Stapfia	80	251-265	5.7.2002

Molecular characterization of zucchini yellow mosaic potyvirus (*Potyviridae*) strains isolated from diseased plants of Austrian oil pumpkin-growing areas

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A b s t r a c t : PFOSSER M. & H. BAUMANN (2002): Molecular characterization of zucchini yellow mosaic potyvirus (*Potyviridae*) strains isolated from diseased plants of Austrian oil pumpkin-growing areas. — Stapfia **80**: 251-265

Isolates of zucchini yellow mosaic virus (ZYMV) were obtained from infected fruits of *Cucurbita pepo* cultivated in the field of all major oil pumpkin-growing areas in Austria and adjacent Slovenia. The partially purified potyvirus isolates have been characterized by RFLP analysis after reverse transcriptase-polymerase chain reaction (RT-PCR) and by DNA sequencing of the coat protein and parts of the cytoplasmic inclusion protein loci of the virus genome. All Austrian isolates appeared to be very similar suggesting a single introduction of the virus with subsequent spreading to all pumpkin-growing areas in Austria. Comparison of the DNA sequences with isolates from Slovenia, Hungary, Germany, Japan, Italy, Israel, Korea, Taiwan, USA, Reunion Island and Singapore revealed highest similarities of the Austrian isolates to the isolates from Slovenia and Hungary. Phylogenetic analysis combines these strains in a highly supported monophyletic group. Sister to this group are isolates from Israel and Germany, and the Japan-M strain. Clearly distinct are strains from Italy, USA, and the Taiwan-NT1 strain. More distantly related are the Korean isolates, most of the strains from Taiwan, as well as the Japan-169 strain. The Reunion and Singapore strains occupy the most basal positions in both the coat protein and cytoplasmic inclusion protein gene trees.

Z u s a m m e n f a s s u n g : PFOSSER M. & H. BAUMANN (2002): Molekulare Charakterisierung von Isolaten des Zucchinigelbmosaikvirus (*Potyviridae*) aus österreichischen Ölkürbisanbaugebieten. — Stapfia **80**: 251-265.

Isolate des Zucchinigelbmosaikvirus (ZYMV) wurden aus infizierten Früchten von *Cucurbita pepo* aus allen wichtigen Ölkürbisanbaugebieten Österreichs und des benachbarten Sloweniens gewonnen. Die grob gereinigten Potyvirusisolate wurden mittels RFLP-Analyse nach reverser Transkription und Polymerase-Kettenreaktion bzw. durch Sequenzieren des Hüllproteingens und des Cytoplasmatischen Inclusion-Proteingens charakterisiert. Da alle österreichischen Isolate genetisch sehr ähnlich waren, wird ein einmaliges Eindringen eines Pathotyps mit nachfolgender rascher Ausbreitung auf alle österreichischen Anbaugebiete angenommen. Phylogenetisch sind die österreichischen Isolate am nächsten verwandt mit Isolaten aus Slowenien und Ungarn. Genetisch verwandt sind weiters die Isolate aus Israel und Deutschland sowie der Japan-M Virusstamm. Deutlich verschieden sind hingegen die Isolate aus Italien, USA, Korea und Taiwan. Die Stämme aus Reunion und Singapur stehen phylogenetisch an der Basis des ZYMV-Stammbaumes.

K e y w o r d s : Zucchini yellow mosaic virus; phylogenetic comparison of isolates; geographical differentiation; coat protein gene; cytoplasmic inclusion protein gene.

Introduction

In 1981 a novel potyvirus that causes zucchini yellow mosaic in diseased zucchini squash was reported almost simultaneously from Italy (LISA et al. 1981) and from France (LECOQ et al. 1981). This virus was shown to be sap-transmitted to fifteen herbaceous species belonging to seven different families and causes severe symptoms in zucchini (*Cucurbita pepo*), cantaloupe (*Cucumis melo*), cucumber (*Cucumis sativus*), and watermelon (*Citrullus vulgaris*). Typically, the virus causes a light green to yellow mosaic on the leaves of diseased plants. Later, the symptoms can become more drastic, resulting in deep foliar serration, blisters, deformations, plants stunting, and may lead to completely unmarketable fruits (Fig. 1). Different strains of this virus were found in infected cucurbits throughout the world including localities in Europe, America, Australia, and Asia (DESBIEZ & LECOQ 1997; LISA & LECOQ 1984; ROBINSON et al. 1993). In 1997 massive virus outbreaks led to substantial losses in oil pumpkin yields in Austria (RIEDLE-BAUER 1998). The virus is highly infectious and can be transmitted not only by aphids but also mechanically or by infected seeds (SCHRIJNWERKERS et al. 1991). Therefore, only two possibilities exist to fight spreading of the disease: (1) artificial infection of young plants with a mild field strain which cross protects plants against severe field strains (GAL-ON 2000), or (2) transfer of resistance against ZYMV, derived from wild sources by conventional breeding (PARIS & COHEN 2000; PROVVIDENTI 1997) or by transgenic approaches (CLOUGH & HAMM 1995; QUEMADA & GROFF 1995). Infection of cucurbits with an engineered ZYMV (ZYMV-AG strain) caused a dramatic symptom change from severe to mild in squash and to a symptom-free appearance in cucumber, melon, and watermelon (GAL-ON 2000). Moreover, cucurbit plants infected with the ZYMV-AG strain were protected against infection by severe strains. However, mechanical inoculation with the recombinant potyvirus is time consuming and therefore only of limited applicability. Furthermore, inoculation of young plants, which are already infected (e.g. from infected seeds) remains ineffective. Breeding for resistance, therefore, may be the only way to obtain disease free plants. No sources of resistance to ZYMV exist within *C. pepo*. Resistant genotypes have been found in several accessions of the cultivated species *C. moschata* and *C. ficifolia* as well as in the wild species *C. ecuadorensis* (HERRINGTON et al. 1988; PARIS et al. 1988; PROVVIDENTI et al. 1984; ROBINSON et al. 1988). Except for a few cases, introgression of resistance from *C. ecuadorensis* into *C. pepo* was unsuccessful (HERRINGTON et al. 1988). More promising results have been obtained with interspecific crossings between resistant genotypes of *C. moschata* x *C. pepo* (PARIS & COHEN 2000; PROVVIDENTI 1997). However, breeding is complicated due to partial cross incompatibility between *C. moschata* and *C. pepo*, resulting in only a few viable seeds in most cross combinations (WHITAKER & DAVIES 1962). Austrian oil pumpkin varieties are characterized by thin coated seeds, a feature being regarded as a very rare case of spontaneous mutation due to the probably oligogenic nature of this genotype (TEPPNER 2000). Crossing with ZYMV-resistant genotypes, therefore, results in any case in the loss of the typical thin-coated seed phenotype. It is therefore necessary to reduce the crossings to the most promising resistant genotypes. Different levels of resistance against distinct ZYMV strains exist in resistant varieties. For example, in a study involving the inoculation of four resistant varieties of *C. pepo* which have obtained the resistance from the *C. moschata* variety "Nigerian Local", a high level of resistance was observed against the Florida isolate of ZYMV. However, three of the

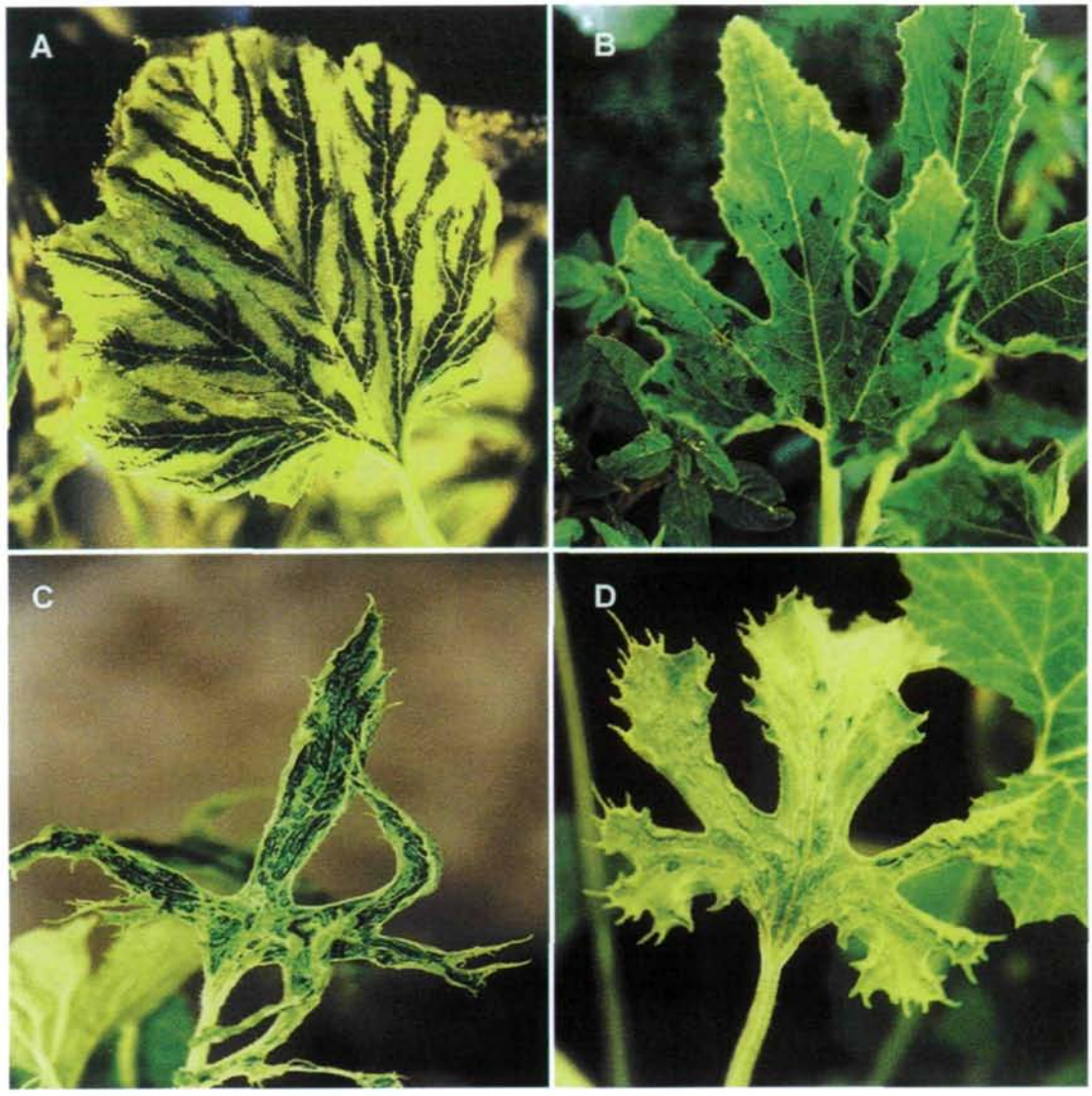

Fig. 1: Symptoms of zucchini yellow mosaic virus infections on leaves of *C. pepo*. A, B, light green to yellow mosaic on leaves of diseased plants. C, D, severe symptoms with deep foliar serration, blisters and deformations.

tested *C. pepo* varieties lacked the high level of resistance to the Connecticut strain of their "Nigerian Local" parent (ROBINSON & PROVVIDENTI 1997). Differences in the response of varieties to inoculations with different ZYMV strains are therefore likely and have to be considered in choosing the most promising crossing partners. To provide a basis for the selection of resistant genotypes as crossing partners and the establishment of a resistance-breeding program for Austrian oil pumpkins, we performed a phylogenetic characterization of the ZYMV isolates found in oil pumpkin-growing areas in Austria.

Material and Methods

Sample collection

Fruits or leaves with symptoms of ZYMV infections were collected from different regions in Austria and adjacent Slovenia and were stored at –20° C (Tab.1).

Table 1: Virus strains, geographic origin and EMBL accession numbers of coat protein and cytoplasmic inclusion protein genes.

Virus	Geographic origin	EMBL acc. (cp/cip)	References
ZYMV Austria-1	Austria, Styria, Hummersdorf	-/-	this paper
ZYMV Austria-2*	Austria, Styria, Zelting	AJ420012/AJ420021	this paper
ZYMV Austria-3	Austria, Styria, Hürth	-/-	this paper
ZYMV Austria-4	Austria, Styria, Sicheldorf	-/-	this paper
ZYMV Austria-5*	Austria, Styria, Gleisdorf	AJ420013/AJ420022	this paper
ZYMV Austria-6*	Austria, Styria, Gleisdorf	AJ420014/AJ420023	this paper
ZYMV Austria-7	Austria, Styria, Radkersburg	-/-	this paper
ZYMV Austria-8	Austria, Lower Austria, Watzelsdorf	-/-	this paper
ZYMV Austria-9	Austria, Lower Austria, Gettsdorf	-/-	this paper
ZYMV Austria-10*	Austria, Lower Austria, Hadres	AJ420015/AJ420024	this paper
ZYMV Austria-11*	Austria, Lower Austria, Ameis	AJ420016/AJ420025	this paper
ZYMV Austria-12*	Austria, Lower Austria, Zistersdorf	AJ420017/AJ420026	this paper
ZYMV Austria-13	Austria, Lower Austria, Ziersdorf	-/-	this paper
ZYMV Austria-14	Austria, Lower Austria, Zellerndorf	-/-	this paper
ZYMV Slovenia*	Slovenia, Maribor	AJ420018/AJ420027	this paper
ZYMV Italy*	Italy, Piedimonte	AJ420020/AJ420029	this paper
ZYMV Germany*	Germany, Berlin	AJ420019/AJ420028	this paper
ZYMV Hungary-10	Hungary	AJ251527/-	TOBIAS et al. 1998
ZYMV Israel-NAT	Israel	M35095/-	GAL-ON et al. 1990
ZYMV Israel-AT	Israel	S46009/-	GAL-ON et al. 1992
ZYMV Chile	Chile	AF308732/-	PRIETO et al. 2001
ZYMV California	USA, California	L31350/L31350	WISLER et al. 1995
ZYMV Connecticut	USA, Connecticut	D00692/-	GRUMET & FANG 1990
ZYMV Florida	USA, Florida	D13914/-	QUEMADA et al. 1990
ZYMV Japan-169	Japan	AB004640/AB020477	KUNDU et al. 1997; 1999
ZYMV Japan-M	Japan	AB004641/AB020478	KUNDU et al. 1997; 1999
ZYMV Korea	Korea	AF062518/-	YOON & CHOI 1998
ZYMV Taiwan-TN3	Taiwan	AF127929/-	LIN et al. 2000
ZYMV Taiwan-CY2	Taiwan	AF127930/-	LIN et al. 2000
ZYMV Taiwan-TC1	Taiwan	AF127931/-	LIN et al. 2000
ZYMV Taiwan-TNML1	Taiwan	AF127932/-	LIN et al. 2000
ZYMV Taiwan-NT1	Taiwan	AF127933/-	LIN et al. 2000
ZYMV Taiwan-PT5	Taiwan	AF127934/-	LIN et al. 2000
ZYMV Reunion	Reunion Island	L29569/L29569	WISLER et al. 1995
ZYMV Singapore	Singapore	AF014811/AF014811	LEE et al. 1997
ZYMV Australia-A3	Australia	S81377/-	THOMSON et al. 1995
ZYMV Australia-G4	Australia	S81381/-	THOMSON et al. 1995
ZYMV Australia-K	Australia	S81384/-	THOMSON et al. 1995
WMV2 Australia-A6	Australia	S81387/-	THOMSON et al. 1995
WMV2 USA	USA	D13913/-	QUEMADA et al. 1990
SbMV G2	-	S42280/S42280	JAYARAM et al. 1992
PStV	-	U05771/U05771	GUNASINGHE et.al. 1994
PVY-N	-	-/D00441	ROBAGLIA et al. 1989

* Samples used for sequencing

All samples have been tested serologically at the Plant Pathology Department of the Research Center for Agriculture (Vienna, Austria) and showed positive reactions in ELISA tests. Two ZYMV isolates from Germany (PV0466) and Italy (PV0416) were obtained from the "Deutsche Sammlung von Mikroorganismen und Zellkulturen" (Braunschweig, Germany).

Isolation and PCR amplification of viral RNA

Total RNA was isolated from 0.5 mg plant material following the protocol supplied with the RNeasy Plant Mini Kit (Qiagen). Reverse transcription followed by polymerase chain reaction (RT-PCR) was used for amplification of the coat protein gene (cp) and cytoplasmic inclusion protein gene (cip). First strand cDNA was synthesized at 42°C for 30 min from total RNA using Superscript II RNaseH$^-$ (GibcoBRL) and the minus strand oligonucleotide primer CPrev for cp and CIPrev for *cip* (Tab. 2, Fig. 2). Double stranded DNA fragments were generated from the first strand using primer pairs CPfwd and CPrev for cp, CIPfwd and CIPrev for cip. PCR was carried out in a TouchDown thermal cycler (HYBAID) operating in tube control mode during 35 cycles of 94° C 25 sec, 55° C 25 sec, 72° C 70 sec for the cp region and 35 cycles of 94° C 25 sec, 40° C 25 sec, 72° C 40 sec for the cip region.

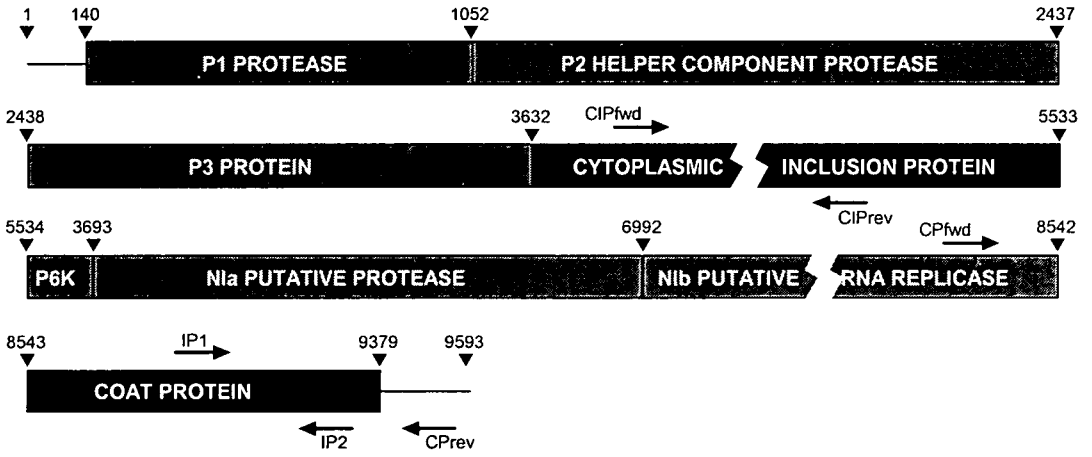

Fig. 2: Gene order of the zucchini yellow mosaic virus genome (California strain). Nucleotide positions of coding regions as well as the positions of PCR primers (arrows) are indicated. The coding regions for cytoplasmic inclusion protein and coat protein analyzed in this study are shown in black.

RFLP analysis of PCR products

RFLP analysis of the cp region was performed using aliquots of the PCR products after over night-incubation with the restriction endonucleases MseI or HpaII at 37° C in a final volume of 20 µl. The digestion products were electrophoretically separated and analyzed in a 2% agarose gel.

Table 2: Oligonucleotides used in the present study.

Primer	Position*	Nucleotide sequence	Reference
CPfwd	8407-8426	5′ - GCT CCA TAC ATA GCT GAG AC - 3′	BARBARA et al. 1995
CPrev	9585-9565	5′ - AAC GGA GTC TAA TCT CGA GC - 3′	BARBARA et al. 1995
IP1	8898-8915	5′ - GGG AGT TGT AAT GAA TG - 3′	this work
IP2	9136-9120	5′ - AAG CAA ACC ATA CCT CG - 3′	this work
CIPfwd	3829-3845	5′ - GAG GAT TGG TGG AAT CG - 3′	this work
CIPrev	4292-4276	5′ - AGT GCA CAA TTG AAA GC - 3′	this work

*Positions of 5′ and 3′ ends of primers according to the complete coding sequence of the California strain of ZYMV (EMBL accession number: L31350).

Dideoxynucleotide sequencing

Two DNA regions of the virus genome were sequenced: the coat protein gene (cp) and part of the cytoplasmic inclusion protein gene (cip). Amplified double-stranded DNA products were sequenced directly using the Dye Terminator Cycle Sequencing Kit (Amersham, UK). Both strands were sequenced in an ABI 377 automated sequencer (Perkin Elmer) using primers CPfwd and CPrev for the cp region and CIPfwd and CIPrev for the cip region. Two additional internal sequencing primers, IP1 and IP2, (Tab. 2, Fig. 2) were designed to resolve ambiguities in the cp region.

Sequence analysis

For comparison, nucleotide sequences of ZYMV viruses from different geographical regions were retrieved from DNA sequence databases (Tab. 1). To root the trees, published nucleotide sequences of potato virus Y strain N (PVY-N), watermelon virus (WMV2 USA), and peanut stripe virus (PStV) were used. DNA and deduced protein sequences were pre-aligned using the PileUp program of the GCG software package (Genetics Computer Group 1994). Final alignment of DNA sequences was done visually. All sequences have been deposited in the EMBL database (for accession numbers refer to Tab. 1). Phylogenetic analysis using distance matrices and neighbor joining was performed with the computer program PAUP version 4.0b4 (SWOFFORD 2000). Ten thousand fast bootstrap replicates (FELSENSTEIN 1985) were used to assess confidence limits for the resulting tree topologies.

Results

For all diseased plant material used in this study we could confirm the presence of ZYMV RNA in crude sap extracts by RT-PCR. Amplification of the cp region using the primers CPfwd and CPrev resulted in a PCR product of approximately 1100 nucleotides (nt). Amplification of a nucleotide sequence close to the 3′ end of the cip gene resulted in a PCR fragment of 440 nt (primers CIPfwd and CIPrev). All PCR primers have been designed specifically for the amplification of ZYMV nucleic acids. Mismatches to other potyvirus sequences have been maximized by comparison of putative primer sequences with homologous sequences of WMV2, PVY, SbMV, and

PStV. Finally, primer sequences have been chosen, which do not contain mismatches to known sequences of ZYMV, but do contain approximately 4-8 mismatches to known sequences of potyviruses other than ZYMV. These precautions practically eliminated the possibility of erroneous amplification of any other viral nucleic acid if eventually present in a sample.

To differentiate among isolates, aliquots of the cp amplification product have been digested with the frequent cutting restriction enzymes MseI or HpaII (Fig. 3A). Both enzymes cut 2 to 4 times within the cp DNA fragments. Screening of all Austrian, the Slovenian, the German, and the Italian isolates, however, resulted in a monomorphic pattern for all strains except for the Italian strain, which showed a different pattern (Fig. 3A, B). The Italian strain obviously lacks one recognition site for HpaII and one for MseI.

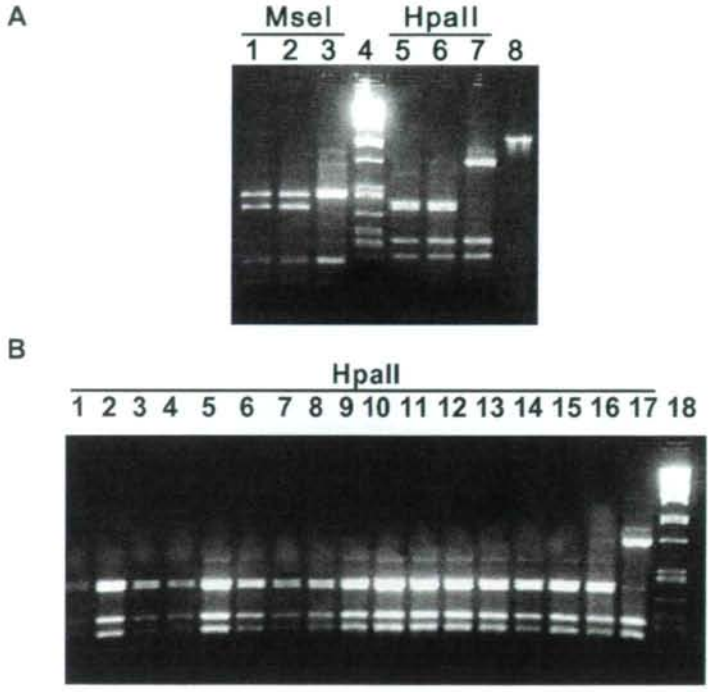

Fig. 3: PCR-RFLP patterns of ZYMV isolates. A, MseI digestion of RT-PCR products of the coat protein gene (lanes 1-3) or digestion with HpaII (lanes 5-7) discriminates the Italian strain (lanes 3, 7) from the German (lanes 2, 6) and Austrian isolates (lanes 1, 5); lane 4, DNA marker λ/PstI; lane 8, undigested PCR product of ZYMV Austria-2. B, Screening of virus isolates with HpaII. HpaII digestion of RT-PCR products of the coat protein results in a monomorphic RFLP pattern for all Austrian ZYMV isolates (lanes 1-14, Austria-1 - Austria-14), the Slovenian isolate (lane 15) and the German isolate (lane 16); lane 17, Italian isolate; lane 18, DNA marker λ/PstI.

Since PCR-RFLP was not sensitive enough to differentiate among strains, six accessions from the Austrian provinces Styria and Lower Austria as well as the Slovenian, the German and the Italian isolate were selected for sequencing the cp and part of the cip genes. DNA sequencing not only allowed us to further discriminate among isolates, but also opened the possibility to compare the Austrian, Slovenian, Italian and German isolates with ZYMV strains found in other geographical regions. Figure 4 shows a phylogenetic analysis of the data matrix composed of cip sequences. In this

analysis all ZYMV strains form a monophyletic group (100% bootstrap support) and are clearly separated from other potyviruses. Within the ZYMV group, the Austrian, Slovenian and German isolates form a highly supported group (100% bootstrap support). Sister to this group is a moderately supported paraphyletic assemblage of the California and the Japan-M strains. Again, the Italian isolate appears to be less closely related to the rest of the European isolates. Even more distinct are the Japan-169, the Singapore and the Reunion strains.

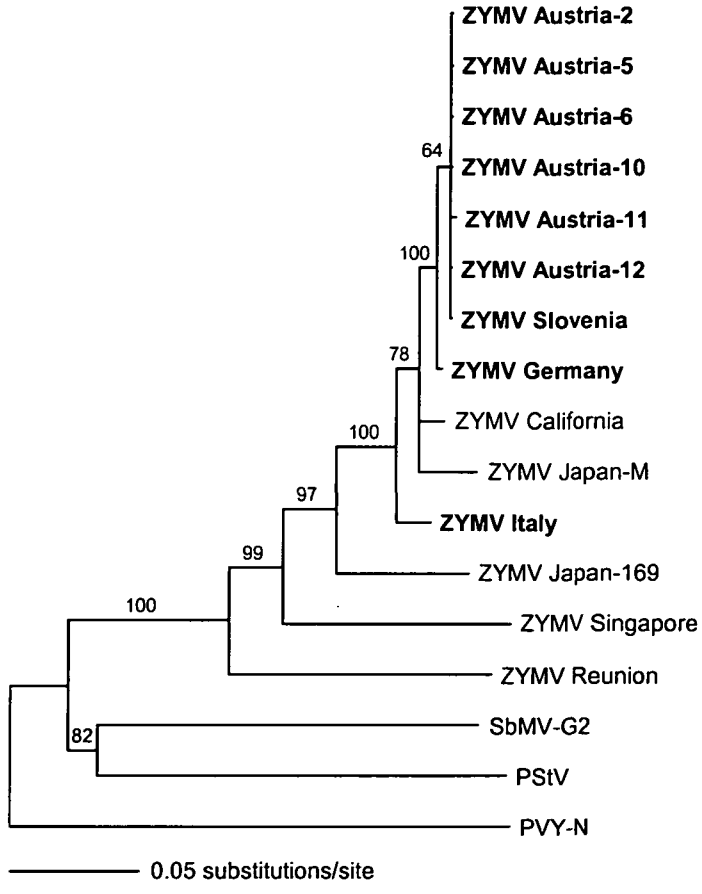

Fig. 4: Neighbor-joining tree based on the cytoplasmic inclusion protein (cip) gene of ZYMV isolates from different geographical regions. The tree is rooted with the cip gene sequence of potato virus Y (PVY-N). Bootstrap support values > 50 % are indicated above branches. European isolates are shown in bold.

Phylogenetic analysis of a data matrix composed of cp sequences generally agrees with the cip tree (Fig. 5). The ZYMV strains are well separated from the outgroup taxa and form a monophyletic group (Fig. 5B). Since more sequences of the cp region than of the cip region are available in public sequence databases, a higher differentiated phylogeny can be obtained within the ZYMV group (Fig. 5A). A highly supported group (100% bootstrap support) with only minor genetic differentiation

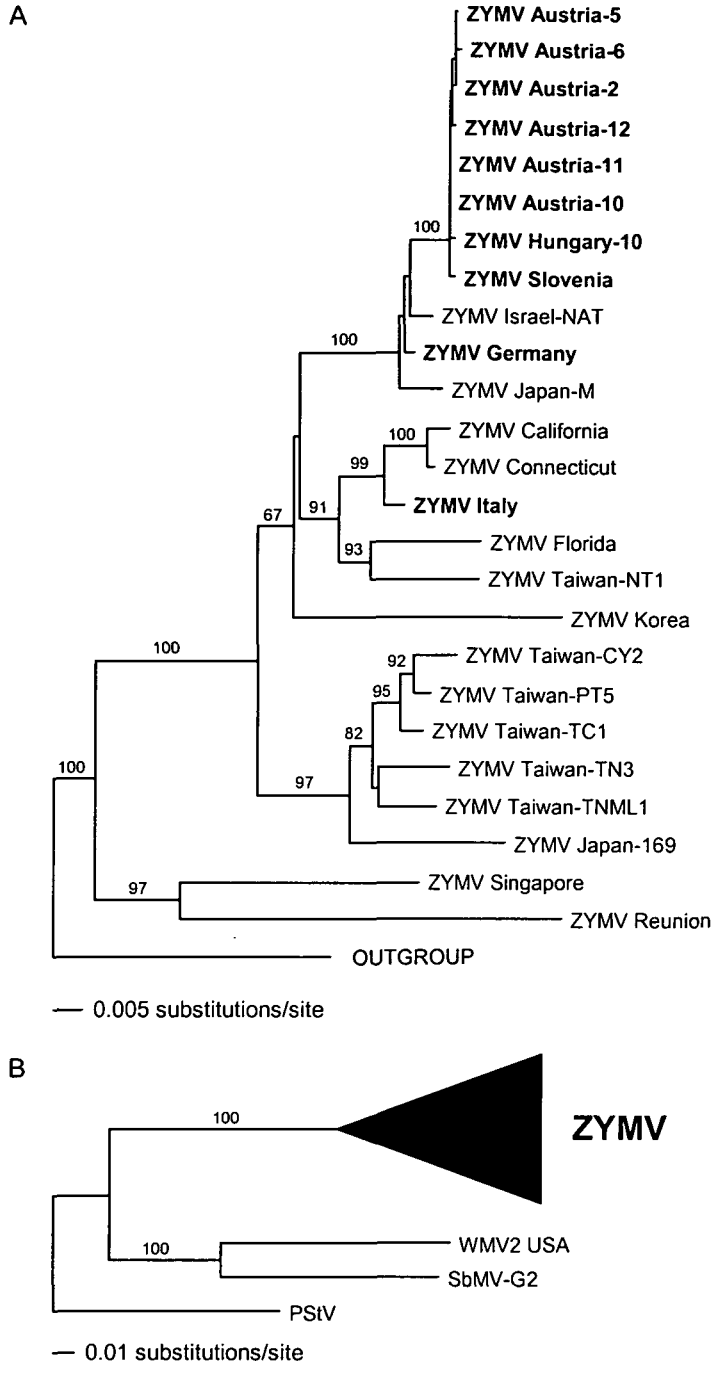

Fig. 5: Neighbor-joining tree based on the coat protein (cp) gene of ZYMV isolates from different geographical regions. The tree is rooted with cp gene sequences of peanut stripe virus (PStV), soybean mosaic virus (SbMV-G2), and one watermelon virus isolate from USA (WMV2 USA). Bootstrap support values > 50 % are shown above branches. European isolates are shown in bold. A, phylogram showing ingroup relationships. B, outgroup relationships, indicating a lower level of genetic diversity within ZYMV when compared with other potyviruses. The horizontal dimension of the triangle representing ZYMV shows genetic diversity in relation to that of the outgroup.

within the group consists of the Austrian, Slovenian and Hungarian isolates. Interestingly, the German isolate, which was together with the Austrian/Slovenian isolates in the cip analysis, is now excluded from this group. It forms a paraphyletic assemblage with isolates from Israel and Japan. The Italian strain is related (99%) to a group formed by isolates from California and Connecticut. Sister to this clade is a group of strains from Florida and Taiwan. A strain isolated in Korea occupies a rather isolated position between this group and an essentially East Asian group composed of five isolates from Taiwan and the Japan-169 strain. Similar to the cip analysis, the Singapore and Reunion strains are basal within ZYMV.

Table 3. Comparison of cDNA and deduced amino acid (AA) sequences of cytoplasmic inclusion (cip) and coat protein (cp) genes of 30 ZYMV isolates (Homology is expressed relative to the ZYMV isolate Austria-2).

	cip DNA % Homology	CIP AA % Homology	cp DNA % Homology	CP AA % Homology
ZYMV Austria-2	100.0	100.0	100.0	100.0
ZYMV Austria-5	100.0	100.0	100.0	100.0
ZYMV Austria-6	100.0	100.0	99.9	100.0
ZYMV Austria-10	100.0	100.0	99.9	100.0
ZYMV Austria-11	99.8	99.3	99.8	100.0
ZYMV Austria-12	100.0	100.0	99.7	100.0
ZYMV Slovenia	100.0	100.0	99.8	100.0
ZYMV Hungary-10	nd	nd	99.8	99.6
ZYMV Italy	97.5	100.0	95.6	98.6
ZYMV Germany	99.8	100.0	98.9	98.6
ZYMV Israel-NAT	nd	nd	98.8	97.8
ZYMV Israel-AT	nd	nd	96.0	88.1
ZYMV Chile	nd	nd	95.4	98.9
ZYMV California	97.7	99.3	94.5	98.6
ZYMV Connecticut	nd	nd	94.7	98.2
ZYMV Florida	nd	nd	93.8	95.7
ZYMV Japan-169	91.1	100.0	92.1	93.9
ZYMV Japan-M	96.8	100.0	98.3	98.2
ZYMV Korea	nd	nd	92.6	92.1
ZYMV Taiwan-TN3	nd	nd	93.2	96.4
ZYMV Taiwan-CY2	nd	nd	93.2	95.3
ZYMV Taiwan-TC1	nd	nd	94.1	96.8
ZYMV Taiwan-TNML1	nd	nd	93.5	96.4
ZYMV Taiwan-NT1	nd	nd	94.0	97.8
ZYMV Taiwan-PT5	nd	nd	93.7	95.7
ZYMV Australia-A3	nd	nd	97.2	90.9
ZYMV Australia-G4	nd	nd	91.7	81.8
ZYMV Australia-K	nd	nd	97.7	90.9
ZYMV Singapore	85.6	98.6	87.9	91.0
ZYMV Reunion	81.5	97.3	85.4	90.0

Comparison of genetic diversity between nucleic acid and deduced amino acid sequences among ZYMV isolates reveals the majority of mutations to be silent substitutions with no effect on amino acid sequence of either cip or cp coding regions (Tab. 3). Homology on the DNA level of cip genes ranges from 81.5 to 100.0% whereas the

corresponding values at the amino acid level range from 97.3 to 100.0%. On the amino acid level five of the Austrian isolates are 100% identical with the Slovenian, German, Italian, and the two Japanese isolates. Within the closely related Austrian isolates there is only a single case (ZYMV Austria-11) where a G->A transition at the second codon position results in an amino acid substitution from Ser to Asn. The same G->A transition is shared by the phylogenetically unrelated ZYMV isolate from Reunion Island indicating that this mutation probably does no effect virus characteristics.

The cp gene appears to be more variable than the cip gene with sequence homology ranging from 85.4% to 100.0% on the nucleic acid level versus 81.8% to 100.0% on the amino acid level. Homology in the highly supported monophyletic group consisting of the Austrian, Slovenian, and Hungarian isolates in the phylogenetic analysis ranges from 99.7 to 100.0%. On the amino acid level this group is 100% identical except for the Hungarian isolate which shows a single amino acid substitution in the coat protein sequence.

Discussion

We have shown, based on molecular analysis, that *Cucurbita pepo* plants from Austrian pumpkin-growing areas with symptoms of ZYMV infections do contain viral nucleic acids. This result confirms previous studies using serological tests to detect ZYMV infections (RIEDLE-BAUER 1998). Since the first massive outbreak of ZYMV infections in Austria occurred in 1997, our work focused on the question, how the virus isolates from Austria are related to isolates from other pumpkin-growing areas. We therefore characterized the Austrian ZYMV isolates and compared them with isolates from other geographical regions including the adjacent pumpkin- and zucchini-growing countries Italy, Slovenia and Germany. The Hungarian isolate has already been characterized (TOBIAS et al. 1998; TOBIAS et al. 1999). Therefore, molecular data for this strain could be retrieved from public databases for comparison.

BARBARA et al. (1995) showed that it is possible to discriminate among closely related strains of ZYMV by a simple PCR-RFLP procedure. A similar analysis of the coat protein gene in our study showed the distinctiveness of the Italian strain, but failed to discriminate among other isolates. Although no sequence information is available for the ZYMV isolates from UK and France which have been investigated by BARBARA et al. (1995) it is possible to compare the RFLP patterns of these two strains with the deduced RFLP patterns of sequenced strains. The distribution of HpaII restriction sites within the cp sequences reveals, that the UK and the French strain share the restriction pattern of the Italian and the Japan-169 strains, respectively, but are not closely related to the Austrian, Slovenian, and Hungarian isolates. Genomic sequencing is the method of choice when closely related strains have to be compared. We have chosen two parts of the ZYMV genome for this purpose: the cip gene has been shown to be useful in classification of potyviruses (LEE et al. 1997), and the cp gene is probably the best candidate for investigating closely related strains, because the coat protein is the only virus gene product which shares little sequence identity with the corresponding protein of other virus groups (BERGER et al. 1997; DOMIER et al. 1987). Tree topologies resulting from the

phylogenetic analyses of these two genes agree with each other in all major points. Smaller differences probably result from different numbers of sequences available for cip or cp genes and a probably higher mutation rate in the cp gene (as judged from genetic diversity measures). In both cases all ZYMV sequences form a monophyletic group and the outgroup relationships show a similar tree topology as in the study of LEE et al. (1996), who analyzed the phylogeny of 15 potyviruses. The cp gene sequences put all Austrian isolates into a highly supported group together with the Slovenian and the Hungarian isolates. Since this group consists only of isolates from geographically closely related areas, a single introduction of ZYMV into this region with subsequent spreading to all pumpkin-growing areas is the most probable explanation. The high bootstrap value for this group practically excludes the possibility that the Italian or German strain has invaded this region. PRIETO et al. (2001) have recently sequenced a fragment of 395 bp in length from the 3′ portion of a Chilean isolate and compared it with 16 different ZYMV isolates, partly overlapping with our sampling. Although some of the clustering in their analysis agrees with our groups, a few discrepancies do exist between the two studies: Our study clearly shows that the Singapore strain (together with the strain from Reunion Island) is basal within the ZYMV group and does not cluster within other clades. The Japan-169 strain appears not isolated as in the PRIETO et al. study, but is basal within a clade of Taiwanese isolates. Unlike the PRIETO et al. analysis, in our study the Korean isolate does not cluster with the strains from Hungary and Israel. These differences probably can be attributed to the more complete sampling in our analysis (25 versus 17 isolates), the use of rooted phylogenetic analysis in our study versus unrooted Neighbor Joining analysis in their study, and the difference in the part of the cp gene sequenced. PRIETO et al. sequenced 395 bp from the 3′ portion of the cp gene only whereas the entire coding region was sequenced in our study (1100 bp).

In viruses, small mutations can have dramatic effects on the virulence of strains. For example, aphid transmissibility is abolished by a single point mutation in the coat protein gene both in ZYMV (GAL-ON et al. 1992) as well as in tobacco vein mottling virus (ATREYA et al. 1990). Likewise, a single amino acid change in a conserved motif in the helper component-protease changes a severe field strain of ZYMV into a mild strain (GAL-ON 2000). This mutation causes a dramatic symptom change from severe to mild in squash and to a symptom-free appearance in cucumber, melon, and watermelon. Among the closely related Austrian, Slovenian, and Hungarian isolates, no differences in virulence of strains have been recorded. The single amino acid mutation in the cip gene product of the Austria-11 isolate is shared by the Reunion strain and most probably does not change the function of the protein. Actually, two conserved motifs in the N-terminal region of the protein (LEE et al. 1997), the nucleotide-binding motif 'GAVGSGKST', as well as the conserved motif 'EPTRPL', are perfectly conserved. Likewise, the single amino acid mutation of the Hungarian isolate occurred in a variable and therefore less functionally conserved region of the coat protein where other strains show mutations, too. However, preliminary results from artificial inoculation experiments with Austrian and Italian ZYMV strains do show differences in host susceptibility among breeding lines of *C. pepo* and cucumber genotypes indicating that even at a homology as high as 98.6% (amino acid sequence) functional differences cannot be excluded (data not shown). This again indicates that

knowledge of diversity and variability within a virus group is of vital interest when virus-resistant cultivars are to be developed.

Acknowledgements

We gratefully acknowledge the help of DI. Riedle-Bauer for sampling diseased plant material. We also thank DI. W. Leonhardt for the photographs of ZYMV symptoms on leaves of *C. pepo*. This work was supported by a grant from the Austrian Federal Ministry of Agriculture and Forestry (Project L-1089).

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