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Full Length Research Paper

Distinguishing proof of the S-genotypes of a few sweet cherry (Prunus avium L.) cultivars by AS-PCR and fertilization

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Sweet cherry cultivars display a self-incompatibility system that restricts self-fertilization and fertilization between cultivars bearing identical S-alleles. PCR-based S-allele typing system for sweet cherry cultivars has been recently developed. It has been reported that all known self-incompatibility (S) alleles of sweet cherry were more than 16 based on the AS-PCR analysis. In this work, two sets of AS-PCR primers that were designed based on conserved domains of cDNA sequences of S-RNases has been used to characterize the S-genotype of 38 sweet cherry cultivars, including 15 cultivars whose S-genotype had not been previously described. Pollination test was also performed to validate the PCR results, and the consistent results were got. A wide variation in the frequency of S-alleles in the Sweet cherry germplasm was observed. S_3 was the most common in the cultivars evaluated, and S_7, S_{10}-S_{16}, S_{23}-S_{25} as rare allele was not found in China geographical areas in our study.

Key words: Self-incompatibility, *Prunus avium*, S-RNase, AS-PCR, pollination test.

INTRODUCTION

Correct assignment of sweet cherry cultivars to cross-pollen S-gene candidate encodes an F-box protein, compatibility groups is important for the efficient which has recently been described in almond (Prunus production of cherry fruit because most of the sweet dulcis) (Ushijima et al., 2003), and in two species of cherry cultivars are self-incompatible and inter- cherry (Yamane et al., 2003). Many worldwide studies on incompatible, that is to say, there is no or close to 0% fruit the cross-compatibility of cherry cultivars have been set unless cross-compatible pollination is provided. Self- carried on (Crane and Brown, 1937; De Vries, 1968; incompatibility (SI) is a widespread mechanism in Tehrani and Brown, 1992). flowering plants that prevents self-fertilization and A classic table assigning some 190 cultivars and a few promotes out-crossing. Sweet cherry exhibits stylar selections to 13 incompatibility groups and a 'universal monofactorial gametophytic locus self-incompatibility donors' group O was established (Matthews and Dow, (Crane and Lawrence, 1929). Several works clearly 1969). Most of the studies above have been done by suggest that two different genes of the S-locus control the controlled pollination tests and/or pollen tube growth tests function of pistil and pollen respectively in GSI (McCubbin that are time-consuming and tend to be affected by and Kao, 2000). The pistil S-gene encodes a family of environmental factors. With the finding that S alleles in ribonucleases (S-RNase) (McClure et al., 1989) and a sweet cherry code for stylar ribonucleases (S-RNase), up to 16 different S-alleles and 27 incompatibility groups have been reported so far using different methods (Tobutt et al., 2001; Sonneveld et al., 2003; Wunsch and Hormaza, 2004a; Boskovic and Tobutt 2001). PCR method using consensus primers, based on conserved

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Table 1. Sweet cherry cultivars used as S-allele standards and their S-genotypes

Cultivar	S-genotype	Publisher (source)
Summit	S_1S_2	EM BC NY
Van	S_1S_3	EM BC NY
Bing	S_3S_4	EM BC NY
Hedelfingen	S_3S_5	EM BC NY MI
Governor Wood	S_3S_6	EM NY
Burlat	S_3S_9	EM BC NY MI

BC: British Columbia (Wiersma et al., 2001).
EM: Easting Malling (Boskovic and Tobutt, 1996; Boskovic and Tobutt, 2001; Boskovic et al., 1997; Sonneveld et al., 2001).
MI: Michigan (Hauck et al., 2001).
NY: New York (Choi. et al., 2000).

Table 2. Fragment lengths generated by PCR of specific S-alleles

Allele	DNA fragment length in base pairs for ASPCR	
	Pru-C2m/Pru-C5m	PruT2/SI32
S_1	886	458
S_2		422
S_3	897	306
S_4	1061	524
S_5		465
S_6	560	521
S_9	780	431

regions (Tao et al., 1999b), or allele-specific primers (Sonneveld et al., 2001, 2003), ploymierase chain reaction-restriction fragment length polymorphisms (PCR-RFLPs) (Yamane et al., 2000) or RFLPs (Hauck et al., 2001) has been used in sweet cherry, which is convenient to have a molecular assay that could be used on vegetative material, independently of age or season.

Most sweet cherry cultivars planted by traditional experience in China have not been genotyped yet. In this study, we utilized the PCR-based S-allele typing system to determine the S-genotypes of 38 sweet cherry cultivars, including 15 sweet cherry cultivars whose S-genotype had not been previously described.

MATERIALS AND METHODS

Plant material

Young leaf tissue or buds of total 38 sweet cherry cultivars were collected from Institute of Forestry and Pomology, Beijing Academy of Agriculture and Forestry Sciences germplasm garden and Shandong Agriculture University. Six self-incompatibility sweet cherry cultivars with known S-genotype, 'Summit' (S_1S_2), 'Van' (S_1S_3), 'Bing' (S_3S_4), 'Hedelfingen' (S_3S_5), 'Governor Wood' (S_3S_6) and 'Burlat' (S_3S_9) (Table 1) were collected and used as standard fragment sizes of their corresponding S-alleles in PCR analysis. These cultivars belong to different self-incompatibility groups and their S-genotype have been confirmed in various different works (Wiersma et al., 2001; Boskovic and Tobutt, 1996; Boskovic and Tobutt, 2001; Boskovic et al., 1997; Sonneveld et al., 2001; Hauck et al., 2001; Choi. et al., 2000).

Isolation of genomic DNA and PCR amplification of S-alleles

Total genomic DNA was isolated from fresh young leaves or buds using the CTAB method described by Stockinger et al. (1996) with a minor modification. The extraction buffer contained 100 mM Tris-HCl (pH 8.0), 20 mM EDTA, 1.4 mM NaCl, 2% CTAB, 2% Polyvinylpyrrolidone (PVP K25) and 1.5% β-mercaptoethanol. The PCR to identify the S-alleles of the different cultivars analysis was carried out using the primer pairs Pru-C2 and Pru-C5 (Tao et al., 1999a) with minor modification, and the combination Pru-T2 (Tao et al., 1999a) and SI32 (Wiersma et al., 2001). Primer sequences were as follows: Pru-C2m (TGGCCAAGTAATTATTCAAACC), Pru-C5m (CAAAATACCACTTCATGTAACAAC), Pru-T2 (GTTCTTGCTTTTGCTTTCTTC) and SI32 (CATAGGCCATGGATGGTG). The PCR amplification was carried out in a total volume of 15 µl using 1 µl DNA about 20-30 ng, reagents included 1.5 U of *Taq* DNA polymerase (Promaga), 1×reaction buffer, 0.2 mM dNTP mix, 1.67 mM $MgCl_2$, 0.17 µM primer. The samples were run in Thermo cycler (PTC-100; MJ Research). Temperature profiles consisted of an initial denaturing of 3 min at 94 °C, then the samples were cycled 35 times through the following steps: denaturing for 1 min at 94°C, annealling for 1 min at 56°C, elongation for 1 min at 72°C, with a final extension of 10 min at 72°C. The PCR product S_1, S_2 of 'Summit'; S_3, S_4 of 'Bing'; S_5 of 'Hedelfingen'; S_6 of 'Governor Wood'; S_9 of 'Burlat'; S_3 of 'Van' were cloned and sequenced.

Detection of PCR products

PCR products were run on 4% denaturing PAGE (polyacrylamide gels) containing 4 M urea. Electrophoresis was performed using 1×TBE buffer on a standard thermoplate sequencer gel-electrophoresis unit (BioRad sequi-GenGT Sequencing Cell). Gels were pre-run for 20 min, and then 4 µl samples were loaded into each well.The samples were denatured at 94°C for 5 min and cool on ice, before loading. Gels were run at 70 W for 3 h, and sliver stained for detection.

Pollination test

Branches bearing buds were cut, stood in water and 'forced' into flower in the lab (about 25°C). Pollen was collected, dry at 37°C oven overnight, then 2 mm mesh was used to separate anthers. Flower on branches in the field selected for crossing, emasculation and hand-pollination were done at the balloon stage and then bagged. Each treatment was comprised 50-100 flowers. Compatibility was classified as positive when final fruit set is 5% or more (Way, 1968). Cultivars that were used in pollination test in this research are list in Table 5.

RESULTS

PCR analysis was conducted using the S-allele consensus primer sets. The known S-genotypes of cultivars represent the S alleles S_1 to S_6 and S_9. Each of

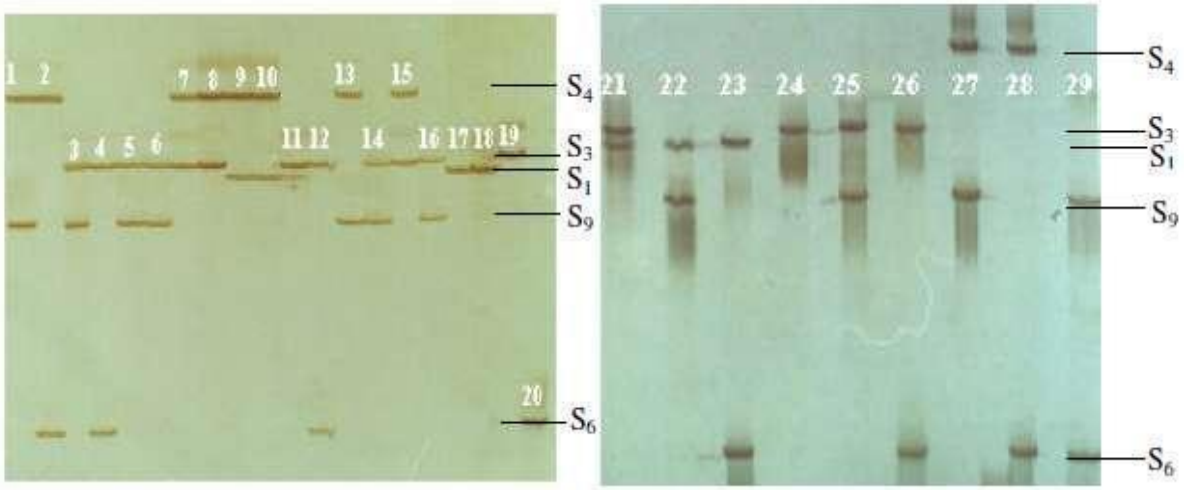

Figure 1. AS-PCR analysis the genotype of different sweet cherry cultivars amplified with primer pair Pru-C2m/Pru-C5m 4,5,7,11,17,24 are the cultivars used as S-genotype standard. _ emphasize the new S-genotype cultivars.
1 Fengjin (S4S9) 2 Elton heart (S4S6) 3 Hongdeng (S3S9**)** 4 Governor wood (S3S6) 5 Burlat (S3S9) 6 Moreau (S3S9) 7 Bing (S3S4) 8 Napolen (S3S4) 9 Rainer (S1S4) 10 Celeste (S1S4) 11 Van (S1S3) 12 Hongmi (S3S6) 13 Youyi (S4S9) 14 Yuzhou(S3S9) 15 Stella (S3S4) 16 Zaohongbaoshi (S3S9) *17* Summit(S1S2) 18 Early Rivers (S1S2) 19 Victor (S2S3) 20 Coleney (S5S6) 21 Gil peck (S1S3) 22 Qihao (S1S9) 23 Lg-1 (S1S6) 24 Hedelfigen (S3S5) 25 Jueze (S3S9) 26 Hongmi (S3S6) 27 Juhong (S4S9) 28 Jiahong (S4S6) 29 Lyons (S6S9)

the two pairs of consensus primers amplified one or two bands of various sizes. With the Pru-C2m and Pru-C5m primer set, S_1, S_3, S_4, S_6 and S_9 had the following sizes: 860, 889, 1117, 668 and 806 bp, respectively (Table 2). An example of the amplification obtained with primers Pru-C2m and Pru-C5m is shown in Figure 1. The PruT2 and SI32 primer set was useful for the identification of alleles S_2 and S_5, which did not amplified with Pru-C2m/Pru-C5m. It was observed that PruT2/SI32 is more efficient since it could amplify all the S alleles we detected. But due to the small size of the fragments that range from approximately 400-550 base pairs, it was not easy to obtain good resolution of the nearly same size bands using 2% standard agarose gels (data not shown). So PAGE (polyacrylamide gels) was used to improve the resolution. Figure 2 shows the high resolution of S_1-S_6 and S_9 allele with primer set PruT2/SI32.

The S-genotype can be recognized by the comparison of amplified results between the known and unknown genotypes of cultivars. The amplified results in Table 2 were used as the standards to deduce the genotype of others. A total of 14 pollen incompatibility groups in sweet cherry were identified in the 38 cultivars by AS-PCR typing analysis. Among the 38 cultivars used in this work, 20 cultivars were assigned to respective S-allele groups I, II, III, IV, VI, VII, IX and XVI, based on analysis which also matched the previous assignments (Table 3). The genotypes of 18 cultivars that had not been described previously for S-alleles were identified in this work (Table 3). China is not the center of origin of sweet cherry. Therefore, it may be impossible to find the putative new S-alleles in sweet cherry due to germplasm diversity. However, a new S-genotype combination S_1S_6 was identified, which should be compatible with cultivars in other groups. Controlled pollination cross was used to validate the genotypes of the new group. The crossing groups of Lg-1 and Juhong are presented in Table 5. The fruit set ratio was used as the criterion of compatibility or incompatibility, and the final fruit set no less than 5% are thought to be compatible. From the results presented in Table 5, we can see that PCR based S-genotype analysis agreed with our controlled pollination data.

The S-genotypes frequency of the 37 cultivars in the 14 groups were different, in which S_3S_9 had the highest ratio of 22%, that is to say, there are 8 cultivars of S_3S_9 S-genotypes in the total 37 cultivars S_3, S_9 are the most-frequent allele; S_1 and S_4 rank the second position (Table 4).

DISCUSSION

Tao et al. (1999b) developed the AS-PCR typing system for sweet cherry based on the cDNA sequences of S-RNase. This AS-PCR technique has been widely used in apple (*Malus domesica*) (Jassens et al., 1995), Japanese apricot (*Prunus mune*) (Burgos et al., 1998) and almond (*Prunus dulcis*) (Tamura et al., 2000). Designing allele-specific primers are the most important part in this AS-PCR typing system. It has been known that there are five conserved region and two hypervariable regions in the sweet cherry cDNA sequence of the S-RNase (Ishimizu et al., 1998; Tao et al., 1999b). Two sets of primers were designed to anneal the conserved coding region during

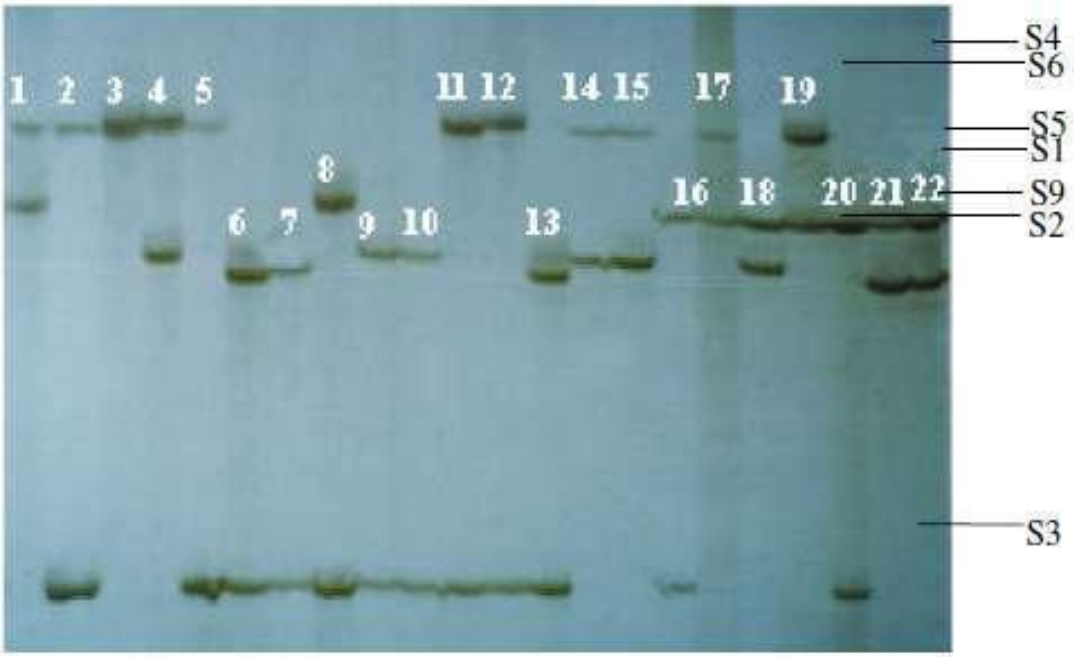

Figure 2. AS-PCR analysis the genotype of different sweet cherry cultivars amplified with primer pair PruT2/SI32. 2,8,9,11,16,21 are the cultivars used as S-genotype standard. _ emphasize the new S-genotype cultivars.

1 Coleney (S5S6) 2 Governor wood (S3S6) 3 Jiahong (S4S6) 4 Juhong (S4S9) 5 Hongmi (S3S6) 6 Victor (S2S3) 7 Vega (S2S3) 8 Hedelfigen (S3S5) 9 Burlat(S3S9) 10 Moreau (S3S9) 11 Governor Wood (S3S6) 12 Stella (S3S4) 13 Viva (S2S3) 14,15 Lyons (S6S9) 16 Van(S1S3) 17 Lg-1 (S1S6) 18 Qihao (S1S9) 19 Rainer (S1S4) 20 Gil peck (S1S3) 21 Summit (S1S2) 22 Early Rivers (S1S2).

PCR in our study which spanned the sequence of the two hypervasions that usually had intron structures. The intron of each allele differed in sequence and size from all other tested alleles (Tamura et al., 2000). The difference makes the distinguishing of the alleles by PCR possible. However, in some genotypes only one allele could be amplified. Several reasons could be given for this lack of amplification. First, the mismatches between conserved primer sequence and S allele sequence site (Wunsch and Hormaza, 2004a). Second, the S-allele has large intron between the primer pairs. The latter could be the case of allele S_2 that can not be amplified with the primer pair Pru-C2m/Pru-C5m. Third, preferential PCR amplification of some regions while allele could also reportedly be resulting in false negatives (Brace et al., 1993). This work clearly indicate's that two PCR reactions with primer pairs Pru-C2m/Pru-C5m and Pru-T2/SI32 are sufficient to identify the S-genotype of sweet cherry cultivars, in most cases due to their high allele polymorphism and high conservation.

Recently the S_7, S_9, S_{10}, S_{11}, S_{12}, S_{13}, S_{23}, S_{24} and S_{25} S-allele have been identified and the sequences of sweet cherry 10 S-alleles (S_1, S_2, S_3, S_4, S_6, S_9, S_{12}, S_{23}, S_{24}, S_{25}) have been published (Tao et al., 1999a, b; Sonneveld et al., 2001; Wunsch and Hormaza , 2004a). With the increase in number of S-alleles, just primer pairs Pru-C2m/Pru-C5m and Pru-T2/SI32 may not be enough to differentiate all alleles and identification can be better achieved complementing the results with other conserve primer pairs or with other molecular methods of sweet cherry cultivar identification such as PCR-RFLPs (Yamane et al., 2000). In fact the number of S-alleles in the sweet cherry cultivars that are grown today is limited, as most of these varieties are highly genetically related and there are S-alleles like S_1, S_2, S_3, S_4, S_6, S_9 that are very common among the cultivated varieties (Wunsch and Hormaza , 2004b; Boskovic, 2001). S_7 was first found in the mazzard rootstock 'Charger', then Boskovic found S_7 in two sweet cherry cultivars, 'Gryall's Seedling' and 'Guigne d'Annonay' (Boskovic, 2001). S_{10}, S_{11} only occurred in the timber clone 'Orleans 171', still have not been found in sweet cherries. Lacis (2008) investigated the S-allele in the Latvian and Swedish germplasm, which appeared to have a high frequency of the S6 allele in both collections, and a relatively high frequency of the S5 allele in Latvian germplasm. In this work, S_3S_9 are the most frequent genotypes. S_3, and S_9 are the most-frequent allele. S_1 and S_4 alleles rank the second position. The high frequency of S_3 maybe due to its linkage with some important commercial properties, and is that the reason why breeders used materials containing S_3 gene as the parents, or the properties of S_3 are easily

Table 3. Incompatibility groups identified by PCR analysis in the sweet cherry cultivars

Incompatibility group	S-genotype	Cultivar
I	S_1S_2	Summit
		Early Rivers
II	S_1S_3	Van
		Gil Peck
III	S_3S_4	Bing
		Napoleon
IV	S_2S_3	Stella (sc), Sunburst (sc)
		Victor
		Vega
		Viva
V	S_4S_5	-
VI		Governor Wood Hongmi
VII	S_3S_6	Hedelfingen
VIII	S_3S_5	-
IX	S_2S_5	Rainier
	S_1S_4	Celeste, Lapins (sc)
		Viscount
X		Lyons
XI	S_6S_9	-
XII	S_2S_7	-
XIII	S_6S_{13}	-
XIV	S_2S_4	-
XV	S_1S_5	Coleney, 6-3
XVI	S_5S_6	Burlat, Moreau, Yuzhou,
	S_3S_9	Zaohongbaoshi, Jueze, Zaojiang, Hongdeng, Hongyan
XVII		Elton heart, Jiahong
XVIII	S_4S_6	Qihao, Zaodaguo
XIX	S_1S_9	-
XXI	S_3S_{13}	Fengjin, Youyi, Juhong,
	S_4S_9	Changbahong
	S_1S_6	Lg-1

The cultivars that had not been descried previously are in bold. sc, self-compatible cultivar.

Table 4. Frequency of sweet cherry S genotype and allelic frequencies

	S_1	S_2	S_3	S_4	S_5	S_6	S_7	S_9	No. alleles	Frequency of alleles
S_1	-	2	2	3	-	1	-	3	12	0.16
	S_2	-	3	-	-	-	-	-	5	0.07
		S_3	-	2	1	2	-	8	20	0.27
			S_4	-	-	1	-	3	12	0.16
				S_5	-	2	-	-	3	0.04
					S_6	-	-	1	7	0.09
						S_7	-	-	0	0.0
							S_9	-	15	0.20
									74	

Table 5. Summarization of controlled pollination cross test results for identificationof the S-genotype.

Cultivars	Tester	Fruit set (%)	Compatibility
Lg-1 (S_1S_6)	Governor wood (S_3S_6)	23	+
	Summit (S_1S_2)	24.4	+
	Rainer (S_1S_4)	18.6	+
	Juhong (S_4S_9)	20.2	+
	Elton heart (S_4S_6)	22	+
	Van (S_1S_3)	19.4	+
	Bing (S_3S_4)	24.3	+
	Moreau (S_3S_9)	14.2	+
Juhong (S_4S_9)	Changbahong (S_4S_9)	1.0	-
	Governor wood (S_3S_6)	13.5	+
	Summit (S_1S_2)	13.9	+
	Rainer (S_1S_4)	10.9	+
	Elton heart(S_4S_6)	15.2	+
	Van (S_1S_3)	23.8	+
	Bing (S_3S_4)	19.5	+
	Moreau (S_3S_9)	14.7	+

'-' incompatible. '+' compatible.

to be inherited in the breeding. The sweet cherry cultivars used by Boskovic were mostly from England, American, and Canada which seldom contain S_9, while most of the cultivars from China and Russia used in our research have proved to have abundant S_9. And the cultivars we collected rarely have S_2. The frequency of S-alleles in geographical areas might reflect the local origin of the ancient cultivars.

There are self-fertile cultivars which are derived from crosses obtained through X-irradiated pollen (Lewis, 1949; Lewis and Crowe, 1954) such as 'Stella', 'Lapins', 'Sunburst' etc. Although the band of S4' appeared the same as S4-allele of other self-incompatibility cultivars in the AS-PCR, it should be possible to select offspring of self-fertile because there is a very tight genetic linkage between the pollen and stylar part genes for Self-incompatibility. So the band S_4' can be used as a linked marker for the important self-fertile trait in the sweet cherry self-compatibility breeding work. For example offspring yielding the S_aS_4' or S_bS_4' bands from $S_aS_b \times S_cS_4$' (c≠4) should be self-compatible, and it also can select offspring of homozygosis S_4'S_4' from $S_aS_4' \times S_aS_4'$. This makes the pre-selection possible, so this technology is promising in the self-compatibility breeding program for its economical and time-saving.

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