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Broad-range detection of different Strawberry Latent Ring Spot Virus-isolates by Immuno Capture-PCR

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Currently one of the most sensitive methods in virus detection is based on the amplification of DNA by Immuno Capture-PCR (IC-PCR). The catalogue for detecting different viruses can be extended by the detection of Strawberry Latent Ringspot Virus (SLRV). Primers were defined according to the existing sequence informations for SLRV. IC-PCR was applied to detect SLRV in different isolates of SLRV found in various crops. Infection by SLRV could be verified by using isolates from chestnut, strawberry, other small fruits and grapevine. The amplified region is located at the 3'-end of SLRV in the non-coding region out of the sequence for the coat protein. The reproduction reproducibility of the results could be improved by using a Poly-T primer instead of the anti-sense primer (3-end) for the reverse transcription. The sensitivity in detection is ten times higher than with ELISA testing. Positive results were even obtained after diluting the plant extract with extraction buffer for 1:5000. The PCR products of different isolates were cloned into the pUC18 vector and sequenced. The homology of the different isolates ranks from 89 % to 94 % by comparing the 400 base pairs. Nevertheless broad-range detection of SLRV by IC-PCR was feasible.

Universaler Nachweis verschiedener Erdbeer-Ringflecken-Virus-(SLRV)-Isolate durch Immunocapture-PCR (IC-PCR). *Zurzeit eine der sensitivsten Methoden in der Virusdetektion ist die Immunocapture-PCR (IC-PCR). Die Liste der Viren, welche mittels dieser Methodik nachgewiesen werden können, ist um das Erdbeer-Ringflekken-Virus (SLRV) zu erweitern. Gemäß der verfügbaren Sequenzinformation wurden Primer für SLRV entworfen. IC-PCR wurde eingesetzt, um SLRV von verschiedenen Isolaten aus unterschiedlichen Pflanzen zu detektieren. Ein Befall mit SLRV konnte bei Isolaten von Kastanie, Erdbeere und anderen Beerengewächsen sowie bei Weinrebe nachgewiesen werden. Die vervielfältigte Sequenz liegt am 3-Ende von SLRV in der nichtkodierenden Region außerhalb des Hüllproteingens. Die Reproduzierbarkeit des Nachweises konnte verbessert werden, indem anstatt der 3-DNA-Sonde eine Poly-T-Sonde für die Reverse Transcription verwendet wurde. Die erreichte Sensitivität der IC-PCR ist um den Faktor 10 höher als beim ELISA-Test. Positiver Nachweis war nach einer 5000-fachen Verdünnung des Pflanzenextraktes noch möglich. Die PCR-Produkte der verschiedenen Isolate wurden in pUC 18 kloniert und sequenziert. Die Homologie der Sequenzen dieses 400 Basen langen Abschnittes reicht von 89 % bis 94 % und lässt die weite Anwendungsbreite dieser Nachweismethode erkennen.*

Décèlement à large spectre de divers isolats du virus des taches annulaires de la fraise (SLRV) par voie de IC-PCR (Immunocapture-PCR). *À l'heure actuelle, la méthode IC-PCR est une des méthodes les plus sensibles de détection de virus. La liste des virus pouvant être décelés à láide de cette méthode peut être élargie au virus des taches annulaires de la fraise (SLRV). Des amorces pour le virus SLRV ont été conçues conformément à l'information disponible sur la séquence. La méthode IC-PCR a été utilisée afin de détecter différents isolats du virus SLRV provenant de différentes plantes. L'infection SLRV a pu être détectée dans des isolats de châtaigniers, de fraises et d'autres plantes à baies ainsi que dans des vignes. La séquence reproduite se situe à l'extrémité 3' du virus SLRV dans la région non codante à l'extérieur du gène protéique enveloppant. Il a été possible d'améliorer la reproductibilité de la*

détection en utilisant une sonde Poly-T au lieu de la sonde 3'-DANN pour la transcription inverse. La sensibilité de la méthode IC-PCR est dix fois plus élevée que celle du test ELISA. Un décèlement positif a encore été possible après avoir dilué 5000 fois l'extrait des plantes. Les produits PCR des différents isolats ont été clonés dans pUC 18 et séquencés. L'homologie des séquences de cette section composée de 400 bases va de 89 % à 94 %, ce qui met en relief l'étendue du champ d'application de cette méthode de détection.

Strawberry Latent Ringspot Virus (SLRV) is a member of the nepovirus (*1*) group despite less homology to all other members of this group and some similarities to Como- or Fabaviruses. SLRV shows isometric particles which encapsidate two single-stranded positive sense RNA molecules. The length of RNA 2 (*2*) of the hitherto sequenced isolates is 3824 nucleotides and contains only one open-reading frame. Sequence homology of the two isolates compared so far was high (97 %) in some parts of the genome (*2, 3*). At the 3-end the 3-terminal coding sequences could not be aligned by computer based comparison due to deficiency in homology. Since SLRV is transmitted by soil-inhabiting nematodes (*Xiphinema diversicaudatum* and *X. coxi*), vegetative propagating material could be infested by this pathogen. The main crops damaged by this pest are small fruits, trees like chestnut, cherry and grapevine. Isolates of all the different groups were involved in developing an IC-PCR test. Symptoms induced by the pathogen especially in grapevine on the leaf surface and at canes are weak and disappear during the growing season. Despite fading symptoms economical damage is high due to highly reduced yield with different crops. Hence a sensitive diagnostic method applicable during the whole season is an immense advantage for the phytopathological evaluation and certification of vegetative propagation material.

Detection of the pathogen is nowadays performed by ELISA testing, different IgGs sera (*3, 4*) were developed and most companies with commercial interest in plant viruses diagnosis offer an ELISA based test system. Detection of the pathogen by ELISA is easily to handle and available established in commercial most laboratories as routine analysis. Unfortunately the sensitivity is not sufficient to reproduce the positive results during the whole vegetational period. Like all other nepoviruses SLRV titer decreases under hot temperatures during summer and therefore infection could be masked. This phenomenon is utilized for the efficient elimination of nepoviruses (*5*) by applying thermotherapy with enclosed meristem regeneration.

The objective of this work was to establish a high-sensitive detection system applicable all over the year and with a broad range of diagnosis.

Material and methods

Preparation of extract

Freshly harvested plant material was extracted by grinding with mortar and pestle and the slurry was resuspended in a general extraction buffer (1 x PBS, 2 % PVP MW 24.000, 0.05 % Tween 20). Isolates of strawberry (strawspak.seq, straw.seq, SLRVF2, SLRV 21, SLRV 24, SLRV16R), chestnut (SLRV-44R, SLRV-46F), cherry (SLRVF1) and small fruits were kept on *Chenopodium quinoa* or on *Cucumis sativa.* The grapevine isolate was directly isolated from the woody material of canes. The woodshavings were grinded together with the grapevine extraction buffer recommended by Fa. BIOREBA AG (Basel, Switzerland) (PVP MW24000 20 g/l, Tris/HCl 37.2 g/l, NaCl 8 g/l, PEG MW6000 10 g/l, Tween20 0.5 ml/l). For evaluating the sensitivity the extract was diluted in PBS buffer 1:5, 1:100, 1:1000, 1:5000 and 1:10000.

Performance of the IC-PCR

Tissue of freshly harvested or frozen plants was used for the detection of SLRV. 100 mg of tissue were resuspended in 2 ml extraction buffer. The crude extract was centrifuged for 3 min at 8000 g. The supernatant was loaded to precoated 0.5 ml-tubes to capture viral antigens out of the extract by immunoactive binding. IgGs reactive to SLRV were purchased from Fa. BIOREBA AG (Basel, Switzerland) and diluted 1:500 in coating buffer (0.1 M Na_2CO_3/$NaHCO_3$ pH = 9.6). Precoating was performed at room temperature for four hours with 100 ul or alternatively with 50 ul. Following the protocol three washes with PBS-Tween buffer were done. Coating proteins were disrupted by incubation with 1 % Triton X-100-solution, heating to 65 °C and stirring at the vortex.

The reverse transcription was performed in buffered solution containing 50 mM Tris/HCl pH = 8.3, 75 mM KCl, 3 mM $MgCl_2$, 1 mM dNTPs 10 nM oligo-dT(16) primer or 10 nM 3'-primer, 10 Units RNase Inhibitor (Fa. BOEHRINGER MANNHEIM, Germany) and 20 Units of Mu-MLV Reverse Transcriptase (Fa.

GIBCO LIFE TECHNOLOGIES, Vienna, Austria). Incubation of the solution (20 µl) at 42 °C for transcription of the RNA sequence to DNA takes 45 min at the minimum.
Amplification was usually done by adding 5 µl of the RT charge to 15 µl of the PCR solution (10 mM Tris/HCl, 50 mM KCl, 15 mM $MgCl_2$, 100nM dNTPs 250nM oligonucleotides (3'- and 5'-primer) and 1 U unit Taq DNA-Polymerase (Fa. PROMEGA, Mannheim, Germany). The reaction was performed by running 35 cycles consisting of 45 sec. at 92 °C denaturing phase, 1 min at 50 °C annealing phase and 1 min at 72 °C extension phase.
As the most appropriate sequence for amplification the following oligomeres were designed:
SLRV sense: 5'GTCCCTTGGTTACTTTTACCTC 22mer (3403 - 3425)
SLRV antisense: 5'TAAGTGCCAGAACTAAACCCGG 22mer (3813 - 3791).

Cloning and sequencing

The DNA fragments were purified by separation on a 2 % agarose gel and preparation using the QuiagenEx (Fa. BIOTRADE, Vienna, Austria) system. The isolated DNA fragment was filled up with nucleotides by the Klenow reaction (Fa. BOEHRINGER MANNHEIM, Germany). The phosphorylation of the 3'-end was performed by applying T4 Polynukleotide Kinase (Fa. BOEHRINGER MANNHEIM, Germany) for 1 h at 37 °C. These fragments were cloned into the Sma I restriction site of the plasmid pUC 18. The ligated plasmids were transferred into the bacterial strain of *Escherichia coli* XLS by electroporation (Fa. BIORAD, Gene Pulser Biorad, Vienna, Austria). Due to cloning into the lac Operon selection of positive clones was easily performable.
The resulting sequences of SLRV 3'-end were derived from two independently isolated clones. Sequence was gained by using the forward universal and the reversed primer for pUC 18 provided with the sequencing kit (Cy5-Fluoro-Sequence, Fa. PHARMACIA, Vienna, Austria). The sequence reaction and analysis were done according to the manual of the kit and the performance recommended for the automated sequencing apparatus (ALF Express, Fa. PHARMACIA BIOTECH, Vienna, Austria)

Results and Discussion

The presented primer pair (see Material and Methods) resulted as the most convincing sequence information for detection of SLRV. Several other sequence locations were not appropriate for PCR detection of several SLRV isolates (data not shown). In combination with IgGs polyclonal SLRV antibodies purchased from Fa. BIOREBA AG all different isolates available for this study could be detected. Sensitivity of the diagnosis was evaluated by diluting the plant extract with extraction buffer. The sensitivity (Fig. 1.) was comparable to a similar approach already shown with GFLV, ARMV and GCMV IC-PCR. Therefore this degree of a 1:5000 dilution of extract seems to be easily detectable with well adapted IC-PCR, while ELISA is 10 times less sensitive. Dilution of more than 1:5000 gave no positive result as revealed in previous studies (*4*). When a

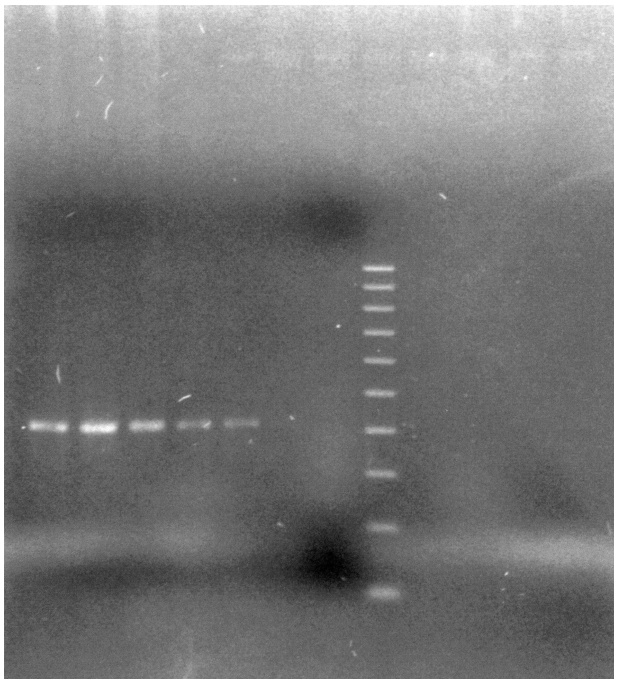

Figure 1: Sensitivity testing by dilution of the plant extract before the IC step - lane 1: undiluted; lane 2: dilution 1:5; lane 3: dilution 1:100; lane 4: dilution 1:1000; lane 5: dilution 1:5000; lane 6: dilution 1:10000; lane 7: dilution 1:20000

Poly-T-primer instead of the 3-end primer was used for reversed transcription sensitivity could be kept at the same range. Moreover, the signal was better to see and the DNA band was at least three times more intense than using the 3'-primer for reverse transcription. Nevertheless both specific primers were required for efficient PCR amplification. Therefore the conclusion

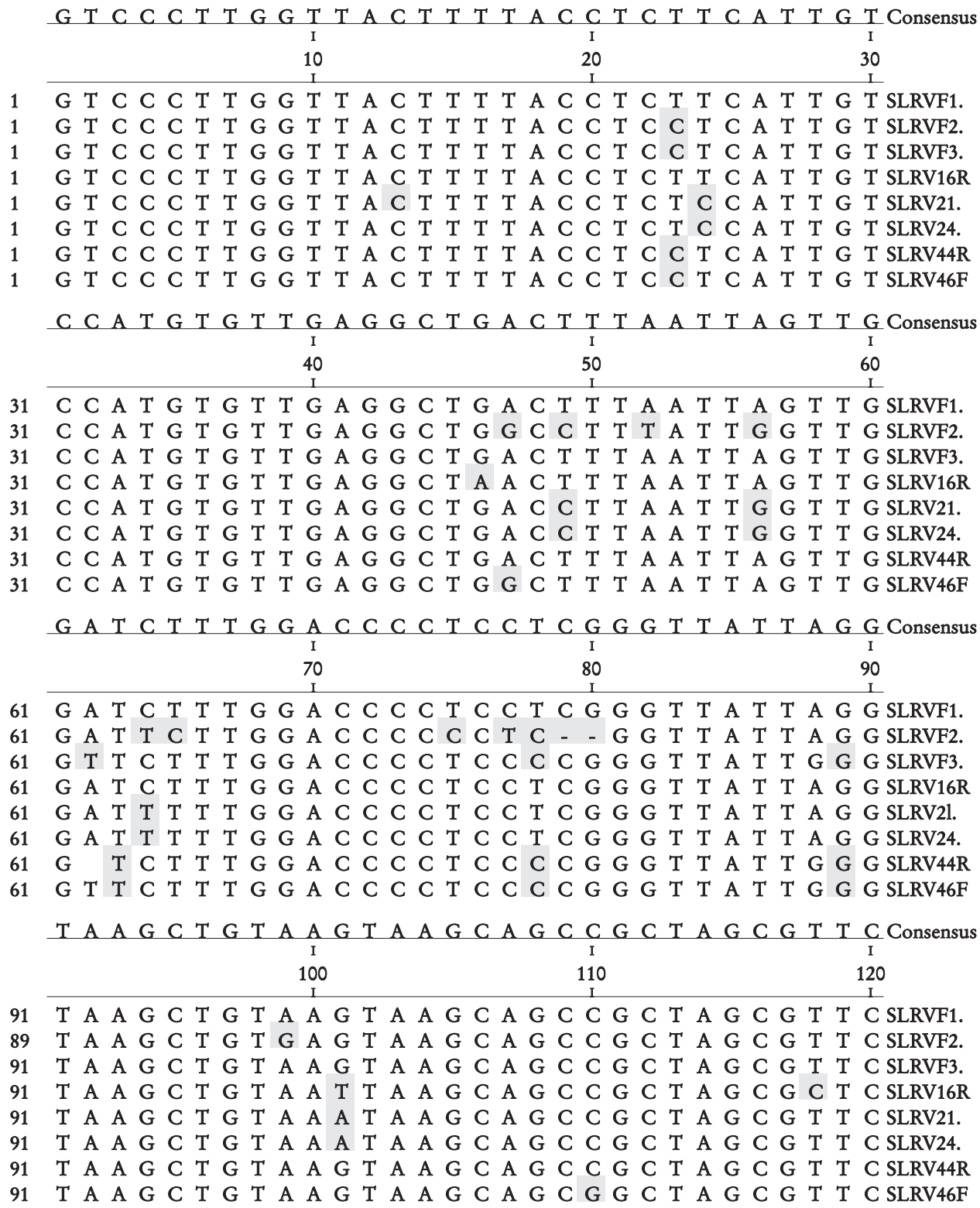

Figure 2: Sequences of the different SLRV isolates gained by IC-PCR, whereas a consensus sequence can be proposed. The following isolates were sequenced SLRV: F1 = cherry, F2 = strawberry, F3 = grapevine, 16R = strawberry, 21 = strawberry, 24 = strawberry, 44R and 46F = chestnut

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     T G G A T T T C C A G G C A T A G T G A G A A T C A G T G G Consensus
                       I                 I                 I
                      130               140               150
                       I                 I                 I
121  T G G A A T T C C A G G C A T A G T G A G A A T C A G T G G SLRVF1.
119  T G G A T T T C C A G G C A C A G T G A G A A T C A G T G G SLRVF2.
121  T G G A T T T C C A G G C A T A G T G A G A A T C A G T G G SLRVF3.
121  T G G G T T T C C A G G C A T A G T G A G A A T C A G T G G SLRV16R
121  T G G A T T T C C A G G C A T G G T G A G A A T C A G T G G SLRV21.
121  T G G A T T T C C A G G C A T G G T G A G A A T C A G T G G SLRV24.
121  T G G A T T T C C A G G C A T A G T G A G A A T C A G T G G SLRV44R
121  T G G A T T T C C A G C C A T A G T G A G A A T C A G T G G SLRV46F

     C A G A T T A G C G A C G C C A C A C G C T T G T A G A C C Consensus
                       I                 I                 I
                      160               170               180
                       I                 I                 I
151  C A G A T T A G C G A C G C C A C A C G C T C G T A G A C C SLRVF1.
149  C A G A T T A A C G A C G C C A C A C G C T T G T A G A C C SLRVF2.
151  C A G A T T A G C G A C G C C A C A C G C T T G T A G A C C SLRVF3.
151  C G IG A T T A G C G A T G C C A C A C G C T T G T A G A C C SLRV16R
151  C A G A T T A G C G A C G C C A C A C G C T T G T A G G C C SLRV21.
151  C A G A T T A G C G A C G C C A C A C G C T T G T A G G C C SLRV24.
151  C A G A T T A G C G A C G C C A C A C G C T T G T A G A C C SLRV44R
151  C A G A T T A G C G A C G C A A C A C G C T T G T A G A C C SLRV46F

     G T A C G A T A G T C T A C C T T T A C A T G C T T T T G T Consensus
                       I                 I                 I
                      190               200               210
                       I                 I                 I
181  G T A C G A T A G T C T A C C C T T A C G T G C T T T T G T SLRVF1.
179  G T A C G A T A G T C T A C T T T T A T A C G C T T T T G T SLRVF2.
181  G T A C G A T. A G T C T A C C T T T A C A T G C T T T T A T SLRVF3.
181  G T A T G A T A G T C T A C C T T T A T A C G C T T T T G T SLRV16R
181  G T A C G A T A G T C T A C T T T T A C A T G C T T T T G T SLRV21.
181  G T A C G A T A G T C T A C T T T T A C A T G C T T T T G T SLRV24.
181  G T A C G A T A G T C T A C C T T T A C A T G C T T T T A T SLRV44R
181  G T A C G A T A G T C T A C C T T T A C A T G C T T T T A T SLRV46F

     A G T T T - A G G G C C T T T G T G A T T T T A C T C T c T Consensus
                       I                 I                 I
                      220               230               240
                       I                 I                 I
211  A G T T T - A G G G C C T T T - T G A T G T T A - T C T C T SLRVF1.
209  A G T T T - A G G G C C C A T T - T G A T G T T A - T C T C T SLRVF2.
211  A G T T T T A G G G C C T T T G T G A T T T T T C T C T C T SLRVF3.
211  A G T T C - A A C G C C T C 1T - T G A T G T T A - T T T C T SLRV16R
211  A G T T T A A G G - - C C C 1T G T G A T A T T A T T - T C T SLRV21.
211  A G T T T A A G G - - C C C T G T G A T A T T A T T - T C T SLRV24.
211  A G T T T - A G G G C C T T T - G T G A T T T T T - C T C T C T SLRV44R
211  A G T T T - A G G G C C T T T - G T G A T T T T T - C T C T C T SLRV46F
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Figure 2 (continued from page 186): Sequences of the different SLRV isolates

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     T A A T T G A G T T - G T A T G T C A C T T G T T A T G C C Consensus
                         I                 I                 I
                       250               260               270
                         I                 I                 I
238  T T A T T G A G T T - G T A C G T C A C T T G T T A C G C C SLRVF1.
236  T T A T T G A G T T - G T A C G T C A T C T G T T A T G C C SLRVF2.
241  T A A T T G A G T T T G T A T G T C A C T C G T T A T G C C SLRVF3.
238  T A A T T G A G T T - C T A C G T C A T T T G T T A T G C C SLRV16R
238  T A A T T A A G T T - G T A T A T C A C T T G T T A T G C C SLRV21.
238  T A A T T A A G T T - G T A T A T C A C T T G T T A T G C C SLRV24.
238  T A A T T G A G T T - G T A T G T C A C T C G T T A T G C C SLRV44R
238  T A A T T G A G T T - G T A T G T C A C T C G T T A T G C C SLRV46F

     A T T T T G C T T T T G T G T G T T T T C T T T G A G C C T Consensus
                         I                 I                 I
                       280               290               300
                         I                 I                 I
267  A C T C T G C T T T T G T G T G T T T T C T - T G A G C C T SLRVF1.
265  A C T C T G C T T T T G T G T G T T T T C T - T G A G C C T SLRVF2.
271  A T T T T G C T T T T G T G T G T T C T T T T T G A G C C T SLRVF3.
267  A T T C T G C T T T T G T G T G T T T T C T - T G A G C C T SLRV16R
267  A T T T T G C T T T T G T G T G G T - T T T C T G A G C C C SLRV21.
267  A T T T T G C T T T T G T G T G T T - T T T C T G A G C C C SLRV24.
267  T T T T T G C T T T T G T G T G T T C T C T T - G A G T C T SLRV44R
267  T T T T T G C T T T T G T G T G T T C T C T T - G A G C C T SLRV46F

     T T T A T G T C T C C T T T T A T T A G G T G T A C G T A A Consensus
                         I                 I                 I
                       310               320               330
                         I                 I                 I
296  T T C A T G T C T T C C T T C A T T G G A T G T A C G T G A SLRVF1.
294  T T T A T G T C T C C T T T C A T T A G G T G T A C G T A A SLRVF2.
301  T T T A T G T C T C C T T T T A T T A G G T G T A C G T A A SLRVF3.
296  T T C A T G T C T T C C T T C A T T G G A T G T A C G T G A SLRV16R
296  T T T A T G T C T C C T T T T A T T A G G T G T A C G T A A SLRV2l.
296  T T T A T G T C T C C T T T T A T T A G G T G T A C G T A A SLRV24.
296  T T T A T G T C T C C T T T T A T T A G G T G T A C G T A A SLRV44R
296  T T T A T G T C T C C T T T T A T T A G G T G T A C G T A A SLRV46F

     T T G T T A T G C T C T T T G C T T T C T T T T G T G T T T Consensus
                         I                 I                 I
                       340               350               360
                         I                 I                 I
326  T T G T T A T G C T C T T T G C T T T C T T T T G T G T T A SLRVF1.
324  T T G T T G T G C T C T T T G C T T T C T T T T G T G T T T SLRVF2.
331  T T G T T A T G C T C T T T G C T T T C T T T T G T G T T T SLRVF3.
326  T T G T T A T G C T C T T T G C T T T C T T T T G T G T T T SLRV16R
326  T T G T T A T G C T C T T T G C T T T C T T T C G T G T T T SLRV21.
326  T T G T T A T G C T C T T T G C T T T C T T T C G T G T T T SLRV24.
326  T T G T T A T G C T C T T T G C T T T C T T T T G T G T T T SLRV44R
326  T T G T T A T G C T C T T T G C T T T C T T T T G T G T T T SLRV46F
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Figure 2 (continued from page 187): Sequences of the different SLRV isolates

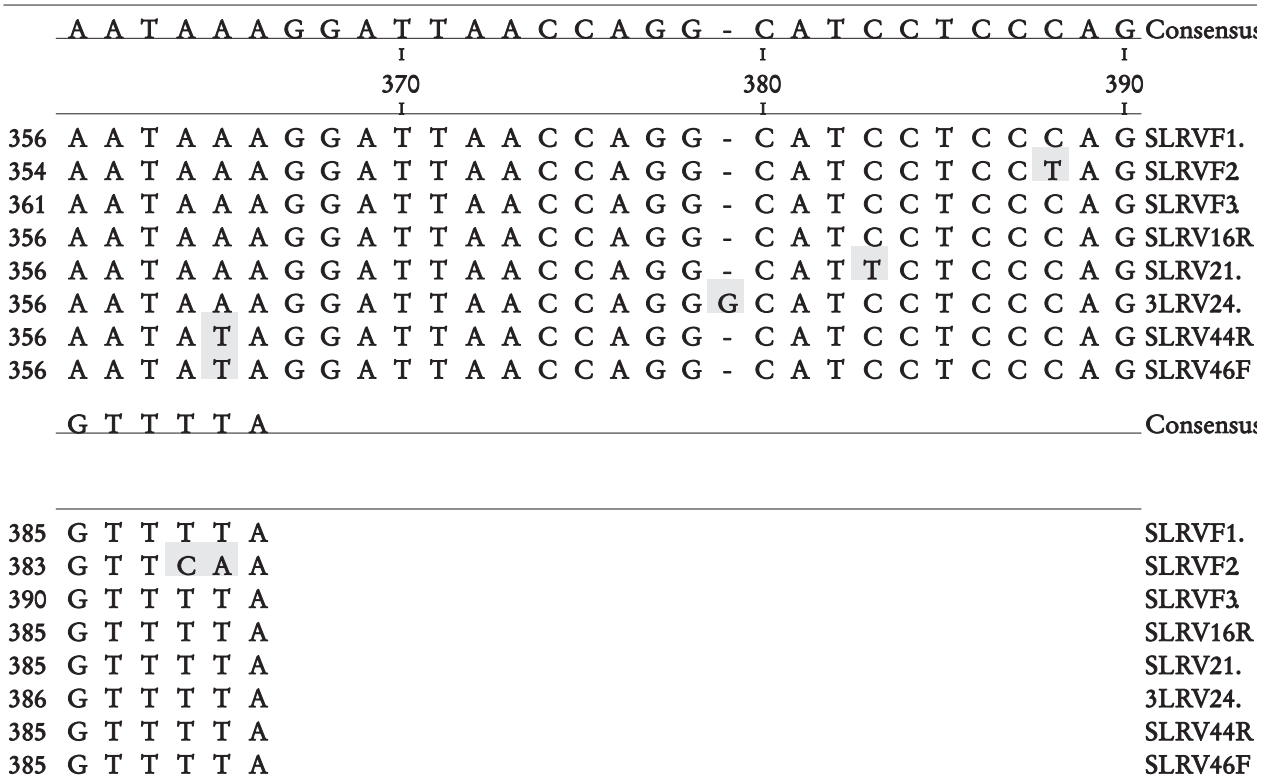

Figure 2 (continued from page 188): Sequences of the different SLRV isolates

Percent Similarity

Percent Divergence	1	2	3	4	5	6		
1		93,1	83,5	85,6	88,5	86,9	1	grapevine seq
2	1,0		87,6	89,0	90,5	91,5	2	chestnut. seq
3	9,2	10,2		86,9	86,9	88,7	3	straw. seq
4	9,2	10,1	11,4		88,7	93,8	4	strawmur. seq
5	7,7	8,3	11,2	11,5		89,0	5	strawspak. seq
6	7,1	8,0	8,9	6,2	10,5		6	cherry. seq
	1	2	3	4	5	6		

Figure 3: Sequence pair distance of all 6 SLRV isolates by using clustal method with weighted residue weight table

is justified that 3'-end annealing is not as efficient as Poly-T-priming in SLRV detection.

All different isolates were sequenced (Fig. 2) and their homology concerning the 3'-end were calculated (Fig. 3). It was interesting to see the high homology between the chestnut and the grapevine isolate whereas strawberry isolates differ due to their local incidence (Fig. 4). For fast differential diagnostics concerning the strains of SLRV a restriction analysis could be proposed. Based on the sequence information we could propose several restriction enzymes for cutting DNA of SLRV for discrimination of the different SLRV strains of this study (Fig. 5). Hinf I and Hae III digestion allowed to differentiate all strains except grapevine and cherry. An additional Sma I or Xma I restriction would be useful to identify the grapevine strain. All these sequence analyses allowed us to demonstrate the universality of the selected primers and to show the heterologous sequence of the different SLRV isolates. Despite the spe-

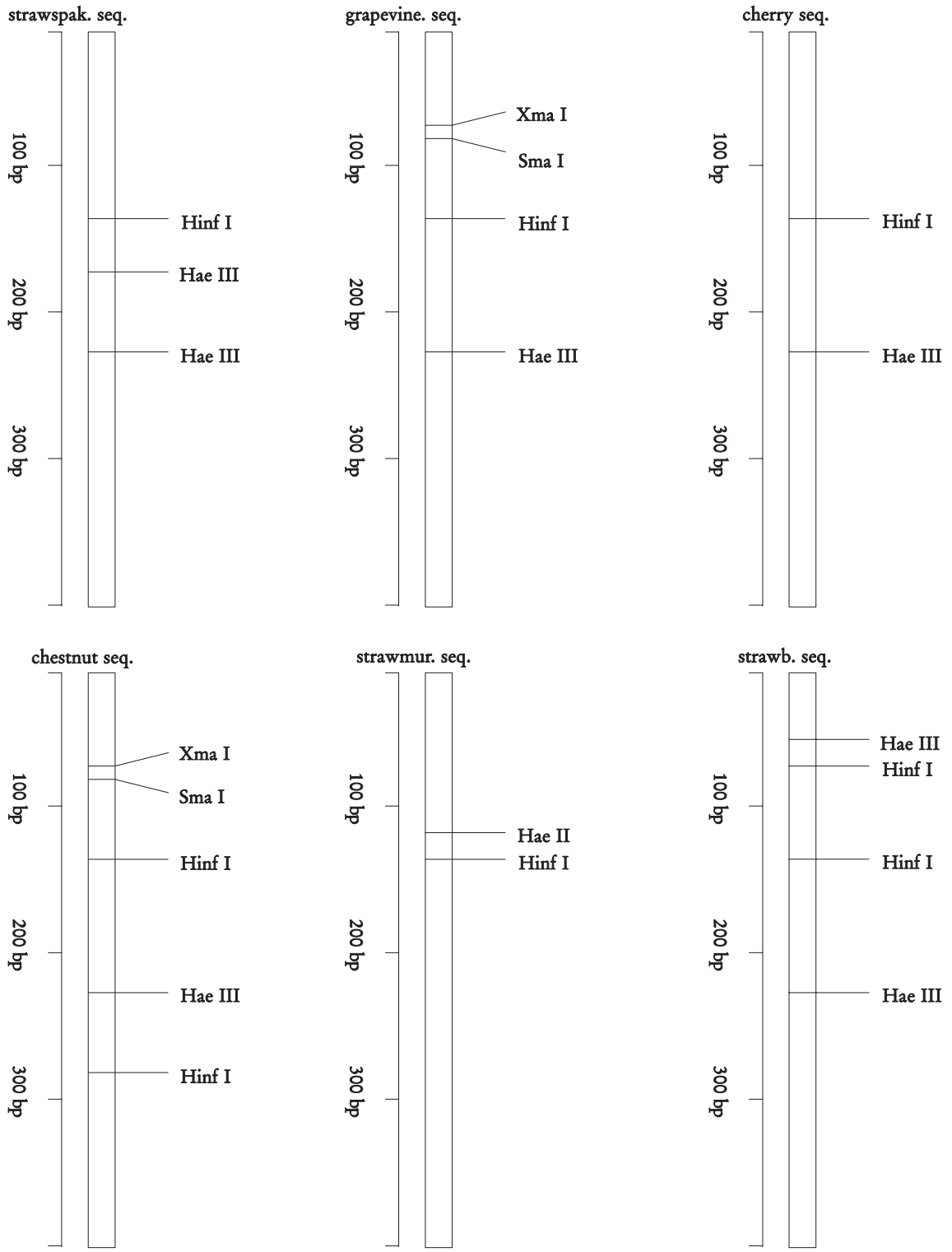

Figure 5: Restriction sides for differential diagnostics of the SLRV isolates

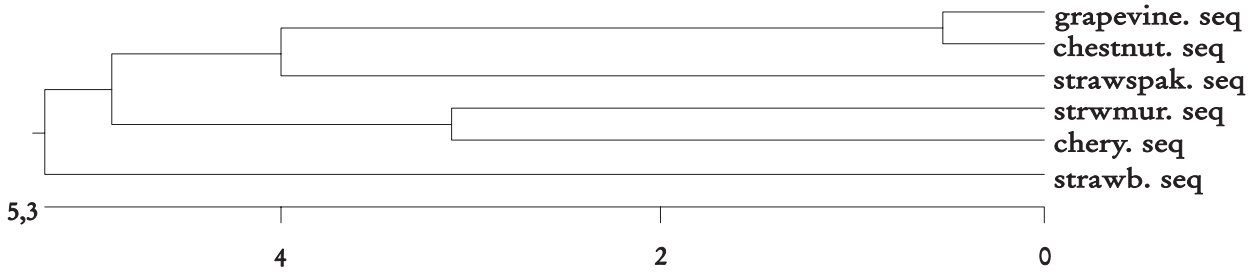

Figure 4: Phylogenetic tree of all 6 SLRV isolates by using clustal method with weighted residue weight table

cificity of PCR diagnosis it should be possible to cover most strains of the viral variability due to an ideal primer designation.

Acknowledgements

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