* isolates which contain RNA 5. *Plant Path.*, 811.

2. HAUSER, S., WEBER, C., VETTER, G., STEVENS, M., BEUVE, M. & LEMAIRE, O. (2000). Improved detection and differentiation of poleroviruses infecting beet or rape by multiplex RT-PCR. *J.*

3. *virol. Methods* **89**, 11-21.

4. HEIDEL, G. B., RUSH, C. M., KENDALL, T. L., LOMMEL, S. A. & FRENCH, R. C. (1997). Characteristics of *Beet soilborne mosaic virus*, a Furo-like virus infecting sugar beet. *Plant Disease*, 1070-1076.

5. JUPIN, I. GUILLEY, H., RICHARDS, K.E. & JONARD, G. (1992). Two proteins encoded by *Beet necrotic yellow vein virus* RNA3 influence symptom phenotype on leaves. *EMBO J.*, 11, 479-488.

6. KOENIG, R., HAEBERLE, A. M. & COMMANDEUR, U. (1997). Detection and characterization of a distinct type of *Beet necrotic yellow vein virus* RNA 5 in a sugarbeet growing area in Europe. *Arch. Virol.*, 1499-1504.

7. KOENIG, R. & LENNEFORS, B. L. (2000). Molecular analyses of European A, B and P type sources of *Beet necrotic yellow vein virus* and detection of the rare P type in Kazakhstan. *Arch. Virol*, 1561-1570.

8. KOENIG, R., LÜDDECKE, P. & HAEBERLE, A. M. (1995). Detection of *Beet necrotic yellow vein virus* strains, variants and mixed infections by examining single-strand conformation polymorphisms of immunocapture RT-PCR products. *J. gen. Virol.*, 2051-2055.

9. KRUSE, M., KOENIG, R., HOFFMANN, A., KAUFMANN, A., COMMANDEUR, U., SOLOVYEV, A. G., SAVENKOV, I. & BURGERMEISTER, W. (1994). Restriction fragment length polymorphism analysis of reverse transcription PCR products reveals the existence of two major strain groups of *Beet necrotic yellow vein virus*. *J. gen. Virol.*, 1835-1842.

10. MIYANISHI, M., KUSUME, T., SAITO, M. & TAMADA, T. (1999). Evidence for three groups of sequence variants of *Beet necrotic yellow vein virus* RNA5. *Arch. Virol*, 879-892.

11. TAMADA, T. & BABA, T. (1973). *Beet necrotic yellow vein virus* from rhizomania-affected sugar beet in Japan. *Ann. Phytopathol. Soc. Japan*, 325-332.

12. TAMADA, T., SHIRAKO, Y., ABE, H., SAITO, R., KIGUCHI, T. & HARADA, T. (1989). Production and pathogenicity of isolates of *Beet necrotic yellow vein virus* with different numbers of RNA components. *J. gen. Virol.*, 3399-3409.

Table 1: primers used for multiplex IC-RT-PCR (RNA-2 and -5) and for high fidelity RT-PCR for 3 and -5 sequencing

RNA-2 AMPLIFICATION: Forward (F) and Reverse primer (R)

BN2/FI: 5' ATG TCG AGT GAA GGT AGA TAT ATG 3'

BN2/R2: 5' ATC TGC TAA CCC TGA ATC AGT TAA 5'

RNA-3 AMPLIFICATION: Forward (F) and Reverse primer (R)

BN3/F2: 5' GTT GTT GTG TTT TCT GAT CAT CAT T 3'

BN3/R2: 5' GTG TTG TTG AAA TTG TGA TAA CTC 3'

RNA-5 AMPLIFICATION: Forward (F) and Reverse primer (R)

BN5/F1: 5' GTT TTT CCG CTC GCA CAA GCG 3'
BN5/R1: 5' CGA GCC CGT AAA CAC CGC ATA 3'

Figure 1: Immunocapture RT-PCR of RNA-2 and -5 enabling the distinction between P and A or B types, used in the molecular epidemiology study

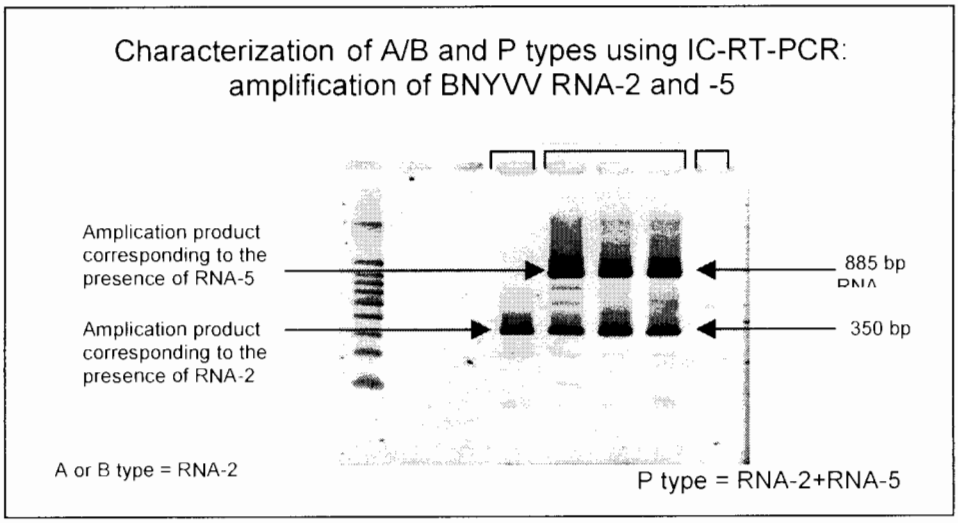

Figure 2: European distribution of the BNYVV P and A or B types in 2002

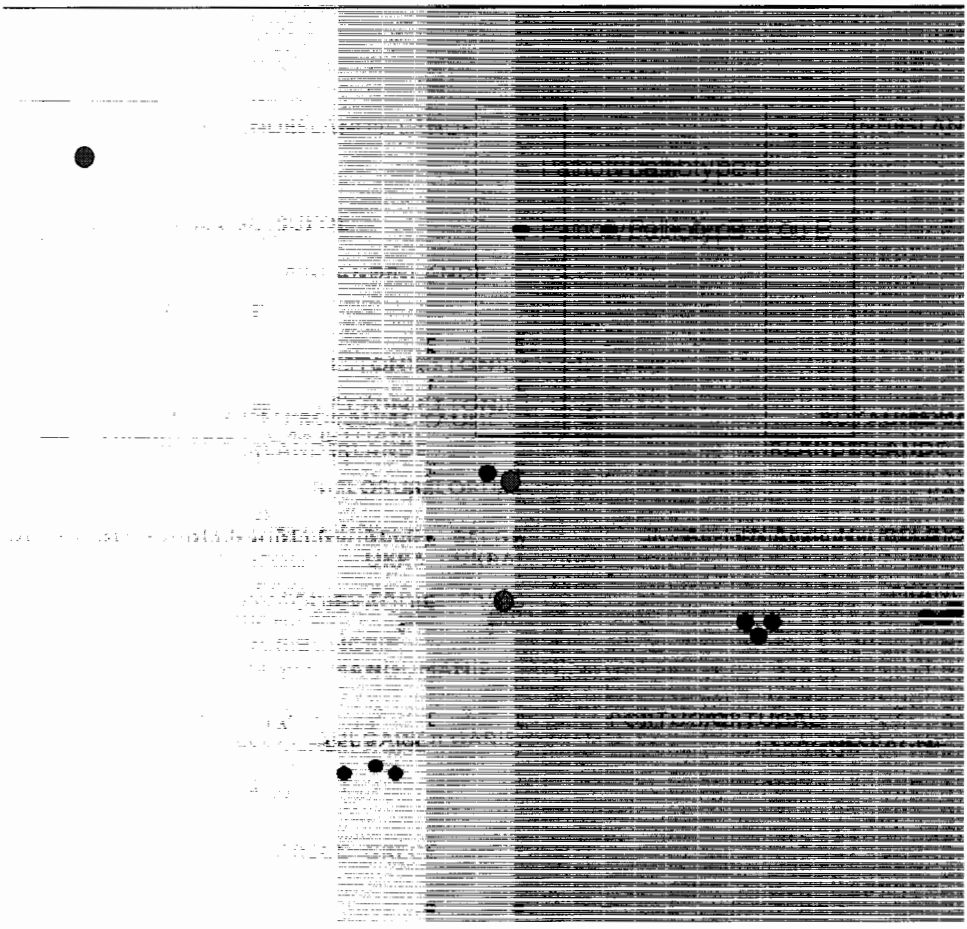

Figure 3: European localization of the 3 BNYVV types in 1997, and distribution map of the BNYVV types around the Pithiviers site according to the IC RT-PCR results from 57 locations analyzed to study the P type spread in 2002

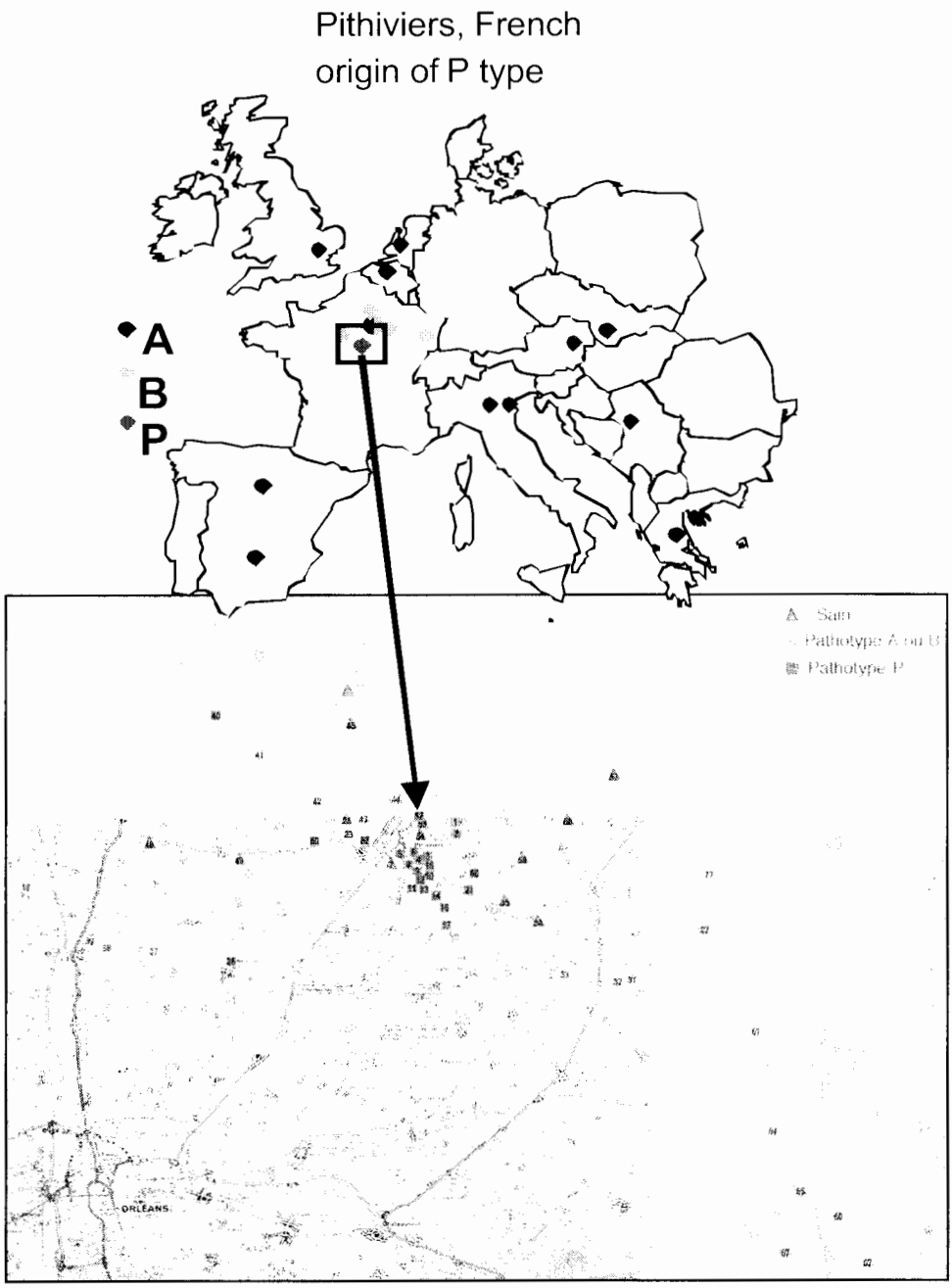

Figure 4: P-type BNYVV lacking RNA-5. In a plot around Pithiviers, a putative P-like BNYVV type lacking RNA-5 (PΔ5 type), according to its RNA-3 sequence, has been identified in infected sugar beets, although BNYVV containing RNA-5 was detected in neighbouring beets from the same field

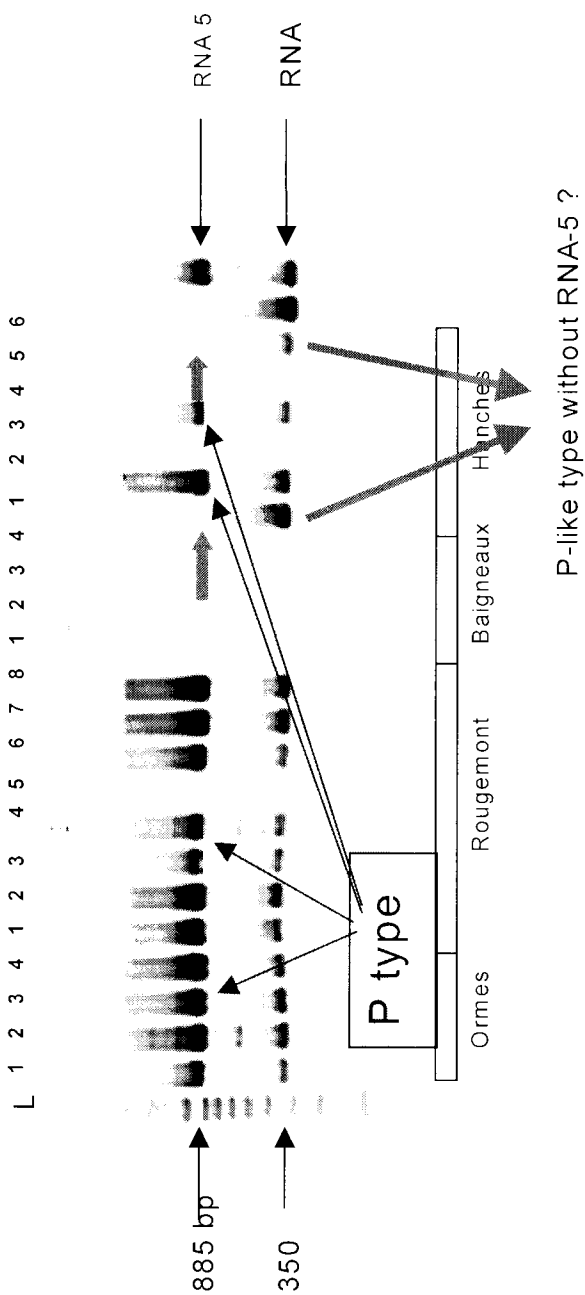

Figure 5 : Phylogenetic tree obtained from the P25 sequence alignments (RNA-3) using ClustalW, and constructed by the Neighbor Joining method using 1000 bootstrap replicates. Clusters were determined according to the length of the branchs and the boostrap values

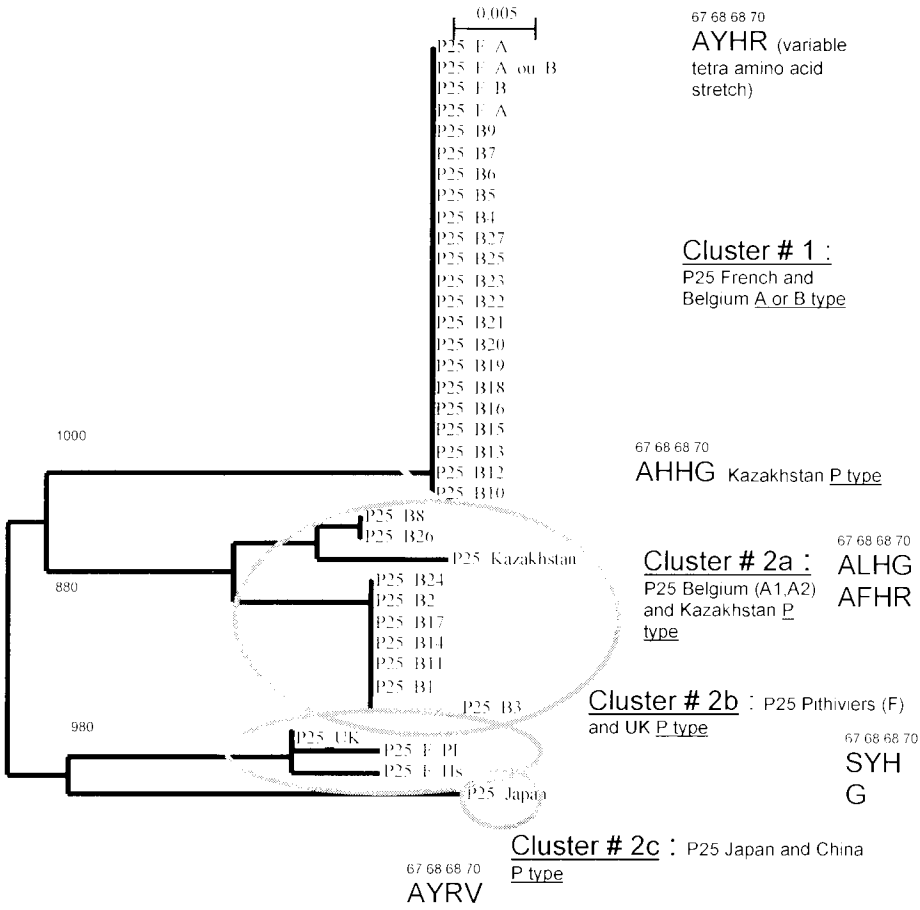

Figure 6 : phylogenetic tree obtained from the P26 sequence alignments (RNA-5) using ClustalW, and constructed by the Neighbor Joining method using 1000 bootstrap replicates. Clusters were determined according to the length of the branchs and the boostrap values

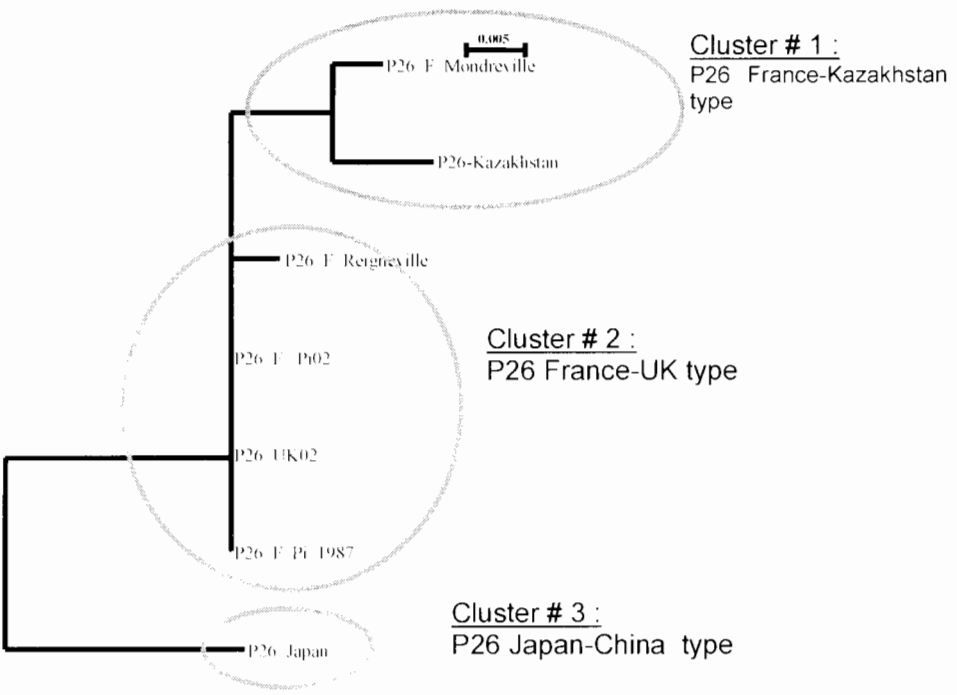

Figure 7 : combination of the P25 and P26 phylogenetic trees pointing out putative interactions between these proteins from geographically-associated isolates