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Interaction between tobacco Ribulose-l,5-biphosphate Carboxylase/Oxygenase large subunit (RubisCO-LSU) and the PVY Coat Protein (PVY-CP)

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Abstract

A 54 kDa band (P54) was continually detected with the 30 kDa viral capsid protein (CP) on the SDS-PAGE migration profile of purified potato virus Y (PVY). P54 was observed following the use of two different procedures for the purification of the PVY from infected tobacco. It was a constitutively expressed tobacco protein. The analysis of the PVY preparation showed that P54 has aggregation properties and precipitates, thus pulling down the virus. We used an enzyme-linked immunosorbent assay (ELISA) to study the relationship between P54 and the PVY particles. We performed an inhibition test with monoclonal antibodies (mAb) directed against the PVY-CP, to show that these two components interact. This result was confirmed by western blot. The internal sequence of five major peptides, obtained by C-lysine endoprotease digestion of the P54 followed by HPLC separation, showed 100% homology with the large subunit of the ribulose-1,5-biphosphate carboxylase/oxygenase (RubisCO-LSU) of tobacco. MAb directed against RubisCO-LSU were produced and used to reveal the RubisCO-LSU/PVY complex in infected tobacco extracts. A phage library displaying random heptapeptides was used to isolate several peptides that specifically bound to the native form of the PVY. The sequences of thirty-three phage-displayed peptides, which bound specifically to this virus, present further discontinuous sequence homologies with the RubisCO-LSU. Five peptides (p1 to p5) corresponding to the RubisCO-LSU homologous regions were used for a bacterial two-hybrid system to confirm *in vivo* direct interactions between the selected RubisCO-LSU regions and the PVY-CP. We propose that the PVY-CP may be involved in the production of mosaics and yellowing symptoms in tobacco through its interaction with RubisCO-LSU.

Abbreviations: AC – adenylate cyclase; CP – coat protein; cabp – carboxyarabitinol-l,5-biphosphate; mAb – monoclonal antibodies; LSU – large subunit; PVY – potato virus Y; RubisCO – Ribulose-1, 5-biphosphate Carboxylase/Oxygenase; SSU – small subunit; VMD – visual molecular dynamics.

Introduction

Potato virus Y (PVY) is the type member of the *Potyvirus* genus belonging to the *Potyviridae* family (Shukla et al., 1994). It consists of flexuous virions with a 10 kb single-stranded, positive-sense genomic RNA. This genomic RNA contains a single open reading frame, encoding a polyprotein that is processed into functional viral proteins by three virus-encoded proteases (P1, HC-Pro and NIa)

(Riechmann et al., 1992). PVY is naturally transmitted by 40 aphids species in a non-persistent manner as well as via the plant tuber. It causes diseases in potato, tomato, pepper, tobacco, nightshade and other solanaceous plants (De Bokx and Piron, 1978). The symptoms of PVY are variable depending on the host cultivar and virus strain. For example, PVY^O and PVY^C cause mosaic symptoms in tobacco plants but are responsible for tissue necrosis, mottling, yellowing, leaf drop and occasionally premature death in potato. Conversely, PVY^N causes necrotic symptoms in tobacco and very mild symptoms in potato (De Bokx and Huttinga, 1981). The phenotypic expression of the viral infection is associated to several regions of the virus genome (Rodríguez-Cerezo et al., 1991; Atreya and Pirone, 1993; Klein et al., 1994; Johansen et al., 1996; Chu et al., 1997; Simón-Buela et al., 1997). The susceptibility of the host plant to viral infection depends on the association between the host plant and the viral factors for the replication of the viral genome, the expression of the viral genes and the movement of the virus throughout the plant (Zaitlin and Hull, 1987). Consequently, plant and viral component interactions are presumed to play a pivotal role in disease development. Nevertheless, the general pathway by which the virus symptoms are formed is still unknown as are most of the host proteins that interact with the viral molecules. A viral protein which is supposed to interact with the host components is the coat protein (CP). The PVY-CP is a three-domain protein: the N- and C-terminal ends are exposed on the surface of the virion, and the central domain forms the core subunit structure (Shukla and Ward, 1989). The primary function of the CP is to encapsidate the viral RNA, a process that probably requires the highly conserved central and C-terminal regions of the potyviral CPs (Shukla and Ward, 1989). Mutations in the CP region result in defective virus assembly, limit virus movements (Dolja et al., 1994, 1995; Rojas et al., 1997; López-Moya and Pirone, 1998) and reduce genome amplification. Moreover, an amino acid triplet, Asp-Ala-Gly (DAG), near the surface-exposed N-terminus of the CP (Shukla and Ward, 1989) is highly conserved among aphid-transmissible potyviruses and involved in the interaction with the helper component (HC) during aphid transmission (Atreya et al., 1995; Blanc et al., 1997). The PVY symptoms might be explained by the presence of CP, HC and RNA of this virus in chloroplasts of infected leaves (Gunasinghe and Berger, 1991), which might interfere with the chloroplastic function including RubisCO photosynthetic activity (Miziorko and Lorimer, 1983; Suzuki, 1987; Rival et al., 1996). This coincides with the fact that transgenic plants expressing PVY-CP in their chloroplasts lose their green color and grow poorly (Naderi and Berger, 1997). These plants have the same phenotype as the *N. tabacum* mutant that expresses low RubisCO levels associated with chlorophyll loss (Dulieu, 1974; Nguyen-Quoc et al., 1989). Conversely, symptom expression seems to be a complex processes that may involve different mechanisms. It has been shown that the basis of virus-induced diseases in plants may be explained by an interference with microRNA-controlled development pathways that share components with the antiviral RNA-silencing pathway (Kasschau et al., 2003;Chen et al., 2004). In the present study, we have identified a 54 kDa tobacco protein which interacts with PVY-CP. Our results may help to explain the appearance of symptoms, such as chlorosis, mosaic, mottling and yellowing in tobacco plants.

Materials and methods

PVY strain

The PVY-P21 strain was isolated from pepper in the Cap Bon region of Tunisia. Biological characterization using test plants showed that this isolate belongs to the PVY ordinary strain (PVY^0). This isolate was propagated in *Nicotiana tabacum cv. Xanthi,* which was cultivated in an insect-proof greenhouse at 25 °C

PVY and plant protein purification

The PVY was purified as described by Dougherty and Hiebert (1980a and b) with some modifications. After the clarification stage, the supernatant was applied to a 20% sucrose cushion and centrifuged at 23,000 rpm (SW25 rotor) for 150 min. The pellet, corresponding to elements sedimenting in these conditions was immediately recovered in sterile distilled water. The same procedure has been used starting from the healthy tobacco (*N. tabacum cv.* Xanthi) leading to a protein preparation. The PVY and the tobacco protein preparation were divided

into aliquots and stored at −20 °C. For immediate use, the PVY preparation was stored at 4 °C. In these conditions a precipitate corresponding to the major part of contaminants was observed and only the supernatant containing the PVY particles was recuperated.

SDS-PAGE and western blotting

SDS-PAGE was performed as described by Laemmli (1970). The PVY and the preparation obtained from healthy tobacco plants subjected to PVY purification procedure were mixed with an equal volume of SDS-buffer, boiled at 100 °C for 2 min and loaded onto a 12% acrylamide gel. For western blotting, the membranes were blocked with buffer A (3% BSA in phosphate buffer saline (PBS)) and followed (or not, depending on the case) by 1 h incubation at room temperature with the supernatant PVY preparation diluted in buffer A. After washing (PBS, 0.1% tween), the membranes were incubated for 1 h, respectively, with anti-PVY-CP mAb (Sanofi) or with polyclonal antibodies prepared against a 54 kDa tobacco protein band (P54) extract from SDS-PAGE gel. The membranes were washed and incubated for 1 h at room temperature in alkaline phosphatase-conjugated sheep anti-mouse immunoglobulins (Sigma). The reaction was revealed by incubating the membranes with 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium (Sigma) and stopped by washing them in water.

ELISA

Binding of the PVY particles to the tobacco protein preparation

Microtiter plates were coated overnight at 4 °C with serial dilutions of the tobacco protein preparation obtained from healthy plants using the PVY purification procedure (20 to 0.5 μg ml^{-1}, in PBS). After saturation for 1 h at room temperature with buffer B (PBS, 1% gelatin, 0.1% tween). The coated plates were incubated at 37 °C for 2 h with 5 μg ml^{-1} of the supernatant PVY preparation diluted in buffer C (PBS, 0.5% gelatin, 0.1% tween). The plates were incubated for 1 h at 37 °C with anti-PVY-CP mAb (Sanofi) and followed by an incubation with peroxidase-conjugated sheep anti-mouse immunoglobulins diluted in buffer C. The reaction was revealed with citrate buffer (citrate 0.1 M, pH 5.2; 0.2% orthophenylenediamine; 0.03% H_2O_2) and stopped by the addition of HCl (3 M). The absorbance was measured at 492 nm.

Inhibition of anti-PVY-CP mAb binding to the PVY by the tobacco protein preparation

Microtiter plates were coated with the supernatant PVY preparation (5 μg ml^{-1} in PBS) overnight at 4 °C. The coated plates were blocked with buffer B and incubated for 2 h at 37 °C with serial dilutions of the healthy tobacco preparation or with BSA (20–0.5 μg ml^{-1} in buffer C). After washing, plates were incubated for 90 min at 37 °C with anti-PVY-CP mAb (Sanofi) diluted in buffer C. Reaction was revealed by peroxidase-conjugated sheep anti-mouse immunoglobulins diluted in buffer C and inhibition was calculated as following: $I\% = 100 \times (l_O - l_c)/l_O$, ($l_O$ and l_c are respectively the absorbance measured at 492 nm without and with inhibitor).

RubisCO-LSU/PVY complex detection. DAS ELISA was performed: microtiter plates were coated using different concentrations of anti-PVY-CP mAb (0 to 20 μg ml^{-1} diluted in buffer C. After saturation with buffer B, the PVY-infected-tobacco-extract was added. The presence of the complex is revealed by anti-RubisCO-LSU mAb diluted in buffer C.

Preparation of samples for the P54 microsequencing

The healthy tobacco protein preparation was subjected to SDS-PAGE on a 10% gel, transferred to PVDF membrane and stained with amidoblack. After excision from the membrane, the 54 kDa band (P54) was denatured and reduced, and cysteines were alkylated (Brune, 1992). Protein digestion was performed by incubation of the P54 with C-lysine endoprotease for 18 h at 35 °C. The resulting peptide fragments were separated by reverse phase HPLC, sequenced and used to search a database protein.

Production of anti-RubisCO-LSU mAb

BALB/c mice were immunized subcutaneously (s.c.) with 50 μg of the P54 (extract from SDS-PAGE gel) in 100 μl of sodium chloride (150 mM), and emulsified with 100 μl of complete Freund's

adjuvant. Three s.c. booster injections were given 1, 3 and 5 weeks later in incomplete Freund's adjuvant. At this stage, a blood sample was taken and the corresponding polyclonal antibodies were extracted and tested using the P54 as antigen, then stored for a further use. Thirty days later, mice received the last intra-peritoneal booster of the P54 and hybridomas were established (Köhler and Milstein, 1975). Specificity of hybridomas and clones was screened by ELISA as well as by western blotting using wild-type and RubisCO low expression mutant *N. tabacum* as an antigen.

Library screening and phage characterizations

The PhD-7 Phage Display kit (New England Biolabs) contains a library of recombinant phages expressing seven amino acids peptides displayed on the surface of the M13 bacteriophages. The biopanning experiments were performed essentially as recommended by the manufacturers. The supernatant PVY preparation (100 μg ml^{-1} in carbonate buffer: 0.1 M $NaHCO_3$, pH 8.6) was used as ligand. The biopanning procedure was repeated twice and bound phages were eluted by adding 0.2 M glycine-HCl, pH 2.2; 1 mg ml^{-1} BSA for 10 min. Finally, to avoid false positive phage-peptides, two rounds of counter panning were performed with the healthy tobacco protein preparation. From this counter selection, unbound phages were collected and characterized by ELISA. This step was followed by cloning and amplification of each positive clone.

For manual dideoxy sequencing with ^{35}S of the selected clones, DNA was prepared as described by the PhD-7 Phage Display kit (New Englands Biolabs) and employed as a template. The PhD-96 primer (5′-G CCC TCA TAG TTA GCG TAA CG-3′) and a T7 sequencing kit (Amersham Pharmacia Biotech, France) were used in the procedure.

The specificity of the binding of the selected clones has been analyzed by an inhibition assay. Hence, various amounts of the supernatant PVY preparation (0, 1.25, 2.5, 5, 10 and 20 μg in PBS) used as inhibitor were incubated overnight at 4 °C with a 10^{10} phages of each clone. Each mixture was transferred into a well of a microtiter plate coated with PVY preparation supernatant. The plates were incubated for 30 min at 4 °C. Bound phages were detected by peroxydase-conjugated anti-M13 mAb and the percentage of inhibition was calculated as described in ELISA section.

Bacterial two-hybrid assay

In vivo protein–protein interactions between the PVY-CP and the tobacco RubisCO-LSU were studied using the bacterial two-hybrid system based on the complementation of two fragments (T25 and T18) of the catalytic domain of *Bordetella pertussis* adenylate cyclase (AC) (Karimova et al., 1998).

Starting from RNA purified from the Tunisian PVY-P21 isolate, the PVY-CP coding region was amplified by RT-PCR using the following oligonucleotides as primers: AS (5′-CT GCA GCA AAT GAC ACA ATT GAT GC-3′) and S (5′-GGATC CAT GTT CTT GAC TCC AAG TAG-3′). The restriction sites included in the PCR primers are underlined. The PCR product was cleaved with *Pst*I and *Bam*HI and cloned into the *Pst*I and *Bam*HI sites of pKT25 (Karimova et al., 2001). The resulting plasmid pKT25-CP encodes a CP fused in frame with the T25 fragment of AC. On the other hand, five oligonucleotides encoding selected peptides of the tobacco RubisCO-LSU (p1 to p5) and including *Bam*HI and *Pst*I sites (Table 1) were cloned into pUT18C plasmid. The set of plasmids obtained (pUT18C-p1 to pUT18C-p5) express the T18 fragment fused in frame with one of these five selected peptides. To test the interactions, pKT25-CP was co-transformed with each of the pUT18C-Px plasmids into BTH101 (an *E. coli cya* strain deficient in endogenous AC) (Karimova, unpublished). Transformed cells were plated on MacConkey agar medium (Miller, 1992) containing 1% maltose, 0.5 mM isopropyl-β-D-galactopyranoside (IPTG). 100 μg ml^{-1} ampicillin and 50 μg ml^{-1} kanamycin, and were grown for 6 days at room temperature. To confirm the interactions, double hybrids were plated on a minimal medium supplemented with maltose, X-gal, IPTG, ampicillin and kanamycin.

Molecular visualization

The relative locations of the five RubisCO-LSU peptides tested for their interaction with the PVY-CP were identified by Raster 3D (photorealistic molecular graphics) and VMD (visual molecular dynamics) programs (Merrit and Bacon, 1997).

Table 1. Primer sequences and corresponding RubisCO-LSU peptides (p1 to p5) used for the bacterial two-hybrid assays

Primers	Sequences	Peptides
RUB1:	5′-ACTTATTATACTCCTGAGTACCAAACCAAGGATACG-3′	p1: TYYTPEYQTKDT
RUB1 reverse	5′-GATCCGTATCCTTGGTTTGGTACTCAGGAGTATAA-TAAGTCCTGCA-3′	
RUB2:	5′-CGAGTAACTCCTCAACCTGGAGTTCCACCTGAA-G-AAGCG-3′	p2: RVTPQPGVPPEEA
RUB2 reverse	5′-GATCCGCTTCTTCAGGTGGAACTCCAGGTTGAGGAGT-TACTCGCCTGCA-3′	
RUB3:	5′-ACTTCCATTGTAGGTAACGTATTTGGGTT-CAAAGCCCTGCGG-3′	p3: TSIVGNVFGFKALR
RUB3 reverse	5′-GATCCCGCAGGGCTTTGAACCCAAATACGTTACCTA-CAATGGAAGTCCTGCA-3′	
RUB4:	5′-GGTATTCACGTTTGGCATATGCCTGCTCTGACCGA-GATCTTTGGGGATGATTCG-3′	p4: GIHVWHMPALTEIFGDDS
RUB4 reverse	5′-GATCCGAATCATCCCCAAAGATCTCGGTCAGAGCA-GGCATATGCCAAACGTGAATACCCCTGCA-3′	
RUB5:	5′-GGACATCCTTGGGGTAATGCGCCG-3′	p5: GHPWGNAP
RUB5 reverse	5′-GATCCGGCGCATTACCCCAAGGATGTCCCCTGCA-3′	

Restriction sites are underlined.

Results

Presence of 54 kDa band in the PVY preparation

Starting with the healthy and PVY-infected tobacco extracts, we performed a purification procedure according to the method of Dougherty and Hiebert (1980a and b) that has been modified. SDS-PAGE migration followed by coomassie blue staining revealed the presence of a 54 kDa band (P54) in both preparations. A 30 kDa band, which is a characterization of PVY was only present in the infected extract (Figure 1). This indicates that under our experimental conditions some plant proteins contaminated the viral preparation. After a few days storage at 4 °C, the contaminant P54 protein precipitate and disappear from the supernatant (data not shown).

Binding of the P54 to the PVY particles by ELISA and western blot

In order to investigate the hypothesis that the tobacco protein preparation would display a property allowing its co-purification with the PVY particles, we performed ELISA using plates coated with different dilutions of the protein preparation obtained from healthy plants subjected to PVY purification procedure. The supernatant PVY preparation was added to each well. Bound virus

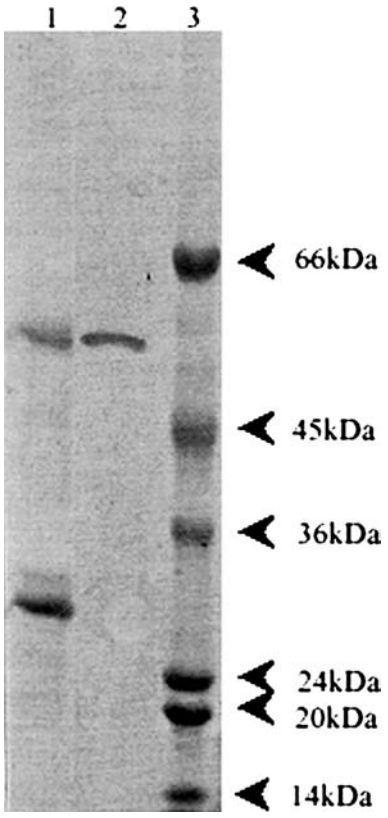

Figure 1. Coomassie blue stained 12% SDS-PAGE gel of preparations obtained from infected (lane 1) and healthy (lane 2) tobacco extracts following a modified procedure described by Dougherty and Hierbert (1980a and b). Lane 3: molecular weight.

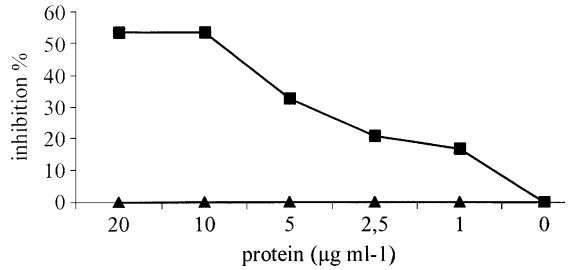

Figure 2. The P54 inhibits the fixation of anti-PVY-CP mAb to the PVY particles. Different concentrations of the P54 ([■]) or BSA ([▲]) were incubated with PVY-coated microtiter plates. Then the anti-PVY-CP mAb was added. Bound antibodies were revealed with peroxidase-conjugated sheep anti-mouse immunoglobulins.

particles were revealed by anti-PVY-CP mAb, suggesting that the PVY particles would bind to the protein preparation (data not shown). This result was confirmed by the inhibition of the anti-PVY-CP mAb binding to homologous antigen by high concentrations of the healthy tobacco protein preparation (Figure 2). By western blot analysis, we revealed that the interaction of the healthy tobacco proteins with the PVY particles involves more precisely a 54 kDa protein (Figure 3). This size corresponds to the one of the RubisCO-LSU which is expected to be the major component of the P54 band. We suggest that this molecule could interact with the PVY particles. However, it cannot be excluded that another 54 kDa protein present in this band is responsible for the binding to the PVY.

Presence of the RubisCO-LSU in the P54

In order to identify the major protein present in the tobacco protein preparation, we sequenced the P54 band extracted from the SDS-PAGE gel without carrying out any further purification. After reduction and alkylation, the P54 was digested with C-lysine endoprotease. The resulting peptides were separated by HPLC and, from the peaks obtained, the five major ones were sequenced. When compared to protein sequences available in data banks, the peptides sequenced (Seq1 to Seq5) were 100% homologous with RubisCO-LSU (Figure 4). This would indicate that the RubisCO-LSU is the major contaminating component of the viral preparation.

Presence of the RubisCO-LSU/PVY complex in the infected tobacco extract

In order to confirm the RubisCO-LSU/PVY interaction, we raised mAb against the P54 band.

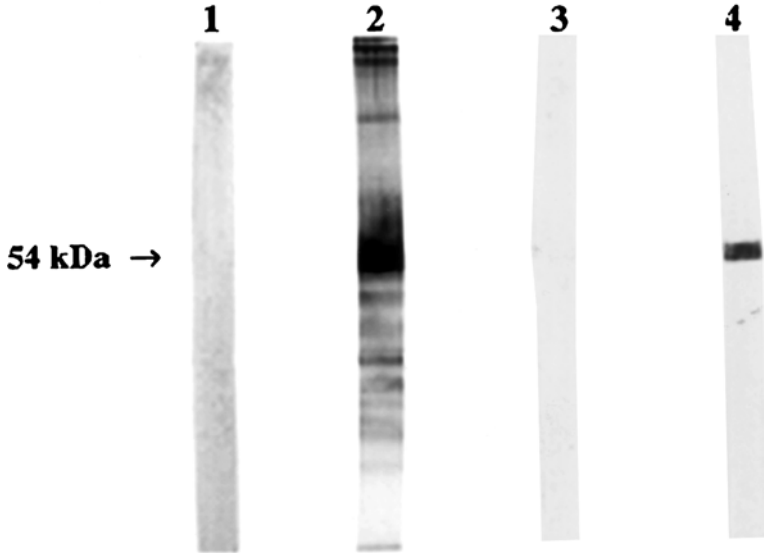

Figure 3. Interaction between the P54 and the PVY-CP. The healthy tobacco protein preparation was separated by SDS-PAGE and analysed by western blot. The nitrocellulose membrane was incubated with (lane 4) or without (lane 3) the supernatant PVY preparation and probed with anti-PVY-CP mAb. Lane 2 was probed with polyclonal antibodies prepared against the P54 band and lane 1 represents negative control incubated only with conjugate.

MSPQTETKASVGFKAGVKEYKLTYYTPEYQTKDTDILAAFRVTPQPGVPPEEAGAAVAAESSTGTWTT

VWTDGLTSLDRYKGRCYRIERVVGEKDQYIAYVAYPLDLFEEGSVTNMFTSIVGNVFGFKALRALRLE

DLRIPPAYVKTFQGPPHGIQVERDKLNKYGRPLLGCTIKPKLGLSAKNYGRAVYECLRGGLDFTKDDEN

VNSQPFMRWRDRFLFCAEALYK**AQAETGEIK GHYLNATAGTCEE**MIKRAVFARELGVPIVMHDYL

Seq1 **Seq2**

TGGFTANTSLAHYCRDNGLLLHIHRAMHAVIDRQKNHGIHFRVLAKALRMSGGDHIHSGTVVGKLEGE

RDITLGFVDLLRDDFVEQDRSRGIYFTQDWVSLPGVLPEASGGIHVWHMPALTEIFGDDSVLQFGGGTL

GHPWGNAPGAVANRVALEACVK**ARNEGRDLAQEGNEI**IREACK**WSPELAAACEV WKEIVFNFAAV**

DVLDK **Seq3** **Seq4** **Seq5**

Figure 4. The entire protein sequence of *N. tabacum* RubisCO-LSU. Residues in bold (Seq1 to Seq5) correspond to the alignment between RubisCO-LSU and the five major peptides obtained after the P54 digestion.

The ELISA positive clones were tested by western blotting using the immunizing preparation and were shown to recognize the P54. Assuming the antigen preparation heterogeneity, we undertook a better characterization of the anti-P54 mAb obtained, with the objective to distinguish those which are directed against RubisCO-LSU. To this aim, we analyzed the mAb binding to mutant *N. tabacum* extract as compared with the wild tobacco extract (data not shown). Indeed, this mutant which has been previously described (Dulieu, 1974; Nguyen-Quoc et al., 1989) shows, as the main defect, low RubisCO levels that decrease with age. To perform extractions from this mutant, we used old leaves that have lost RubisCO expression. The three anti-P54 mAb tested (A4-1, E1-1 and G3-2) show high fixation levels to the wild extract and absence of binding to the mutant extract (data not shown). This result, along with the previous data, suggests that these mAb are directed against the RubisCO-LSU. Indeed, the probability that they could recognize another 54 kDa tobacco protein which is present in the immunizing preparation and absent from the mutant, appears very weak, though cannot be totally excluded.

Without any further characterization, we used these mAb as tools to investigate an involvement of their homologous antigen in the interaction with the PVY particles. We performed two kinds of DAS ELISA to reveal the RubisCO-LSU/PVY complex. In the first test, anti-PVY-CP mAb-coated plates were used to bind the RubisCO-LSU/PVY complex present in the infected tobacco extract, whereas the labeled A4-1 mAb was used for the detection (Figure 5). In the second test, we performed the opposite process using the A4-1 for coating the plates and anti-PVY-CP mAb for the complex detection (data not shown). In both cases, binding was observed with the PVY-infected tobacco extract and not with the healthy tobacco extract. This result is in agreement with an interaction that takes place in our experimental conditions between the PVY particles and the tobacco RubisCO-LSU.

Binding of isolated phage-peptides to the PVY particles

Following a different methodological approach, we isolated peptides that specifically bind to the

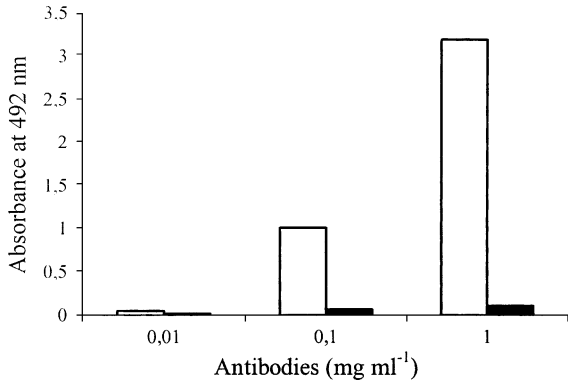

Figure 5. Detection of the RubisCO-PVY complex by ELISA. Microtiter plates were coated with a different concentration of anti-PVY-CP mAb. Incubation of healthy (black) or PVY infected tobacco extract (white) is followed by addition of the labeled A4-1 mAb directed against the tobacco RubisCO-LSU.

PVY particles, starting from a phage library displaying unconstrained heptapeptides associated, in a fused protein, with the pIII minor CP of bacteriophage M13 (Barbas et al., 1991). First, three rounds of biopanning were performed against the supernatant PVY preparation. Colony blotting analysis of the eluted phages from the third round revealed weakly positive clones. To increase the number of specific phages, two additional rounds of counter panning were carried out against the tobacco protein preparation that contains plant proteins usually contaminating PVY preparation. After these two additional rounds, phages with peptides specific to contaminating tobacco proteins should significantly decrease from the phage library. Indeed, the phage population was significantly enriched in peptides binding to the supernatant PVY preparation as compared with the initial phage library (data not shown). We then performed a cloning step of the enriched phage population followed by an ELISA test to confirm their individual binding capacity. Thirty-three phage-displayed peptides gave a strong positive signal in ELISA. Among these 7-mer-thirty-three phages, the specificity of six representative clones (C2, C3, C13, C21, C139, C339) selected by alignment with RubisCO-LSU was assessed by the binding inhibition of each one to the PVY-coated-plate, using the homologous PVY preparation as inhibitor. The fixation inhibition rates of these six clones by the supernatant PVY preparation (2.5 μg ml^{-1}) was ranged between 15.3 and 73% (data not shown). The sequences of the 33 peptides were determined and their alignment revealed homologies that enabled to carry out consensus groups (data not shown). These recurrent peptides selected among 10^{11} phage-peptides could be considered as the result of specific interactions with given structures present in the supernatant PVY preparation. These structures might belong to the viral particles since the PVY is the main component of this preparation. Moreover, the two counter panning steps should have eliminated most of the peptides that could bind to any contaminating protein.

Alignment of isolated peptide with the tobacco RubisCO-LSU

As our previous results suggested an interaction between the PVY particles and a 54 kDa tobacco protein (P54), that could be RubisCO-LSU, we looked for homology between the thirty-three identified phage-peptides and the tobacco RubisCO-LSU primary sequence. Analyses showed that the isolated peptide sequences could be aligned with three main RubisCO-LSU domains (R1: 23–50, R2: 117–162, and R3: 362–415) on a discontinuous manner considering primary sequence (Figure 6) and grouped as epitopes on the tertiary structure of RubisCO-LSU (data not shown). Interestingly, some homologous motifs to the selected peptide (located in R1 and in R2) were found at the N-terminus of the RubisCO-LSU, the region which contains residues involved in active site forming of the enzyme (Hartman and Harpel, 1993). The remaining clones are aligned with the third identified domain (R3). Since the peptides studied here have been selected for their affinity toward the supernatant PVY preparation, it appears that the homologous domains of the tobacco RubisCO-LSU could similarly interact with the PVY particles. However, since this molecule constitutes the main contaminating plant protein of the PVY preparation, one could not exclude that some of these peptides could have been selected against the tobacco RubisCO-LSU itself even after two counter panning rounds. It is known, that RubisCO-LSU acts as a dimer and the amino acids involved in the protein aggregation are located in the previously identified R2 domain (Guetteridge and Guatenby, 1995). Hence, peptides homologous to this region might be self reactive. Moreover, these results have been obtained *in vitro*, in conditions very distinct from *in vivo*.

In vivo *interaction of RUBISCO-LSU with the PVY-CP*

To show *in vivo* direct interaction between the PVY-CP and the tobacco RubisCO-LSU, we applied the bacterial two-hybrid system which allows identification of interacting partners in *E. coli* cells (Karimova et al., 1998). In this bacterial two-hybrid assay, the interaction between two proteins of interest results in functional complementation between two fragments (T25 and T18 of the *B. pertussis* adenylate cyclase (AC)) and, therefore, restores a Cya$^+$ phenotype of the host *E. coli cya* cells. The Cya$^+$ phenotype can be easily visualized either on a MacConkey/maltose medium (Cya$^+$ cells form large, red colonies, whereas Cya$^-$ cells

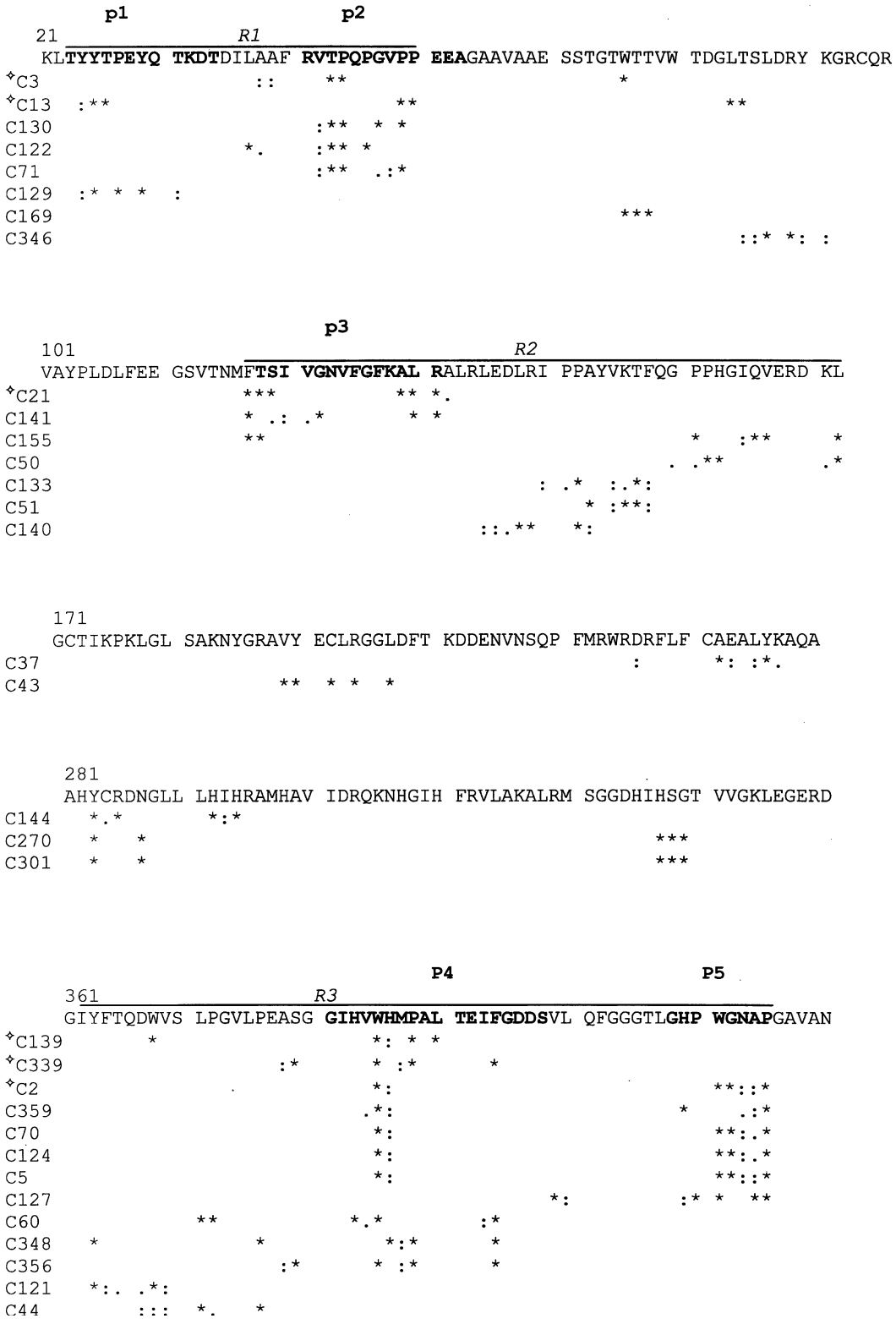

Figure 6. Sequence alignment of the 33 phages-peptides presenting affinity to the supernatant PVY preparation: (∗) denotes identical residues, (:) very similar residues, (·) similar residues. (◇) corresponds to the representative phage-peptide clones. The peptide sequences selected for the bacterial two-hybrid test (p1 to p5) are in bold.

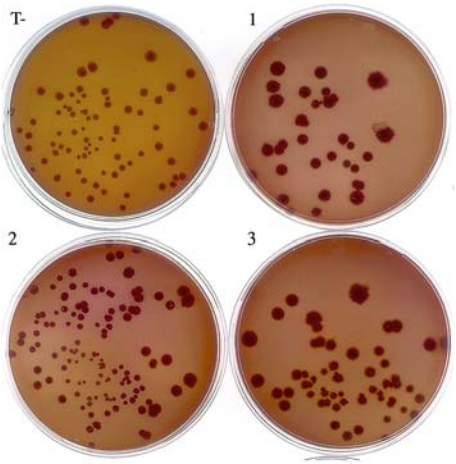

Figure 7. The bacterial two-hybrid test. *E. coli cya* BTH101 co-transformed with 1: pUTl8C-p1 and pKT25-CP; 2: pUT18C-p2 and pKT25-CP; 3: pUT18C-p3 and pKT25-CP; T (Negative control): pUT18C and pKT25-CP. The test was performed on MacConkey/maltose/X-gal/IPTG/kanamycin and ampicillin medium.

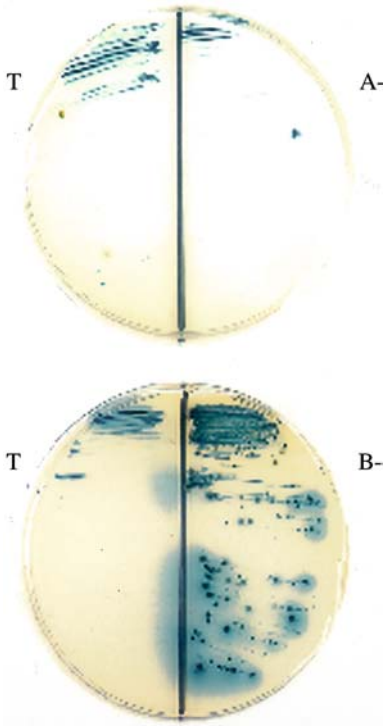

Figure 8. The *E. coli cya* BTH101 co-transformed with A: pUT18C-p4 and pKT25-CP; B: pUT18C-p3 and pKT25-CP; T (Negative control): pUT18C and pKT25-CP. The test was performed on a minimum medium/maltose/X-gal/IPTG/ampicilline and kanamicine.

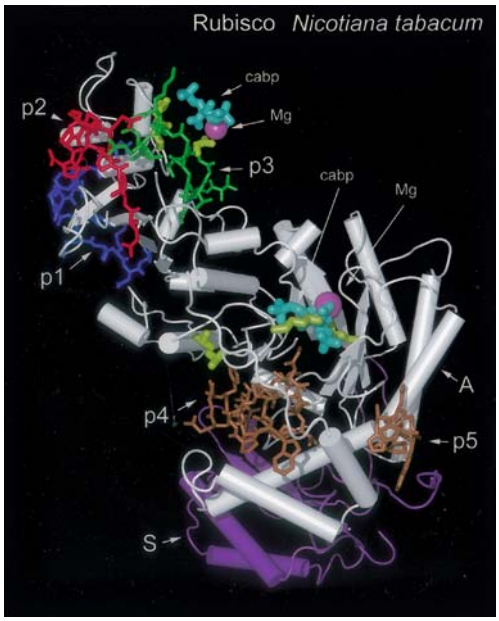

Figure 9. Space-filling view of tobacco RubisCO-LSU (white) and -SSU (violet) which shows the LSU-three peptides that interact with the PVY-CP (p1: blue, p2: red, p3: green), the LSU-two peptides that do not interact with PVY-CP (p4 and p5 in brown), the residues that form the enzyme active site (yellow); cabp (turquoise) and Mg (pink).

remain colorless) or on a minimal medium supplemented with maltose as the sole carbon source on which only cells presenting wild phenotype as the result of interaction can grow.

In order to study the interaction between the PVY-CP and the tobacco RubisCO-LSU using the bacterial two-hybrid system, we constructed several plasmids. The pKT25-CP plasmid expresses a hybrid protein consisting of the N-terminal T25 fragment of AC fused in frame with the PVY-CP. In parallel, we constructed a set of plasmids (pUT18C-p1 to pUT18C-p5) expressing five regions of the tobacco RubisCO-LSU (p1 to p5) fused to the C-terminal TI8 fragment. These RubisCO-LSU regions p1 (residues 23–34), p2 (residues 41–53), p3 (residues 118–131), p4 (residues 381–398) and p5 (residues 408–415) were determined on the basis of discontinuous alignments with the selected phage-displayed peptides (Figure 6). Each of the pUT18C-px plasmids was co-transformed with the pKT25-CP into *E. coli cya* strain BTH101 and the bacteria were plated on MacConkey/maltose agar medium supplemented with the relevant antibiotics.

The BTH101 cells co-expressing T25-CP and T18C-p1, T25-CP and T18C-p2, or T25-CP and T 18C-p3 hybrid proteins, formed red colonies on MacConkey/maltose agar medium (Figure 7). In contrast, no complementation was observed between the hybrid proteins T25-CP and T18C-p4 or T18C-p5. BHT101 cells co-expressing these fusion proteins remained colorless like the control cells expressing T25-CP and T18C proteins after 6 days of incubation at room temperature (Figure 7). Moreover, BTH101 cells co-expressing T25-CP and TI8C-p1, T25-CP and T18C-p2, or T25-CP and T18C-p3 hybrid proteins were able to grow on minimal medium supplemented with maltose as a unique carbon source (example of the peptide p3 shown in Figure 8). One has to notice that the bacterial two-hybrid system used in this study does not give false positive results as described for some other systems, since the reporter gene is indirectly activated. Moreover, T18C-p4 or T18C-p5 constructions could be considered as good negative controls of the experiment which give strength to the positive results obtained with p1, p2 and p3 peptides. These results give a strong argument in favor of the PVY-CP/ RubisCO-LSU interaction.

Molecular visualization

The location of the five peptides on the tobacco RubisCO-LSU was further analyzed via molecular visualization. At present, more than twenty different RubisCO three-dimensional structures have been deposited within the protein data bank. In our molecular visualization study, the tobacco RubisCO-LSU and -SSU, and cabp located bounding were considered. The three peptides p1 (residues 23–34), p2 (residues 41–53) and p3 (residues 118–131), involved in the interaction with the PVY-CP and the amino acids associated with the active site are represented in Figure 9. Interestingly, hydrophobicity analysis of the tobacco RubisCO-LSU has shown that p1, p2, and p3 were located in a region accessible to solvents and hence at the surface of the molecule. Furthermore, the p3 (residues 118–131) peptide includes Asn-123, which is essential for the formation of the RubisCO active site. It also appears that the peptide p2 (residues 41–53) is close to the residues Glu-60 and Thr-65, which are also important for the formation of the RubisCO active site (Guetteridge and Gatenby, 1995). However, the two peptides p4 (residues 381–398) and p5 (residues 408–415), which do not interact *in vivo* with the PVY-CP, are not located near the active site. These observations indicate that if the PVY/RubisCO-LSU interaction takes place *in vivo*, it could have great consequences on the function of tobacco RubisCO.

Discussion

In this study, it has been shown that the major component of the 54 kDa band that is continually detected in the PVY preparation is tobacco RubisCO-LSU. The RubisCO holoenzyme consists of eight LSU associated in the chloroplast with eight SSU (L_8S_8) without involvement of any disulfide bridges (Schneider et al., 1986, 1990). It appears to be in a solution within the chloroplast stroma and also adsorbs to the thylakoid membranes without being anchored to them. Moreover, RubisCO represents a high proportial of the soluble proteins in a photosynthetic plant (25 to 50%) (Chua and Schmidt, 1978). Therefore if the high molecular concentration of this holoenzyme is the only parameter explaining contamination, both of SSU and LSU should be present and at the same level in the PVY preparation. However, this is not the case, as no band corresponding to the RubisCO-SSU was detected. Hence, RubisCO-LSU must have structural properties enabling it to be purified in the presence or absence of the PVY, probably through auto-aggregation (Miziorko and Lormier, 1983), and to co-precipitate spontaneously with the virus after storage at 4 °C (data not shown). Several independent approaches have been performed and their results are consistent with RubisCO-LSU/PVY-CP interaction *in vitro* and *in vivo* experimental conditions. By ELISA, we observed the inhibition of the relevant mAb binding to the PVY by the P54. The anti-PVY-CP mAb detected the PVY/RubisCO-LSU complex in PVY-infected tobacco extracts. Using a phage display library, we were able to select phage peptides on the basis of their affinity to the PVY. These peptides present discontinuous homology with the tobacco RubisCO-LSU, but exist in grouped epitopes on the surface of the LSU conformational structure. Moreover, using the bacterial two-hybrid system, we demonstrated that at least three RubisCO-LSU peptides located within functional domains could interact with the PVY-CP.

Our observations could be related to mosaics and yellowing, which are the major symptoms during the PVY infection and which are consistent with perturbed photosynthetic activity (Naidu et al., 1984) and deteriorated chloroplast structure and function (Culver et al., 1991). These symptoms might be explained by the presence of the PVY-CP in chloroplasts of infected leaves (Gunasinghe and Berger, 1991), since transgenic plants expressing the PVY-CP in their chloroplasts loose their green color and grow very badly (Naderi and Berger, 1997). These plants have the same phenotype as the mutant *N. tabacum* that expresses low RubisCO levels associated with chlorophyll loss (Dulieu, 1974; Nguyen-Quoc et al., 1989). This effect suggests that these transgenic plants have analogous altered chloroplast functions. These functions might include RubisCO activity, which is known to play an important role in photosynthesis and in photorespiration (Miziorko and Lorimer, 1983; Suzuki, 1987; Rival et al., 1996). On the other hand, if this interaction is related to symptom formation, one has to propose that in plants with mild symptoms, as are seen in potato cultivar infected by PVY^N isolates, this interaction would not occur or would be weaker. Nevertheless, to have a biological significance, this interaction, shown in experimental conditions, must also be demonstrated within tobacco plant as well as using other PVY strains and other host plants. Despite the fact that some members of the *Potyvirus* genus induce structural changes in chloroplasts (Brunt et al., 1976; Fraser et al., 1979; Russo et al., 1987), only one interaction of a chloroplast protein and a member of this genus has been reported. This interaction involves a 37 kDa chloroplast protein and the TMV-CP (McClintock et al., 1998). The role of this interaction in viral replication and in symptom expression is still unknown.

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