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RESEARCH ARTICLE

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Detection and molecular characterization of *Polymyxa betae*, transmitting agent of sugar beet rhizomania disease, in Iran

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Abstract

The plasmodiophorid *Polymyxa betae* is considered as the only natural transmitting agent of *Beet necrotic yellow vein virus* (BNYVV), the most devastating agent of sugar beet fields throughout the world. To evaluate for the first time the genetic diversity of *P. betae* isolated from different autumn and spring sugar beet fields, and also to detect the presence of virus in these isolates, susceptible sugar beet plants (cv. Regina) were grown in soil samples collected from 10 different regions of Iran. *P. betae* detection was carried out using root microscopic observations, DAS-ELISA, and PCR-based methods. Results showed the presence of plasmodiophorids in all soil samples. Complementary assays also revealed the presence of viruses in soil samples collected from Khorasan Razavi, Fars, Hamadan and Kermanshah regions. The genetic diversity was evaluated through comparing glutathione-S-transferase nucleotide sequences amplified by PCR with the Internal Transcribed Spacers region. Results showed no significant differences in nucleotide sequences between virus-bearing and virus-free isolates of *P. betae*.

Additional key words: *Beta vulgaris*; cystosori; ITS; GST; BNYVV; virus-bearing; virus-free.

Introduction

Rhizomania, which causes both abnormal rootlet proliferation and sugar yield loss, is the most destructive disease in sugar beet (*Beta vulgaris* L.) growing areas in Europe, Asia, and America (McGrann *et al*., 2009). The causal agent of the disease is *Beet necrotic yellow vein virus* (BNYVV) and the natural vector of virus is *Polymyxa betae* Keskin (Fujisawa & Sugimoto, 1977). *Polymyxa* belongs to the order Plasmodiophora containing many genera, among which *Plasmodiophora brassicae* (the causal agent of brassica club root), *Spongospora subterranea* (the causal agent of powdery scab of potato), and *P. betae* are of agro-economic importance as pathogens or virus vectors (Kanyuka *et al.,* 2003; Rush, 2003). The vector can survive in the soil for years and transmit other different viruses including *Beet soil-borne mosaic virus* (Wisler, 2001), *Beet soil-borne virus*, and *Beet virus Q* (Prillwitz & Schlösser, 1992).

With no acceptable pesticides available to restrict the vector, the control of rhizomania is now achieved almost exclusively through using resistant cultivars. A single dominant resistance gene, *Rz1*, has been used to manage the disease worldwide in recent years, although this gene confers only partial resistance (McGrann *et al*., 2009). New variants of BNYVV have evolved which are able to cause significant yield penalties on resistant cultivars (McGrann *et al.,* 2009). A breeding strategy for durable resistance to BNYVV is to combine virus resistance genes (*e.g., Rz1, Rz2)* with resistance genes to *P. betae* vector (Barr *et al.,* 1995; Asher *et al.,* 2009; Pavli *et al.,* 2011). Combined virus and vector resistance, achieved either by

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Abbreviations used: BNYVV (Beet necrotic yellow vein virus); ELISA (enzyme-linked immunosorbent assay); GSH (thiol group of the glutathione); GST (glutathione-S-transferase); ITS (internal transcribed spacers); PCR (polymerase chain reaction).

conventional or transgenic breeding, offers a new approach in its continuing struggle against rhizomania to the sugar beet industry (McGrann *et al.,* 2009). Germplasm screening for resistance to *P. betae* infection demands a quick diagnostic test to select those plants that show low symptoms of carrying the parasite when grown in infested soil (Mutasa *et al.,* 1993).

Polymyxa species are obligate parasites of plant roots and their detection involves skilled and tedious microscopic examination (Mesbah *et al.,* 1997). In recent years, molecular techniques have been developed to study and detect *Polymyxa* species (Mutasa *et al.,* 1993, 1995, 1996; Ward *et al.,* 1994, 2004). Such procedures also have the potential to distinguish isolates of the parasite from different host plant or virus transmission specificities. Through comparing internal transcribed spacers (ITS) sequences of *Olpidium brassicae*, *S. subterranea*; *P. brassicae, Ligniera* spp. and *Polymyxa graminis*, Legrève *et al.* (2002) clearly distinguished *P. graminis* from the rest. The specific rDNA sequence type of *P. graminis* varies depending on the host range, temperature requirements, and geographical origin. Therefore, Legrève *et al.* (2002) suggested the classification of these sequences into different subgroups (*formae speciales)*.

Glutathione-S-transferase (GST) is an enzyme which has been found in all living species, including plants, animals, fungi, and bacteria. It belongs to a super family of multi-functional enzymes that detoxify endobiotic and xenobiotic compounds by conjugating glutathione (GSH) to a hydrophobic substrate (Öztetik, 2008). Previous study showed that GST was expressed at high levels in *P. betae*, and could overcome host plant defense mechanisms (Mutasa-Gottgens *et al.,* 2000). Kingsnorth *et al.* (2003) found that *P. betae* GST is an immunogenic protein. Safarpour *et al.* (2012) cloned the coding sequences of the GST gene from an Iranian *P. betae* isolate and developed an efficient procedure for producing a polyclonal antibody against this Iranian isolate.

The objectives of this study were: (i) to detect *P. betae* in spring and autumn sowing sugar beet fields based on quantitative ELISA, specific PCR assay, and microscopic observation; (ii) to investigate the presence of BNYVV in *Polymyxa*-infected isolates using the ELISA method; iii) to determine variability of Iranian *P. betae* isolates (virus-bearing and virus-free) based on rDNA and GST sequences.

Material and methods

Detection of *Polymyxa betae* in infested soil samples

In order to detect *P. betae*, susceptible sugar beet plants, cv. Regina (as a bait plant) were grown in soil samples collected from 10 different regions of Iran including Markazi, West Azarbaijan, Khoozestan, Golestan, Hamadan, Alborz, Kerman, Kermanshah, khorasan Razavi, and Fars (Fig. 1). Soil samples were taken from five different parts of the field in each region and were mixed thoroughly. To provide favorable growth conditions for *P. betae*, the soil samples (250 cm^3 in a pot) were dried and sieved to 3 mm, then were heated to 40°C for 15 h to encourage *P. betae* germination (Beemster & De Heij, 1987). Five weeks after cultivation, microscopic examination was conducted to confirm the infection. Rootlet samples were taken out from each plant and were washed to remove soil debris, then stained with lactophenol containing 0.1% cotton blue or acid fuchsine. Infection of *P. betae* was checked by observing the amount of resting spore clusters in pieces of the rootlets (about 1 cm long) of each plant under a light microscope.

Detection of *P. betae* and BNYVV by ELISA

To detect *P. betae* and BNYVV, DAS-ELISA test was carried out as described by Kingsnorth *et al.* (2003) and Asher *et al.* (2002) respectively, with slight modifications. Lateral root samples collected from Regina plants were used for *P. betae* detection and BNYVV quantification. The plant samples were prepared by crushing 0.2 g fresh roots in liquid nitrogen followed by 10-fold dilution (w/v) in extraction buffer (PBS buffer containing 2% PVP-25 and 0.2% skimmed dried milk). Specific detection of *P. betae* was achieved with polyclonal antibody against recombinant expressed protozoan GST (Safarpour *et al.,* 2012). Also the BNYVV was specifically detected using anti-BNYVV polyclonal antibodies (kindly provided by Mr. Saeed Darabi, Research Center for Agriculture and Natural Resources, Fars Province, Iran). DAS-ELISA tests were conducted using negative (recombinant immunodominant membrane protein; 100 μg mL^{-1}, kindly provided by Dr. Fatemeh Shahryari, Agricultural Biotechnology Research Institute of Iran) and positive (GST recombinant protein; 100 μg mL^{-1}) controls for *P. betae*. Soil samples

Number	I	II	III	IV	V	VI	VII	VIII	IX	X
Province	Alborz	West Azerbaijan	Kermanshah	Hammaadan	Markazi	Golestan	Khoarasan Razavi	Khoozestan	Fars	Kerman
Cultivation (ha)	2496	25791	11682	5511	3508	0*	59604	7920	21266	3482

Figure 1. Geographical distribution of sugar beet fields where the *Polymyxa betae* samples used in this study were collected. * Only a research field.

Table 1. Quantification of *P. betae* using ELISA technique

	GST recombinant protein (μg mL^{-1})								**Negative control (IMP*)**
	0.5	**1**	**2**	**3**	**4**	**6**	**8**	**10**	**10**
ELISA ($A_{405\ nm}$)	0.11	0.15	0.23	0.78	1.25	1.74	2.10	2.45	0.04

* IMP: immunodominant membrane protein. **Average of three replications.

collected from Shiraz, Iran, contained the A type of BNYVV (Amiri *et al.*, 2003). Sugar beet Regina's cultivar was used as both negative and positive controls in all tests. To produce negative control plants, seedlings of the cultivar Regina were transplanted into the sand and for positive controls, the seedlings were transplanted into a uniform mixture of infested soil and sand (3:7 v/v). All samples were tested in triplicate. To quantify the expressed GST from *P. betae*, a standard curve with 3500 μg mL^{-1} of purified recombinant GST (10 to 0.5 μg mL^{-1} Tris-HCl buffer) was plotted in triplicate and was analyzed in parallel (Table 1). Total protein concentration of the GST standard was determined using the Bradford (1976) assay.

DNA extraction

DNA was extracted from the dried lateral root plants, grown in each soil of the different origins, according to Liu *et al.* (2000) with slight modifications. Lateral roots (1 g) were frozen in liquid nitrogen and were dis-

rupted to a fine powder in lysis buffer [400 mM Tris-HCl (pH 8.5), 60 mM EDTA (pH 8.5), 150 mM NaCl, 1% SDS] using mortar and pestle. All centrifugation steps were conducted at 11000 × g for 5 min at 4°C.

Cloning and sequencing of the *P. betae* GST gene

PCR (polymerase chain reaction) amplification of GST gene was performed using forward (5'-CAACGT CGACAAGGGACCAAGGTCAATGC-3') and reverse (5'-CAACGCGGCCGCTTATTTTGGACCGGCTGC-3') primers developed by Safarpour *et al.* (2012). PCR was performed in a volume of 50 µL containing 0.2 µM primers, 1U Taq DNA polymerase (Promega, Madison, USA), 0.2 mM dNTPs (Promega, Madison, USA), 10X PCR buffer (Promega, Madison, USA) and 23 mM $MgCl_2$. The cycle parameters in the PCR program were as follows: 95°C for 5 min; 35 cycles of 95°C for 1 min, 55°C for 1 min, 72°C for 90 s; a final extension at 72°C for 5 min. PCR products were analyzed on 1% agarose gel and DNA was then eluted from the gel using the Agarose Gel DNA Extraction Kit (Roche, Ind, USA). PCR products were subjected to ligation with a TA cloning vector pTZ57R/T, 2886 bp (Fermentas, Lithuania). The ligated product was transformed into *E. coli* DH5α and plated onto LB agar Amp-X gal-IPTG plates. Plates were left at 37°C for 12-14 h. On the next day, blue and white colonies were observed and positive white colonies were further grown for plasmid isolation. Intact clones containing the right sequence were selected after digestion, PCR amplification, and subsequent sequencing analysis using M13 sequencing primers.

PCR amplification, cloning and sequencing of *P. betae* rDNA

A fragment of rDNA between ITS1, 5.8s gene and ITS2 regions from *P. betae* was amplified using specific primers for *polymyxa* detection [Psp1 (5´-TAGACGCAGGTCATCAACCT-3´) and Psp2rev (5´-AGGGCTCTCGAAAGCGCAA-3´)] as described by Legrève *et al.* (2003). PCR was performed in a volume of 20 µL containing 1 µL DNA template (30 ng), 1 µL of each forward and reverse primers (30 ng), 11.2 µL ddH_2O, 2 µL 10X PCR buffer, 1.6 µL 10 mM dNTP, 2 µL 25 mM $MgCl_2$ and 0.2 µL Taq DNA polymerase (Promega). Cycling conditions were as follows: 95°C for 2 min; 36 cycles of 96°C for 45 s, 50°C for 45 s and 72°C for 1 min; and a final synthesis at 72°C for 10 min. The PCR products were run on 1% agarose gel followed by recovery using DNA Extraction Kit (Vivantis, Malaysia). PCR products were then subjected to ligation with a TA cloning vector pTG19/T (Vivantis, Malaysia). The ligated products were transformed into *E. coli* DH5α and intact clones containing the right sequence were selected by colony PCR using M13 universal primers. Finally, subsequent sequencing analysis was conducted as mentioned above.

P. betae phylogenetic tree based on rDNA region

Sequence polymorphism of *P. betae* isolates ribosomal DNA, originated from each soil sample, was analyzed by CLUSTALW and was adjusted manually. The most appropriate model was determined using Bayesian Information Criterion (BIC) implement in MEGA version 5.0 program. The alignments selected by the lowest BIC were used to calculate genetic distance by k2+G method (Tamura *et al.,* 2011). Phylogenetic analyses were done using the neighbor-joining method within the MEGA 5.0. Robustness of inferred evolutionary relationships were assessed by 1,000 bootstrap replicates. *P. betae* sequence [GenBank Acc. No. Y12827 (Ward & Adams, 1998)] was compared with *P. betae* isolates collected from the different geographical regions of Iran. Several *P. graminis* subspecies and other plasmodiophorids were also used for sequence comparison including: *P. graminis* f. sp. *temperata* (Y12824), *P. graminis* f. sp. *tropicalis* (Y12825), *P. graminis* f. sp. *colombiana* (AJ010424), *P. graminis* f. sp. *tepida* (Y12826), *Ligniera* sp. (AJ010425), and *Plasmodiophora brassicae* (Y12831)].

Comparison of GST gene in virus-bearing and virus-free *P. betae* isolates

The amino acid sequence of a virus-bearing *P. betae* isolate originated from Fars and a virus-free isolate originated from Golestan province, were aligned with the published sequence of *P. betae* (AJ132355.1). Next, the coding sequence of *P. betae* GST protein selected from isolates from Fars (AGC94564.1) and Golestan (AGC94563.1) provinces were aligned using the neighbor joining method and GTT + G model with 1000

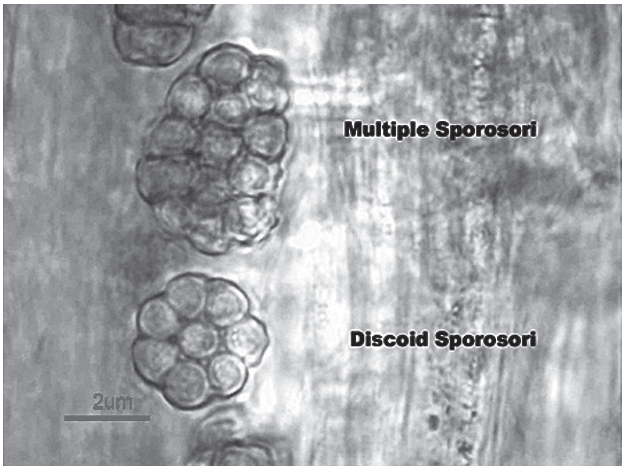

Figure 2. Photomicrographs of sporosori of *P. betae* detected in roots of sugar beet 'Regina' planted in the soil sample from Fars.

bootstraps (MEGA 5.0 software) against GST gene sequence from *Pseudomonas* sp. DJ77 (AF001103), *Sphingomonas paucimobilis* (AF001779), *Escherichia coli* (P0A9D3), *Proteus mirabilis* (P15214), *Haemophilus influenza* (P44521), *Rhizobium leguminosarum* (X89816), the unicellular blue green algae *Cocomyxa* sp. (Q42706) and human (UB6529).

Results

Observation of *P. betae* sporosori in rootlets of sugar beet

Based on cystosori morphology (irregular clusters) and cystosori individual size (4-7 μm diameter), the parasite was identified as *P. betae* (Fig. 2). *P. betae* cystosori were greatly detected in the roots of 'Regina' plants grown in sand and soil mixture, but were absent in roots of the plants grown in sand alone.

ELISA- based assay of *P. betae* and BNYVV

To determine *P. betae* concentration and BNYVV content using ELISA method, lateral roots of the test plants were used. The ELISA displayed significant differences in *P. betae* concentration and BNYVV based on optical density (OD) in different soil samples. The ELISA results showed the presence of *P. betae* in all soil samples (Fig. 3). Soil samples collected from Khorasan Razavi, Fars, Golestan and Kerman showed

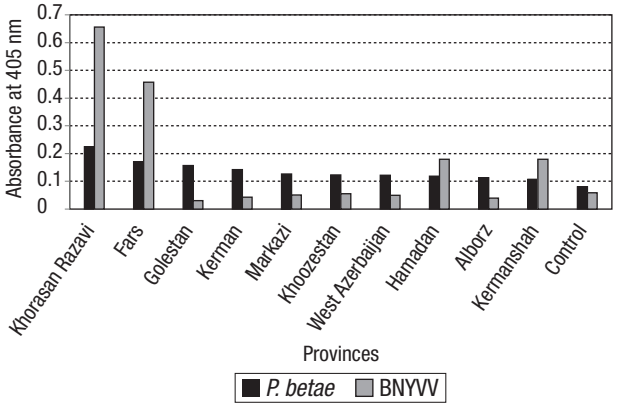

Figure 3. Detection of virus-bearing and virus-free *P. betae* using DAS-ELISA.

the highest *P. betae* contamination. Using specific BNYVV antibodies, ELISA results confirmed the presence of virus in samples collected from Khorasan Razavi, Fars, Hamadan and Kermanshah, but other samples were detected as virus-free (Fig. 3).

P. betae variability in rDNA sequence and phylogenetic analysis

Using *P. betae*-infected sugar beet DNA and Psp1 and Psp2rev primers, PCR fragments of about 454 bp were produced. ITS1 + 5.8S + ITS2 rDNA sequences from all *P. betae* isolates (A, AZ, D, G, H, K, KE, KH, M and S) were determined and aligned with rDNA sequence of *P. betae* isolate from the UK (Acc. No. Y12827, Ward & Adams, 1998). Results showed a very high homology among them (Fig. 4). A few single nucleotide changes were detected in ITS1+5.8S+ITS2 region leading to a very close phylogenetic distance among different *P. betae* isolates of Iran. However, *P. betae* could be distinctly separated from *P. graminis* (Group 2). Within Group 2, two subgroups were detected: one comprised of f.sp. *temperata* and f.sp. *tropicalis* and the other comprised of f.sp. *colombiana* and f.sp. *tepida*. Two other plasmodiophorids including *Ligniera* sp. and *Plasmodiophora brassicae* were considered as out-group.

Comparison of *P. betae* isolates using GST sequence

Using all DNA samples, GST-specific primer set produced a PCR product of about 903 bp length. The

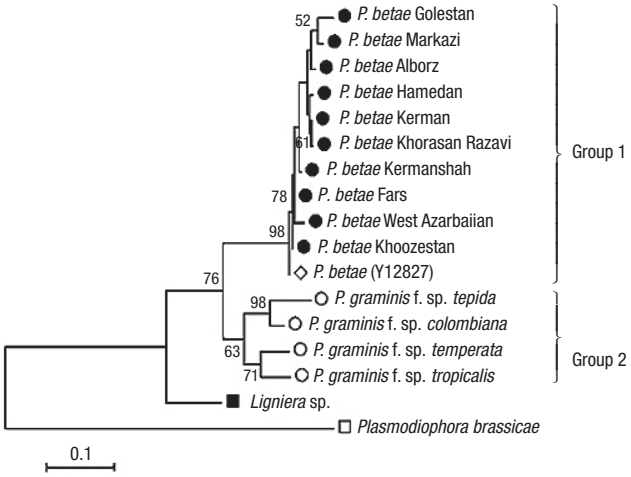

Figure 4. Phylogenetic analysis of different Plasmodiophoromycetes based on rDNA ITS1+5.8S+ITS2 gene.

sequence of two isolates were determined and deposited in Genbank (AGC94564.1 [Fars] and AGC94563.1 [Golestan]). After comparing with other GST genes in the Genbank, two exon regions between 1-343 and 655-903 nucleotides with an intron of 312 bp between them were identified. Because of no homology in blast searches, no difference was observed between the two Iranian sequences in GST exon and intron regions, but their exon regions differed in three nucleotides and five amino acids compared to other *P. betae* GST isolates in the GenBank (AJ132355). There was little sequence homology among *P. betae*-GST gene and other organisms such as *Pseudomonas* sp. DJ77T, *Sphingomonas paucimobilis*, *Proteus mirabilis*, *Haemophilus influenza*, *Rhizobium leguminosarum*, and blue green algae *Cocomyxa* sp.

Discussion

Rhizomania is one of the most widespread sugar beet diseases which is caused by BNYVV and results in severe yield loss in the absence of effective control methods (Pavli *et al*., 2011). Natural virus transmission occurs through the root parasite *P. betae*, a protist that dwells in the soil and survives in the form of sporosori (Fujisawa & Sugimoto, 1977). The virus is carried internally by 10%-15% of *P. betae* resting spores in the soil (McGrann *et al.,* 2009). Currently, management of rhizomania disease is based on genetic resistance to BNYVV. However, for durable disease management, the decrease in inoculum potential of the vector as well as genetic resistance to the virus should be considered. The major objective of the present study was the detection and characterization of *P. betae* in 10 sugar beet sowing regions in Iran. Results confirmed the presence of this plasmodiophorid in all collected samples. Microscopic observation of cyctosors in infected roots was used for preliminary studies. Although it is a simple method for *P. betae* detection in plants, it is also time consuming and inaccurate. In an earlier study (Mesbah *et al.,* 1997), this method was used for detection and quantification of *P. betae*.

To overcome these limitations, complementary approaches such as DAS-ELISA and PCR amplification analysis were performed on these samples. Results obtained from DAS-ELISA method confirmed the initial results obtained using microscopic observations. ELISA is ideal for usage in *P. betae* screening in sugar beet germplasm. Unlike PCR-based methods, antibodies detect the actual amount of antigen without amplification, and in addition are more likely able to differentiate between live and dead pathogen biomass (Kingsnorth *et al.,* 2003). ELISA sensitivity, as determined by an ability to detect low levels of *P. betae* infection, enhances their utility for screening field samples. Complementary analysis for detection of BNYVV in selected samples confirmed the presence of virus in the fields of Fars, Khorasan Razavi, Kermanshah, and Hamadan regions while remaining regions were virus-free. Therefore, to prevent the spread of virus into clean areas, both soil and plant residues translocation from infected lands should be prohibited.

Genetic variation of the collected *P. betae* isolates, including virus-bearing and virus-free, was compared with another isolates of *P. betae* and *P. graminis* by comparing the sequence of rDNA (ITS1 and ITS2) regions and GST gene. Result showed a high level of homology among virus-bearing and virus-free *P. betae* isolates from different regions in Iran. However, only few nucleotide changes could be detected in sequenced *P. betae* ITS1 + 5.8 + ITS2 from different locations. Apparently, such deletions, insertions, and other mutations, which could appear due to higher genomic diversification, were not detected and as a result were not correlated to their geographic spread. Therefore, the lack of rDNA genetic diversity does not exclude differences in the ability to multiply, take up and transmit BNYVV which indicates more work is required to unravel variation in these virus vector properties. These results also showed major phylogenetic distance between the two plasmodiophorid isolates that may be re-

lated to their different hosts (as the main hosts for *P. graminis* are Poaceae which separates them from *P. betae*) which corresponds with Legrève *et al.* (2003) study. *P. graminis* isolates were very diverse in their ITS rDNA sequences (Legrève *et al*., 2002; Kanyuka *et al*., 2003). The lack of diversity in this region of *P. betae* has already been observed in other studies (Pferdmenges & Varrelmann, 2009; Smith *et al*., 2011, 2013). Our work with Iranian isolates supports the finding that this part of the genome is highly conserved in *P. betae*.

Result showed that specific GST primer can be used for quick detection of *P. betae* using PCR method. Analyses of PCR product on agarose gel showed that the size of GST gene was 903-bp. Indeed, the results showed that *P. betae*-GST sequence was quite different from fungal GSTs sequence (Cavalier-& Chao, 2003).

In conclusion, based on the results obtained from DAS-ELISA analysis, *P. betae* was detected in all autumn and spring sowing sugar beet regions as well as newly cultivated regions (Golestan). On the other hand, phylogenetic analysis revealed that there is low genetic diversity among virus-bearing and virus-free *P. betae* isolates collected from different regions of Iran.

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