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Genetic Relatedness among the Pathogenic Variants in *Fusarium oxysporum* Causing Wilts of Cucurbits

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Abstract

Strains of 5 cucurbit-infecting formae speciales of Fusarium *oxysporum* were collected from various locations in Japan. The assay for pathogenicity showed the complexity of their infection spectra. In some cases, a single forma specialis infected multiple genera or species of the family Cucurbitaceae, and different formae speciales shared the same host plants. In other cases, a single forma specialis contained subgroups, designated as races, differing in pathogenicity to a set of differential cultivars within the same plant species. Genetic relatedness among strains of the cucurbit-infecting F. *oxysporum* was determined by DNA fingerprinting with the fungal nuclear repetitive DNA sequences. Four repetitive DNA sequences used as probes were chosen from a genomic library of *F. oxysporum* f. sp. *lagenariae* and designated as FOLR clones. DNA fingerprinting of total DNA of strains from the cucurbit-infecting formae speciales after hybridization with FOLR sequences enables to distinguish strains not only at the forma specialis level but also at the race level. On the basis of DNA fingerprint profiles, phylogenetic relationships among the strains were analyzed by parsimony and UPGMA methods. The analyses indicated that the formae speciales and the races are intraspecific variants specialized in DNA level as well as host range, and that FOLR DNA fingerprinting can be used for the differentiation of these pathogenic veriants.

Discipline: Plant disease
Additional key words: pathogenicity test, forma specialis, race, FOLR clones, genetic population structure

Introduction

Fusarium oxysporum Schlechtend.: Fr. is a soil-borne fungus occurring in soils all over the world. *F. oxysporum* contains phytopathogenic strains which cause destructive vascular wilts on many important crops. However, individual pathogenic strains have a limited host range[19]. Strains with similar or identical host ranges are assigned to intraspecific groups, designated as formae speciales (f. sp.)[1,19]. The formae speciales are distinguished by the ability to cause wilt diseases in a limited taxonomic range of host plants. Some of the formae speciales are further divided into subgroups, designated as races, on the basis of pathogenicity to a set of differential cultivars within the same plant species[1,19].

More than 10 cucurbit plant species and a number of their cultivars are grown in Japan[8]. Corresponding to these diverse plant species and cultivars, pathogenic variation of *F. oxysporum* on the cucurbit plants has been categorized into 6 formae speciales: f. sp. *cucumerinum* on cucumber (*Cucumis sativus* L.), f. sp. *melonis* on muskmelon (*Cucumis melo* L.), f. sp. *lagenariae* on bottle gourd [*Lagenaria siceraria* (Molina.) Standley], f. sp. *niveum* on watermelon [*Citrullus lanatus* (Thumb.) Matsum. et Nakai], f. sp. *luffae* on loofah (*Luffa cylindrica* Roem), and f. sp. *momordicae* on balsam pear (*Momordica charantia* L.)[1,20].

We outline here the pathogenic variation in *F. oxysporum* causing wilts of cucurbit plants and the genetic relatedness among the pathogenic variants determined by DNA fingerprinting with nuclear repetitive DNA sequences.

Host range of the cucurbit-infecting formae speciales

The forma specialis concept was proposed by

Table 1. Host range of cucurbit-infecting formae speciales of *Fusarium oxysporum*

Forma specialis	Plant						
	Cucumber	Muskmelon	Bottle gourd	Malabar gourd	Pumpkin	Balsam pear	Watermelon
cucumerinum	++	−	−	−	−	−	−
melonis	−	++	−	−	−	−	−
lagenariae	−	−	++	++, +, −	++, +, −	−	−
momordicae	−	−	+	+	+	++	−
niveum	−	−	−	−	−	−	++

++ : Highly virulent, + : Weakly virulent, − : Non-pathogenic.

Snyder and Hansen[19] for *F. oxysporum* on the basis of strict host selectivity of the strains. The formae speciales pathogenic to cucurbits are basically host-specific and distinguishable based on host species. However, notable exceptions to the initial concept of forma specialis have been reported in these pathogens[4,7,10,16]. McMillan[10] reported that isolates of *F. oxysporum*, which were obtained from wilted cucumber plants in the Bahamas, were also pathogenic to muskmelon and watermelon. Similar isolates were detected by Kim et al.[4]. Martyn & McLaughlin[7] found that some isolates of the forma specialis *niveum* infected some summer squash cultivars. Nomura[16] showed that the forma specialis *lagenariae* was pathogenic not only to bottle gourd but also to pumpkin and malabar gourd.

To reassess the host specificity of the cucurbit-infecting formae speciales, we collected *F. oxysporum* strains causing wilts of cucurbit plants from various locations in Japan and examined their pathogenicity to 10 cucurbit plant species[12]. Pathogenicity of the strains was assayed by the root dip method using plant seedlings with fully expanded leaves. The roots were dipped in a spore suspension (10^7 conidia per ml) for 15 s. The inoculated seedlings were transplanted to plastic pots filled with sterilized soil and placed in a greenhouse. External symptoms and vascular discoloration were scored 28 days after inoculation, and the pathogen was re-isolated from vascular bundles of inoculated plants.

The host range of the Japanese strains of cucurbit-infecting formae speciales is listed in Table 1. Strains of the formae speciales *cucumerinum* and *niveum* were pathogenic only to their original hosts, cucumber and watermelon, respectively. While these formae speciales have been reported to contain pathogenic variants which infect cucurbit plants other than the original hosts[4,7,10], such variants were not detected in our samples.

Strains of the forma specialis *melonis* were only pathogenic to muskmelon among the cucurbit plants used. However, the forma specialis was found to carry pathogenic variants differing in pathogenicity to oriental melon[13,14]. The details will be described later.

The forma specialis *lagenariae* contained 3 pathogenic variants that differed in pathogenicity to pumpkin and malabar gourd in addition to the original host, bottle gourd: (1) pathogenic only to bottle gourd, (2) highly virulent to bottle gourd and weakly virulent to pumpkin and malabar gourd, and (3) highly virulent to all the 3 plants[13].

The forma specialis *momordicae*, the causal agent of balsam pear wilt, was found to cause disease also in bottle gourd, pumpkin, and malabar gourd, showing that the formae speciales *lagenariae* and *momordicae* have common host plants[13]. The formae speciales that attack related host plants may be closely related genetically and share characteristics necessary for pathogenicity. On the basis of the cross-infectivity of these formae speciales, the genetic relationships within and among these formae speciales have been investigated.

Genetic relatedness among the cucurbit-infecting formae speciales

Over the past several years, genetic population structure in *F. oxysporum* has been examined by using various genetic markers, such as VCG[6], isozyme profiles[2] and RFLPs in mitochondrial and nuclear DNA[3,9,11]. Of these procedures, RFLP analysis has the advantage of potentially detecting numerous polymorphisms at the DNA level.

DNA fingerprinting with nuclear repetitive DNA sequences has recently been employed to distinguish strains of *F. oxysporum* belonging to different pathogenic variants[4,5]. We used this sensitive method to assess the genetic diversity within the cucurbit-infecting formae speciales and to determine the

genetic relatedness among the formae speciales[14].

Four genomic clones, designated as FOLR clones, were isolated from a genomic library of *F. oxysporum* f. sp. *lagenariae* strain MAFF 305118 for use as DNA fingerprinting probes. FOLR clones were identified to carry moderately repetitive DNA sequences dispersed in the fungal chromosomes. Total DNA of 50 strains representing 5 cucurbit-infecting formae speciales and 6 strains pathogenic to plants other than cucurbits was digested with *Eco*RV and hybridized with ^{32}P-labeled FOLR DNA.

DNA fingerprints of total DNA of representative strains after hybridization with FOLR4 probe are shown in Plate 1. Major bands in the fingerprints were shared by strains within a single forma specialis, while minor bands were polymorphic among the strains. Strains within a single forma specialis had generally similar banding patterns and were distinguished from strains of other formae speciales. Extensive polymorphisms in the fingerprints were observed not only in minor bands but also in major bands between strains of different formae speciales. Thus it was easy to distinguish strains at the forma specialis level on the basis of the fingerprint profiles.

Forty-five fingerprint types were detected among the 50 strains from cucurbit-infecting formae speciales by using all FOLR probes. The similarity coefficient for all possible pairs of fingerprint types from their hybridization profiles was estimated by the method of Nei & Li[15]. A dendrogram was constructed from the similarity coefficient data using the unweighted pair group method with arithmetic average clustering (UPGMA)[18]. The dendrogram identified 6 genetic groups within the cucurbit-infecting strains, corresponding to the forma specialis classification, at the similarity level of more than 75% (Fig. 1). Each population of the formae speciales *lagenariae*, *cucumerinum*, *niveum* and *momordicae* clustered into a single group. *F. oxysporum* strains pathogenic to plants other than cucurbits were distinguished from one another and also from the strains of the cucurbit-infecting formae speciales on the dendrogram (data not shown). Parsimony analysis also indicated the presence of forma specialis-dependent grouping. These results suggest that the cucurbit-infecting formae speciales are intraspecific variants that can be distinguished in their host range and at the DNA level.

The formae speciales *lagenariae* and *momordicae* have common host plants, bottle gourd, pumpkin, and malabar gourd. However, the strains were easily distinguished, corresponding to their forma specialis classification, by FOLR DNA fingerprinting: each population of these formae speciales was grouped into a distinct genetic cluster by phylogenetic analysis (Fig. 1).

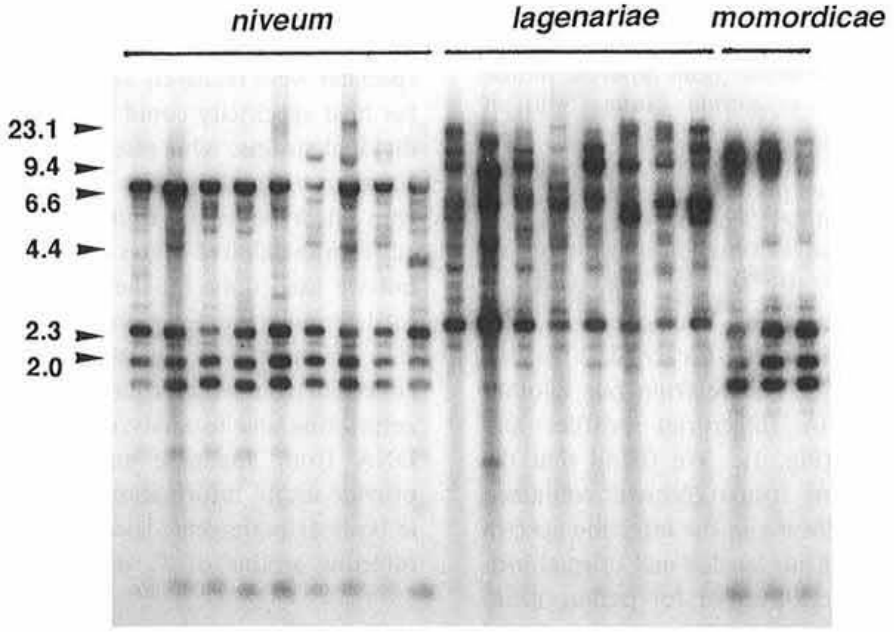

Plate 1. FOLR4 DNA fingerprints of representative strains from the formae speciales *niveum*, *lagenariae* and *momordicae* of *F. oxysporum*
The sizes of marker DNA fragments (*Hin*dIII-digested λ DNA) are indicated on the left in kilobase.

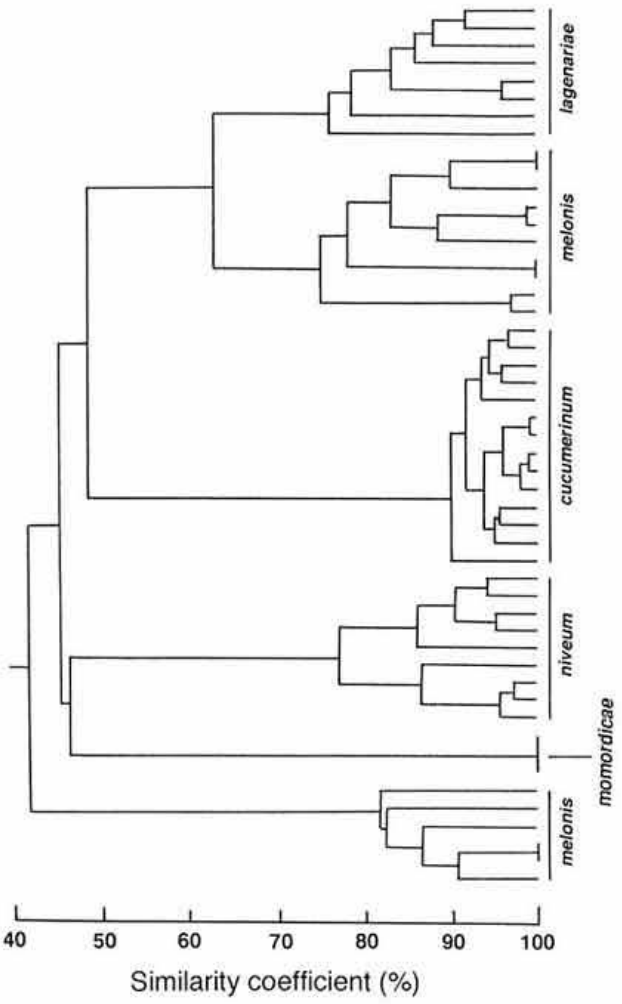

Fig. 1. UPGMA dendrogram showing the levels of genetic relatedness of 50 strains from different formae speciales of *F. oxysporum* causing wilts of cucurbits

The 50 strains were divided into 45 genetic types on the basis of the fingerprints probed with FOLR1 to FOLR4. Similarity coefficients were calculated from the fingerprints by the method of Nei and Li (1979)[15].

The forma specialis *melonis* carried 2 genetic groups that were distinguishable from one another on the basis of DNA fingerprint profiles and phylogenetic analysis (Fig. 1). We found that the Japanese strains of this forma specialis contained pathogenic variants differing in the infection spectra on Japanese cultivars of muskmelon and oriental melon. The 16 strains were assayed for pathogenicity to 2 cultivars each of muskmelon and oriental melon[14]. The assay divided these strains into 2 groups that corresponded to the groups identified by FOLR DNA fingerprinting. One group was pathogenic to both muskmelon and oriental melon, and the other group was pathogenic only to muskmelon. Further analyses of pathogenic and genetic variation within this forma specialis using additional strains will be described later.

Recently, it has been demonstrated that hypervariable repetitive DNA in fungal chromosomes may be useful for the differentiation of strains belonging to a particular pathotype or from a particular locale[3,9,11]. Kistler et al.[5] used nuclear repetitive DNA to predict the genetic relationship among 3 crucifer-infecting formae speciales of *F. oxysporum*. The analysis indicated that there was a close relationship among members of the same forma specialis. These results are similar to our findings with FOLR probes used to determine genetic relationships among the cucurbit-infecting formae speciales.

Kim et al.[4] determined the genetic divergence and the relatedness of 5 formae speciales within the cucurbit plants based on RFLPs of mitochondrial DNA. They identified a close relationship between the different formae speciales and presented evidence for genetic similarity. Both cluster and parsimony analyses of the mitochondrial DNA RFLPs indicated that all of the *F. oxysporum* formae speciales in cucurbits are closely related and that in some cases, isolates of different formae speciales were genetically more similar than isolates of the same forma specialis. Based on the complexity of infection spectra of the formae speciales on cucurbits and RFLP analysis of mitochondrial DNA, Kim et al.[4] assumed that the genetic differences between the formae speciales were relatively small and that determinants for host specificity could be combined or lost in individual strains. Our results, based on FOLR DNA fingerprinting of nuclear DNA, are not consistent with this hypothesis. Kim et al. used strains collected from the United States and a few other countries, but we used only Japanese strains. It is necessary to determine the genetic diversity and the relationship among the *F. oxysporum* strains on cucurbits collected from other countries by FOLR DNA fingerprinting and to analyze RFLPs of mitochondrial DNA from Japanese strains. Such studies will provide useful information for unraveling the genetic basis of pathogenic specialization in the cucurbit-infecting strains of *F. oxysporum*.

Pathogenic variation within the forma specialis *melonis*

A number of muskmelon cultivars are bred and cultivated in Japan. The varieties within the same

species, oriental melon (*C. melo* var. *makuwa*) and oriental pickling melon (*C. melo* var. *conomon*), are also cultivated[8]. As mentioned above, the Japanese strains of the forma specialis *melonis* carried pathogenic variants which differ in pathogenicity to oriental melon[14]. To further characterize pathogenic variation within the forma specialis[13], additional strains of the forma specialis were collected from various locations in Japan and tested for their pathogenicity to several cultivars of muskmelon and oriental melon.

Forty-one strains tested were divided into 3 major pathogenic groups (groups I, II and III), based on the differences in pathogenicity to 2 cultivars (Amus and Ohi) of muskmelon and one cultivar (Ogon 9) of oriental melon. The cultivars Amus and Ohi have been generally used as susceptible and resistant cultivars, respectively, against the forma specialis *melonis* in Japan. Strains of group I, however, were pathogenic to both muskmelon cultivars and also to oriental melon cultivar Ogon 9. Surprisingly, strains of this group were found to be pathogenic to all of the 24 cultivars of muskmelon and 7 cultivars of oriental melon examined. Such strains have not been reported in Japan so far. Strains of group II were pathogenic to the muskmelon cultivar Amus and oriental melon cultivar Ogon 9, but not to the muskmelon cultivar Ohi. Strains of group III were pathogenic only to the muskmelon cultivar Amus. These results indicate that at least 3 pathogenic variants are distributed in the Japanese population of the forma specialis *melonis*.

Risser et al.[17] reported that the forma specialis *melonis* was divided into 4 races (races 0, 1, 2 and 1,2). The race description was based on the pathogenicity to 3 muskmelon cultivars Charentais T, Doublon, and CM17187. Cultivars Doublon and CM17187 harbor single dominant resistance genes *Fom1* and *Fom2*, respectively. The races are designated on the basis of the resistance genes which they overcome. Race 1,2 is subdivided into wilt (race 1,2w) and yellows (race 1,2y) types, depending on the symptomatology. The Japanese strains were subjected to race identification in accordance with the method of Risser et al.[17].

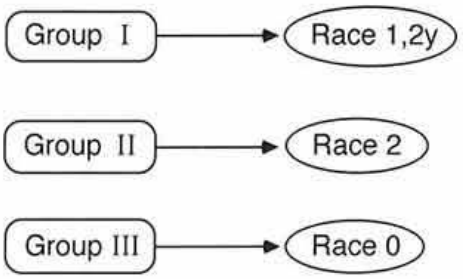

Fig. 2. Race classification of pathogenic groups (Groups I to III) in the Japanese strains of *F. oxysporum* f. sp. *melonis*

Since strains of group I caused firstly yellows and finally death of all the differential cultivars, they were identified as race 1,2y. Strains of group II were pathogenic to both Charentais T and CM17187, and classified as race 2. Strains of group III were classified as race 0, pathogenic only to Charentais T (Fig. 2). These results show that our grouping based on the pathogenicity test using Japanese cultivars of muskmelon and oriental melon corresponds to the race description proposed by Risser et al.[17]. In Japan, it is difficult to obtain seeds of the race differential cultivars reported by Risser et al.[17], because they are not commercially distributed. We assume that the cultivars Amus, Ohi and Ogon 9 can be used as substitutes for the race differential cultivars.

Genetic relatedness among the races of the forma specialis *melonis*

FOLR DNA fingerprinting was used to examine the genetic diversity within the races and to predict genetic relatedness among the races[13]. Total DNA from the 41 Japanese strains was digested with *Eco*RV and hybridized with each of 4 FOLR probes. These probes detected 36 fingerprint types among the 41 strains. DNA fingerprint profiles could differentiate strains at the race level. FOLR3 DNA fingerprints of representative strains from the 3 races are shown in Plate 2. Strains within each race 2 (group II) and race 1,2y (group I) showed similar banding patterns. Strains of race 0 (group III) were divided into 2 groups, whose DNA fingerprints were obviously different.

A cluster analysis of the fingerprint data was employed to calculate the similarity coefficient between the fingerprint types, and a dendrogram was constructed by using UPGMA (data not shown). The dendrogram identified 4 genetic groups (data not shown). The fingerprint types detected in races 2 and 1,2y were grouped into distinct single clusters. Strains of race 0 were divided into 2 genetic groups. These results suggest that race 0 population contains pathogenic variants which are different in pathogenicity to untested host cultivars. The races differed

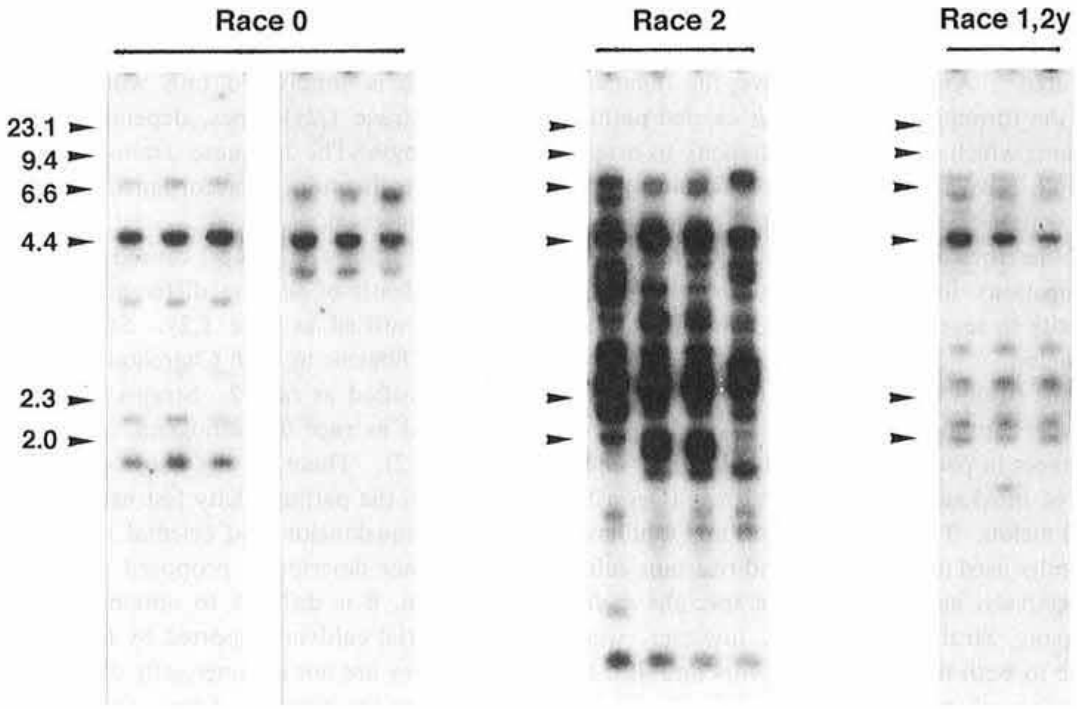

Plate 2. FOLR3 DNA fingerprints of representative strains from different races of *F. oxysporum* f. sp. *melonis*
The sizes of marker DNA fragments (*Hin*dIII-digested λ DNA) are indicated on the left in kilobase.

in pathogenicity to cultivars of muskmelon and oriental melon. Muskmelon and oriental melon have different geographic origins and different histories of cultivation in Japan[8]. Thus, we assume that genetic differences between these pathogenic variants are due to the geographic isolation of the pathogen population during their establishment and prior to their dispersal throughout the world.

Conclusion

The nomenclature of the pathogenic variants in *F. oxysporum*, i.e. formae speciales and races, has considerable and practical value for plant pathologists. However, some of the difficulties arise from assigning a designation to a strain on the basis of pathogenicity tests. As reported repeatedly, the infection spectra of the cucurbit-infecting formae speciales were complex: in some cases, a single forma specialis infected multiple genera or species of the family Cucurbitaceae; in other cases, different formae speciales shared the same host plants. These phenomena indicate that pathogenic and genetic variants are present within single formae speciales, and that the formae speciales which attack related host plants may be closely related genetically and share characteristics required for pathogenicity. However, phylogenetic analysis based on DNA fingerprinting with nuclear repetitive DNA sequences enables to readily distinguish the cucurbit-infecting strains not only at the forma specialis level but also at the race level. FOLR DNA fingerprinting provided a basis for the identification of such pathogenic variants and for studying the potential development of pathogenic specialization in *F. oxysporum*, taking into account the impact of coevolution with host plants.

Characterization of pathogenic and genetic diversity within the species *F. oxysporum* could provide a better understanding of this economically important species and contribute to more effective management of the diseases for which it is responsible.

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