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بررسی ژن *p25* در سه جمعیت ایرانی ویروس رگبرگ زرد نکروتیک چغندرقند (*Beet necrotic yellow vein virus*) و نقش ارقام حساس و مقاوم در تنوع ژن *p25**

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چکیده

ویروس رگبرگ زرد نکروتیک چغندرقند (*Beet necrotic yellow vein virus*, BNYVV) در چغندرقند بیماری مهم و خطرناکی بنام ریشه ریشی (root beardiness) یا ریشه گنائی (rhizomania) ایجاد می کند و دارای گسترش زیادی در اکثر نقاط دنیا است. این ویروس دارای چند قطعه آر ان ای ژنومی می باشد و آران‌ای شماره ۳ آن یک پروتئین ۲۵ کیلودالتونی که مسئول بیماری‌زایی در گیاه چغندرقند است را رمز گذاری می‌کند. موقعیت‌های آمینواسیدی ۶۷ و ۶۸ این پروتئین نقش مهمی در شکستن مقاومت ارقام مقاوم دارند. در این مطالعه p25 در سه جمعیت BNYVV از مغان، لرستان و فارس در ارقام حساس و مقاوم چغندرقند مورد بررسی قرار گرفت. دو جمعیت لرستان و فارس در چهار گذرش پیاپی به ارقام مقاوم و حساس مایه‌زنی شدند و ژن p25 ویروس در آنها بررسی شد. بر اساس نتایج حاصله انتقال پیاپی ویروس در گیاهان مقاوم منجر به تغییراتی در موقعیت‌های آمینواسیدی ۳۰، ۴۹، ۶۷، ۶۸، ۱۲۹، ۱۶۳ و ۱۹۸ در p25 شد، در حالی که در گیاهان حساس تغییرات آمینواسیدی دیده نشد. بیشتر تغییرات تتراد (آمینواسیدهای ۷۰-۶۷) در موقعیت آمینواسیدی ۶۷ مشاهده گردید. وجود بیوتیپ P در مغان و لرستان تشخیص داده شد. از آنجا که انتخاب تحت فشار ارقام مقاوم می‌تواند نقش مهمی در ظهور بیوتیپ P ایفا کند، بنابراین حضور بیوتیپ P در دو منطقه در ایران ممکن است به علت کشت ارقام مقاوم بوده باشد. برخی پارامترهای تکاملی و موتیف‌های p25 مورد بحث قرار گرفته است.

کلیدواژه: ریشه گنائی، چغندر قند، شکستن مقاومت

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Analysis of *p25* in Three Iranian Populations of *Beet necrotic yellow vein virus* and the Role of Susceptible and Resistant Cultivars of Sugar Beet in the *p25* Variation*

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Abstract

Beet necrotic yellow vein virus (BNYVV, *Benyvirus*) is an economically important soil borne pathogen of sugar beet throughout the world. It is a multipartite virus in which RNA-3 encoded p25 protein controls disease symptoms in sugar beet. Under certain conditions, resistance-breaking variants are generated by amino acid changes at positions 67 and 68 in p25. In the present study p25 protein in three populations of the virus from Moghan, Lorestan and Fars were analyzed in susceptible and resistant sugar beet cultivars. Lorestan and Fars populations were serially passaged through resistant or susceptible sugar beet cultivars for 4 generations and p25 was monitored for possible amino acid changes. No significant amino acid changes were observed when the populations were passaged through susceptible cultivars. However, passage of the populations through resistant cultivars resulted in changes of amino acids at positions 30, 49, 67, 68, 129, 163 and 198. Changes were more frequent in position 67 in the tetrad amino acids. Biotype P of BNYVV which was found in Moghan and Lorestan may have appeared as a result of selection under pressure of resistant cultivars. Information on certain evolutionary parameters and motifs of p25 is included.

Keywords: Biotype selection, Genetic variation, Resistance breaking, Rhizomania, Sugar beet

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Introduction

Rhizomania caused by *Beet necrotic yellow vein virus* (BNYVV, *Benyvirus*) is a destructive disease of sugar beet throughout the world (Asher 1993, Izadpanah *et al.* 1996, McGrann *et al.* 2009, Tamada 1999). The causal virus is vectored by the soil-borne plasmodiophorid *Polymyxa betae* (Richards and Tamada 1992). BNYVV is a multipartite virus with a genome consisting of four or five molecules of single-stranded, positive sense RNA. RNA-1 and RNA-2 encode proteins which are essential for replication, encapsidation, and movement, whereas proteins encoded by RNA-3, RNA-4 and isolate specific RNA-5 are involved in pathogenesis, transmission, and suppression of gene silencing. The RNA-3 encoded 25-kDa protein (p25) controls disease symptoms and virulence in sugar beet (Tamada *et al.* 1999). This interaction is controlled by some amino acids (Chiba *et al.* 2008). Resistance-breaking variants of the virus show amino acid changes at positions 67 and 68 of P25 (Chiba *et al.* 2008, Acosta-Leal *et al.* 2008, Liu and Lewellen 2007).

BNYVV isolates were originally classified into two groups, A and B, based on certain properties of the coat protein (CP) gene (Kruse *et al.* 1994, Saito *et al.* 1996, Schirmer *et al.* 2005). A-type BNYVV is distributed worldwide (Schirmer *et al.* 2005) whereas the B-type occurs in limited areas of Europe (Koenig *et al.* 2008), Japan (Miyanishi *et al.* 1999) and China (Li *et al.* 2008). A further group called P-type, because of its original isolation in the Pithiviers area of France, contained an RNA-5 molecule and is closely related to the A-type but with amino acids of resistance breaking isolates (Miyanishi *et al.* 1999, Koenig *et al.* 1997). RNA-5 containing isolates occur in limited areas of Europe (Schirmer *et al.* 2005, Koenig *et al.* 2008, Ward *et al.* 2007) but are widely distributed in Asia (Koenig 2000, Li *et al.* 2008, Miyanishi *et al.* 1999). Isolates with the P-type *p25* but without RNA-5 have been reported from the United States (Liu and Lewellen 2007) and Iran (Mehrvar *et al.* 2009).

In recent years, sugar beet production has become dependent on rhizomania resistant sugar beet cultivars (Asher 1993, Lewellen *et al.* 1987, Rush 2003). The use of such cultivars, however, has become jeopardized by the emergence of resistance breaking isolates of the virus (Acosta-Leal *et al.* 2010, Liu *et al.* 2005, Liu and Lewellen 2007). Such BNYVV isolates often showed changes in amino acid content of p25.

The effect of amino acid changes of p25 on BNYVV content of plants and breaking of resistance has been studied by reverse genetics and sequence analysis of isolates from resistant cultivars (Koenig *et al.* 2009). There is, however, no direct evidence for changes in virus populations by resistant cultivars. The objective of the present study was to experimentally verify the effect of continuous cultivation of resistant cultivars on the sequence of *p25*.

Materials and Methods

Source of virus isolates

Soils were collected from two widely separated fields in Lorestan and Fars provinces of Iran in 2011 and transferred to the greenhouse. A soil sample from Moghan (North West of Iran) was also used for analysis of *p25*. Seeds of rhizomania susceptible (IC and PP8) and resistant (Brigitta and Dorothea) cultivars of sugar beet were obtained from Sugar Beet Research Department, Fars Agricultural Research Center, and planted in the collected soils. Samples of resulting seedlings were tested by ELISA (Clark and Adams 1977) against a locally produced antiserum to verify their infection by BNYVV. The roots of infected seedlings were used as the source of virus populations. The inocula for mechanical transmission of the virus were prepared by grinding root tissues in 5 Vol. of 0.1 M phosphate buffer at pH 7.0.

Serial transfer

Each virus isolate in each of the four beet cultivars was transferred to seedlings of the same cultivar by mechanical inoculation of leaves. Plant to plant passages were made at 40-day intervals. Root samples of source plants were tested by ELISA to verity their infection by BNYVV prior to each transfer. In each transfer, 10 seedlings were inoculated but only two randomly selected seedlings were used for analysis.

cDNA synthesis and PCR

Total RNA was extracted from root tissues using an mRNA capture kit (Roche, Germany) and

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Table 1. Primers used for amplification of BNYVV *p25*

Primer	Sequence 5' to 3'	Expected Product Size (nucleotides)	Reference
NYP25-F1	TTC CTG ACC GAC CAA ATC CA	1250	Acosta –Leal *et al.* 2010
NYP25-R1	GTA AAC GGA CGG GAA CAC CA		
Ab-F	AGT GAC CCC ATC GTT TCA GG	1100	This study
Ab-R	TCT TCT CAC CGA ACA CCG TG		

subjected to RT-PCR using *p25* specific primers listed in Table 1. Primer pair Ab-F/Ab-R was designed using Primer3Web software (WWW.frodo.wi.mit.edu).

PCR was carried out in 25 µL reaction volumes containing 2 µL of cDNA, 1 µL of each 10 µM primer, 0.5 µL of 10 µM dNTPs, 2.5 µL of 10×PCR buffer; 0.75 µL of 50 µM $MgCl_2$, and 0.25 µL (1 U) of Taq DNA polymerase (Cinnagen, Iran). For nested PCR, 0.2 µL of NYP25-F1/ NYP25-R1 PCR product was added to the PCR mix and PCR was carried out using primer pair Ab-F/Ab-R. PCR condition consisted of an initial denaturing at 94°C for 5 min and 35 cycles of 94°C for 1 min, 56°C (for NYP25-F1/ NYP25-R1) or 55°C (for Ab-F/Ab-R) for 30 sec, and 72°C for 1 min. Reactions were completed by a final extension at 72°C for 7 min**.**

PCR and nested PCR products were electrophoresed in a 1.5% agarose gel and visualized under UV light after staining with ethidium bromide.

Viral RNA sequence analyses

PCR products were purified using a gel extraction kit (Qiagen, Germany) and directly sequenced (Bioneer, South Korea). Basic processing of cDNA sequences such as assembling, correction, and alignment was performed with Vector NTI 11 and the chromatograms were inspected by MEGA5 to verify the presence of mutations. Sequence alignments were saved in Fasta format to be exported into different applications used in this study. Phylogenetic trees were reconstructed by the neighbor-joining (NJ) algorithm as implemented in MEGA5. This software was also used to calculate genetic distances between individual sequences and groups of sequences. RDP (Recombination Detection Program) was used to detect possible recombination signals. Motifs were detected by My hit (http://myhits.isb-sib.ch/cgi-bin/motif_scan) software.

Results

PCR Results

Regular PCR using primer pair NYP25-F1/Nyp25-R1 was efficient to detect *P25* in inoculated sugar beet plants in passages 1 and 2. However, it was necessary to use nested PCR with primer pair Ab-F/ Ab-R in order to detect this gene in passage series 3 and 4.

Amino acid analysis

Amino acid sequences of p25 in various stages were depicted from the sequences of PCR products. Table 2 shows change in putative amino acid residues of the isolates in a number of positions. No significant differences were observed in the primary amino acid content of Lorestan (L) and Fars (F) isolates in susceptible and resistant cultivars except for a V instead of I at position 35 in L isolate in resistant Dorothea (D-L-1). Similar results were obtained with series 2 inoculations. Changes were observed in several positions in Lorestan isolate in the series 3 and 4 in Brigitta and in series 4 in Dorothea. In the Fars isolate, amino acid changes were observed at positions 27 and 68 in series 4 in Brigitta and at position 218 in series 2 and 3 in Dorothea. No such changes were found in susceptible cultivars IC and PP8.

Phylogeny of p25 gene of BNYVV series

Phylogenetic analysis of *p25* of world isolates of BNYVV indicates that there are three population clusters corresponding to types A, B and P of the virus. The F and L populations of the present study are clustered with either A or P depending on the host cultivar and inoculation

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Table 2. Amino acid variation in the p25 protein of BNYVV isolates following serial transfers in susceptible and resistant sugar beet cultivars.

	27	28	30	35	36	49	67**	68**	69**	70**	129	132	163	190	191	198	207	213	215	218
B-L-1*	L	T	S	I	C	T	A	Y	H	G	H	L	P	V	W	T	N	D	V	D
B-L-2	L	T	S	I	C	T	A	Y	H	G	H	L	P	V	W	T	N	D	V	D
B-L-3	L	T	***C***	I	C	***S***	***S***	Y	H	G	***N***	L	***S***	V	W	***A***	N	D	V	D
B-L-4	L	T	***C***	I	C	***S***	***S***	Y	H	G	***N***	L	***S***	***E***	***R***	***A***	***Y***	***S***	V	D
D-L-1	L	T	S	V	C	T	A	Y	H	G	H	L	P	V	W	T	N	D	V	D
D-L-2	L	T	S	V	C	T	A	Y	H	G	H	L	P	V	W	T	N	D	V	D
D-L-3	L	T	S	V	C	T	A	Y	H	G	H	L	P	V	W	T	N	D	V	D
D-L-4	L	T	***C***	***I***	C	***S***	***S***	Y	H	G	***N***	***I***	***S***	V	W	***A***	N	D	V	D
I-L-1	L	T	S	I	C	T	A	Y	H	G	H	L	P	V	W	T	N	D	V	D
I-L-2	L	T	S	I	C	T	A	Y	H	G	H	L	P	V	W	T	N	D	V	D
I-L-3	L	T	S	I	C	T	A	Y	H	G	H	L	P	V	W	T	N	D	V	D
I-L-4	L	T	S	I	C	T	A	Y	H	G	H	L	P	V	W	T	N	D	V	D
P-L-1	L	T	S	I	C	T	A	Y	H	G	H	L	P	V	W	T	N	D	V	D
P-L-2	L	T	S	I	C	T	A	Y	H	G	H	L	P	V	W	T	N	D	V	D
P-L-3	L	T	S	I	C	T	A	Y	H	G	H	L	P	V	W	T	N	D	V	D
P-L-4	L	T	S	I	C	T	A	Y	H	G	H	L	P	V	W	T	N	D	V	D
B-F-1	L	T	S	I	C	T	A	Y	H	G	H	L	P	V	W	T	N	D	V	D
B-F-2	L	T	S	I	C	T	A	Y	H	G	H	L	P	V	W	T	N	D	V	***G***
B-F-3	L	T	S	I	C	T	A	Y	H	G	H	L	P	V	W	T	N	D	V	***G***
B-F-4	***S***	T	S	I	C	T	A	***L***	H	G	H	L	P	V	W	T	N	D	V	D
D-F-1	L	T	S	I	C	T	A	Y	H	G	H	L	P	V	W	T	N	D	V	D
D-F-2	L	T	S	I	C	T	A	Y	H	G	H	L	P	V	W	T	N	D	V	***G***
D-F-3	L	T	S	I	C	T	A	Y	H	G	H	L	P	V	W	T	N	D	V	***G***
D-F-4	L	T	S	I	C	T	A	Y	H	G	H	L	P	V	W	T	N	D	V	D
I-F-1	L	T	S	I	C	T	A	Y	H	G	H	L	P	V	W	T	N	D	V	D
I-F-2	L	T	S	I	C	T	A	Y	H	G	H	L	P	V	W	T	N	D	V	D
I-F-3	L	T	S	I	C	T	A	Y	H	G	H	L	P	V	W	T	N	D	V	D
I-F-4	L	T	S	I	C	T	A	Y	H	G	H	L	P	V	W	T	N	D	V	D
P-F-1	L	T	S	I	C	T	A	Y	H	G	H	L	P	V	W	T	N	D	V	D
P-F-2	L	T	S	I	C	T	A	Y	H	G	H	L	P	V	W	T	N	D	V	G
P-F-3	L	T	S	I	C	T	A	Y	H	G	H	L	P	V	W	T	N	D	V	G
P-F-4	L	T	S	I	C	T	A	Y	H	G	H	L	P	V	W	T	N	D	V	G
B-M	L	T	S	***V***	C	T	A	Y	H	G	H	L	P	V	W	T	N	D	V	D
P-M	L	***I***	S	I	C	T	A	***C***	H	G	H	L	P	V	W	T	N	D	V	D
D-M	L	T	S	I	C	***S***	A	Y	H	G	***I***	L	P	V	W	T	N	D	V	D
I-M	***S***	T	S	I	***R***	***S***	***S***	Y	H	G	***I***	***I***	***S***	V	W	***V***	N	D	***I***	D

*Cultivar-isolate-series: B, cv.Brigitta; D, cv. Dorothea; I, cv. IC; P, cv. PP8; L, Lorestan isolate of BNYVV; F, Fars isolate of BNYVV; M, Moghan isolate of BNYVV. Designations 1 to 4 in the left column indicate passage number. The changed amino acids are italicized and underlined.

** Tetrad amino acids of p25.

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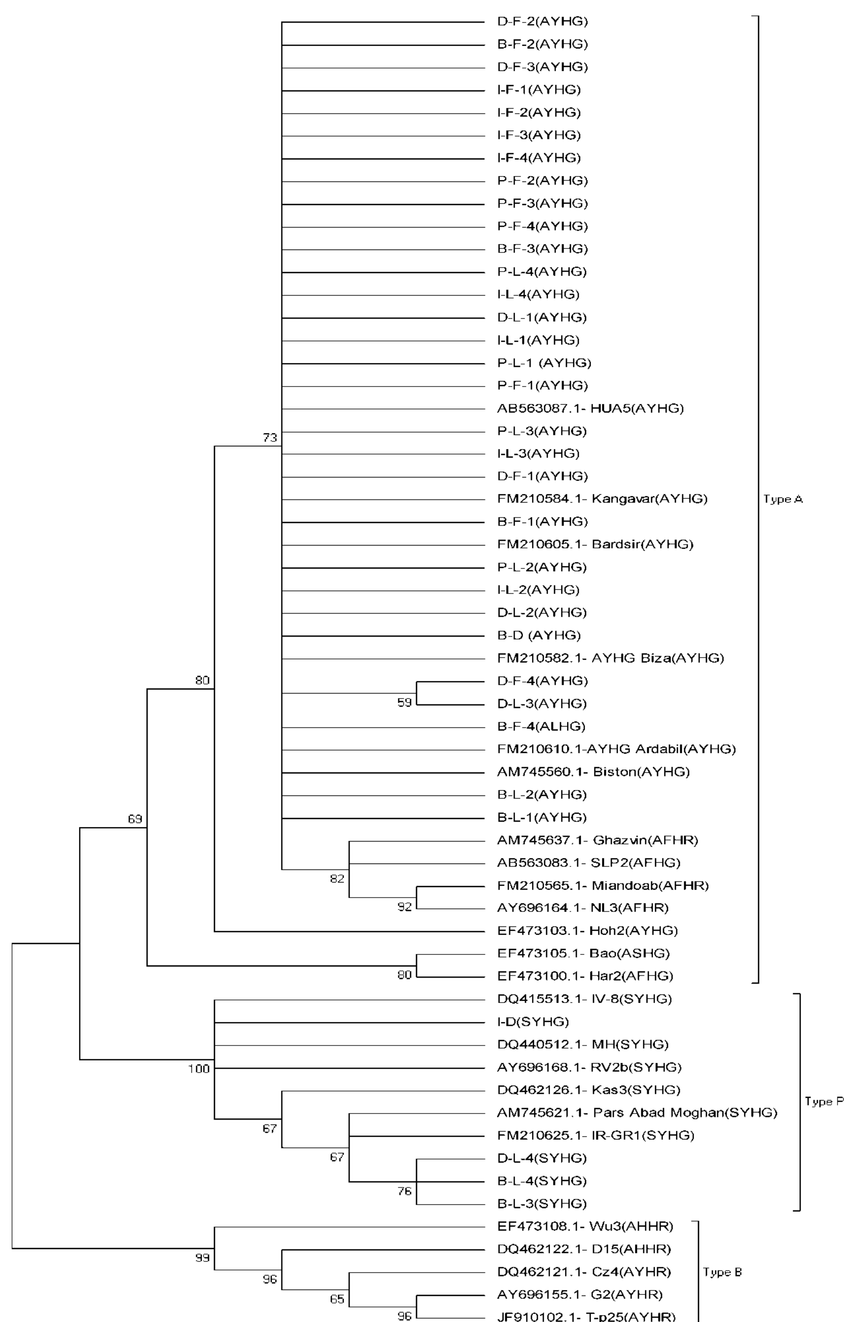

Fig. 1. Phylogenetic clustering of BNYVV isolates and cultivar-passage-specific sequences based on *p25* coding region using neighbor joining method and Kimura 2-parameter model with 100 bootstraps by MEGA 5 software. The tetrad motifs of p25 are indicated in parentheses. See Table 2 for cultivar, isolate and series designations.

series (Fig 1). This is mainly due to the high variability within tetrad amino acids (positions 67-70) which are biotype specific.

Molecular evolution analysis of p25

The ratio of non-synonymous (dN) to synonymous (dS) mutations for P25 was found to be $\omega = 0.63$. This shows that P25 is generally under negative selection pressure. However, the dN for amino acids at positions 68 and 198 was 1 or higher indicating a strong positive selection in particular for the position 68. The overall genetic diversity for P25 was as low as $\pi = 0.008$.

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Table. 3. Amino acid motifs and functions of BNYVV p25 in serial passages

Amino acid position	Motif (Amino acids)	Function	Experimental series	Biotype
46-49	NRTT	Glycosylation	All	A,P
54-57	NNTK	Glycosylation	All	A,P
95-98	NGSR	Glycosylation	All	A,P
156-159	NATN	Glycosylation	All	A,P
21-24	TVYE	CK2*-Phosphorylation	All	A,P
105-108	TRLD	CK2-Phosphorylation	All	A,P
110-113	CVNE	CK2-Phosphorylation	All	A,P
164-167	TTTD	CK2-Phosphorylation	All	A,P
213-216	SGVD	CK2-Phosphorylation	BL3, BL4, DL4	P
63-68	GLLCAY	Myristoylation	All	A,P
195-200	GLYTGD	Myristoylation	All	A,P
56-58	TKR	PKC-Phosphorylation	All	A,P
189-191	TER	PKC-Phosphorylation	BL3, BL4, DL4	P

*CK= casein kinase, PKC= protein kinase C

No recombination events were detected in the *p25* coding region of isolates in this study by RDP software.

Amino acid motifs of p25 were detected by “My hit” software. Four motifs for glycosylation (aa. 46-49, 54-57, 95-98, and 156-159), two motifs for myristoylation (aa. 63-68 and 195-200), four motifs for phosphorylation of casein kinase II (aa. 21-24, 105-108, 110-113 and 164-167) and one motif for phosphorylation of protein kinase C (aa. 56-58) were detected in series corresponding to biotypes A and P. In addition a motif for phosphorylation of casein kinase II (aa. 189-191) and another for phosphorylation of protein kinase C which are specific for biotype P were found in series B-L-3, B-L-4 and D-L-4 (Table 3).

Discussion

Genetic variation of the gene p25

The *p25* is a variable gene compared to other genes in BNYVV, with evidence of strong positive selection in tetrad amino acids (residues 67-70), suggested to be associated with resistance breaking (Chiba *et al.* 2011, Acosta-Leal and Rush 2007). The amino acid 68 in p25 was demonstrated to control certain virus-host-specific resistance interactions (Chiba *et al.* 2008). Furthermore, Acosta-Leal *et al.* (2008) reported positive selection of amino acids 67 and 68 in resistance breaking isolates, although they were not the only p25 amino acids linked to resistance breaking (Acosta-Leal and Rush 2007, Chiba *et al.* 2008, Liu and Lewellen 2007).

Previous studies have shown that p25 amino acids 67-70 can separate the three biotypes of BNYVV (Chiba *et al.* 2011; Mehrvar *et al.* 2009). Therefore, in passages 3 and 4 in the Lorestan isolate on Brigitta cultivar and in passage 4 in Dorothea cultivar, biotype A was changed to biotype P. Alteration of amino acid Y to L at position 68 was found only in passage 4 of Fars population in Brigitta cultivar.

The populations of this study also exhibited overall low genetic diversity averaging 0.008 ± 0.0017. The positions 68 had the highest evolutionary rate as shown by strongly positive selection.

Variation in Lorestan population was higher than in Fars population. Susceptible cultivars showed identical virus content in regard to P25 amino acid sequences. Experiments under greenhouse conditions confirmed existence of different biotypes (A and P) in the Moghan virus population (Table 2). No recombination signal was detected in the sequence.

Genomic RNA typically has a high mutation rate, and individual virus populations consist of a mixture of closely related mutants, with the consensus sequence able to change in response to selection pressure (Garcia-Arenal *et al.* 2001,

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Harrison 2002). Therefore, it is likely that biotypes A and P have been present in the samples collected and the P biotype has been selected by resistant response. Detection of P biotype in a sample from Moghan confirms this conclusion. However, it would be of interest to study the role of resistant cultivars in *de novo* changes in amino acid content of p25.

A number of phosphorylation, glycosylation, and myristoylation were identified in the P25. The role of these motifs in the pathogenicity of BNYVV must be studied further. However, Phosphorylation-dependent regulatory mechanisms are reported to be essential for viral RdRp function and virus replication (Jasnic *et al*., 2006). In geminiviruses, pathogenicity is dependent on a conserved putative protein kinase C (PKC) phosphorylation motif (Chowda-Reddy *et al*. 2008). Two such motifs were identified in the P biotype. Some geminiviruses encode a small protein, AC4, with a role in pathogenesis and suppression of RNA silencing. A putative N terminal myristoylation motif has been predicted in AC4 by several algorithms (Fondong *et al*. 2007). We detected two motifs (63-68 and 195-200) for N-myristoylation in important amino acid position in the resistance breaking type of BNYVV P25.

The results of the present study show that BNYVV can undergo rapid evolution in response to changes in plant host. The outcome of changes depend also on the genetic structure of the initial population. Variation of *p25* in the incompatible cultivars was greater than in the compatible hosts. A virus population includes variants, usually with a dominant (master) sequence, which is more compatible with the host. Use of resistant cultivars results in the change of the master variant, which, in this case, is more virulent. A good understanding of population genetic structure can support design of an effective disease management program.

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