Journal of Sugar Beet
http://jsb.sbsi.ir/
Journal of Sugar Beet 2014, 29(2)

Confirmation of repulsion molecular markers linked to rhizomania resistance gene (*Rz1*) and evaluation of gene dosage effect in sugar beet genotypes

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Norouzi P, Rahmani D, Oroojalian S, Mahmoudi SB, Aghaiezadeh M, Kakueinezhad M, Orazizadeh MR, Vahedi S, Fathi MR. Confirmation of repulsion molecular markers linked to rhizomania resistance gene (*Rz1*) and evaluation of gene dosage effect in sugar beet genotypes. J. Sugar Beet. 2014; 29(2): 71-78.

Received May 5, 2012; Accepted August 18, 2013

ABSTRACT

Rhizomania is the most important disease of sugar beet in Iran and some other parts of the world, and plays an essential role in decreasing sugar yield in fields. The best approach to control this disease is to use resistant varieties. For the involvement of resistance genes in breeding programs, tagging these genes by molecular markers is necessary. In this study, some breeding populations and commercial varieties of sugar beet originated from *Rz1* resistance source were used for validation and repeatability of 6 repulsion molecular markers obtained from last studies. Accordingly, ELISA data related to greenhouse evaluation of rhizomania resistance were used in some breeding populations. DNA was extracted from leaf samples and RAPD-PCR was performed. The PCR products were separated by gel electrophoresis. After electrophoresis, the gel was stained with ethidium bromide and was observed using gel documentation device and finally was scored for the presence or absence of marker bands. In next step, adjustment of the markers with ELISA data in single plants was measured in breeding populations and the presence of markers in commercial varieties was determined. Comparison between ELISA and molecular analysis results showed that repulsion markers PN3 and PN7-2 had acceptable agreement with ELISA (92% and 98%, respectively), susceptible varieties (87% and 90%, respectively), and resistant varieties (75% and 97%, respectively). These markers were considered as the most suitable repulsion markers for identification of *rz1* susceptibility allele. Also, the results of the gene dose effect showed that the ELISA OD mean values of the *Rz1Rz1* genotypes was significantly lower than *Rz1rz1* genotypes in the repulsion markers.

Keywords: Molecular marker, RAPD-PCR, resistance, rhizomania, sugar beet

INTRODUCTION

Importance of sugar beet

Sugar beet is one of the two important sugar production plants in the world planted in over 9 million hectares globally. It allocated more than 34 million tons (29%) of global sugar production in which 27 million tons was produced in Europe, 4.5 million tons in central and North America, 2.5 million tons in Asia, 840000 tons in Africa, and 450000 tons in South America (Draycott 2006). In (2010), approximately 4600000 tons sugar beet was produced in Iran (Ananymus 2011).

Rhizomania disease

Rhizomania is one of the most important and destructive diseases of sugar beet which can destroy the entire product. The disease was first reported in Iran by Izadpanah *et al*. (1996) in Fars province. The disease was subsequently reported

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in most sugar beet planting area (Toodehfallah *et al*. 2000). The casual agent, *Beet Necrotic Yellow Vein Virus* (BNYVV), is transmitted by the soil borne fungus *Polymyxa betae* (Keskin 1964). The only way to protect sugar beet in infected fields is the planting of resistant cultivars. Two rhizomania resistant genes have been identified in sugar beet which have been derived from various sources and have been named as *Rz1* and *Rz2* (Scholten and Lange 2000).

The importance of DNA markers in studying disease resistance source

Since the classical methods of disease resistance selection are based on phenotype assessment (which is dependent on environmental conditions and uniformity of the pollutant sources, occurs at certain season, and some plants escape from pollutant and become relatively resistant) using molecular methods as an alternative or a complementary method, plants carrying resistant genes can be detected in a genotypical level. Thus, DNA markers can be used as a useful tool for selection of resistant genotypes and saving the time and increasing the selection accuracy (Norouzi 2008).

Molecular markers associated with resistance genes to rhizomania

Pelsy and Merdinoglu (1996) used BSA method for identification of RAPD markers linked to rhizomania resistant genes in Holly source. From 160 primers, 19 polymorphic primers were detected and were classified in nine linkage groups. Scholten *et al*. (1997, 1999) suggested *Rz1* for Holly gene and *Rz2* for WB42 gene (s). Amiri (2003) reported that resistant in WB42 source is controlled with dominant gene (*Rz2*) and its distance from *Rz1* is 35 cM in Holly source. They found that resistant genes in Holly source and WB42 are non-allelic and continuous. Amiri (2003) used RAPD technique in F_2 population from a cross between male sterile line 261 and annual beet with resistant Holly source and WB42. He found a repulsion marker with strong linkage (3.6 cM) for *Rz2* gene derived from WB42 source and a pair of marker loci with low linkage for *Rz1* locus derived from Holly source. Lin *et al*. (2007) found that 4 analogous resistant genes cZR-7, cZR-9, cZR-1, and cZR-3 are located on chromosome 3 with relative large gene loci which separate rhizomanian resistance effect. Using similar method and RAPD marker, Nouhi *et al*. (2008) identified two markers including OF-09 with 1150 base pair in paired situation and 27 cM distance from *Rz1* and OP-AN9 with 600 base pair in repulsion situation and 13.7 cM distance from *Rz1*. Mesbah (2007) reported similar results. Using RAPD marker, Norouzi (2008) and Norouzi and Feghhi (2009) identified R1 and r2 markers in 2.32 and 8.3 cM distance from *Rz1* in repulsion phase, and C4 and C1 in 21.4 and 27.5 cM distance from *Rz1* in paired phase. The purpose of this study were 1) to evaluate the reproducibility of repulsion gene markers associated with resistant gene to rhizomania from Holly source, 2) to evaluate the resistant gene dosage through comparison of markers results with Elisa test result, and 3) to determine markers presence in commercial resistant and susceptible sugar beet cultivars for rapid germplasm assessment.

MATERIALS AND METHODS

Plant materials

Different sugar beet genotypes including breeding bulk S1-A (resistant pollinator to rhizomania derived from Shiraz bulk), S2-A (Hamedan bulk pollinators of FC-709-2), S1-B (Resistant O-types), FC (resistant monogerm and multigerm breeding bulks), pollinator 20322 (cultivar HM1990), susceptible commercial cultivars Regina, Shirin, and Rasoul, and resistant cultivars, Flores, Dorouti, Birigita, and Latitia, and tolerant cultivars Zarghan and Jaam were used to confirm six repulsion markers linked to resistant gene to rhizomania. 20-150 plants were selected in most genotypes.

Markers

RAPD repulsion markers linked to resistant gene to rhizomania (*Rz1*) including PN7-2, PN7-1, PN3, PN13-3, PN11-2, and PN10 were selected for confirmation and repeatability of them in internal breeding bulks, and also in resistant and susceptible cultivars to rhizomania. Markers duplication was performed using 10 nucleotides random primers as single or paired. After recording the sequence of these primers in gene bank, they can be used by other researchers.

Elisa test for the measurement of BNYVV virus concentration

Enzyme-linked immunosorbent assay (ELISA), especially the double antibody sandwich from (Clark & Admas 1977) was performed in Plant Pathology laboratory of Sugar Beet seed Institute in Karaj (Amiri *et al*. 2003).

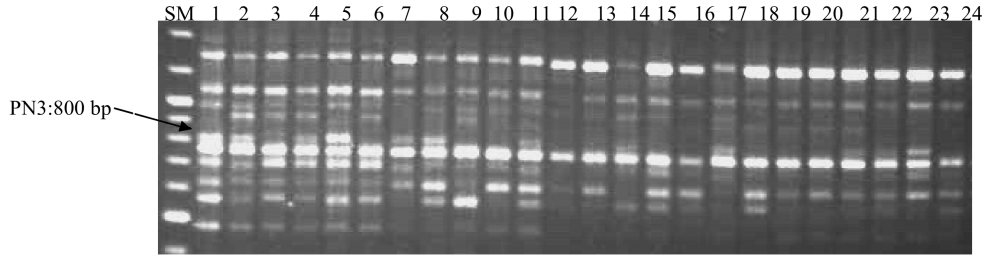

Fig. 1. Electrophoresis pattern of repulsion marker PN_3 in breeding bulks S1-A (columns 1-11) and S1-B (columns 12-24). Columns 1, 2, 5, 8, 17, and 23 are related to resistant cultivars, 3, 4, 7, 11, 14, 21, and 22 are related to resistant heterozygous plants, and 6, 9, 10, 12, 13, 15, 16, 18, 19, 20 and 24 are related to resistant homozygous cultivars. 800 base pair band (related to susceptible allele) was found in susceptible or heterozygous plants, SM = molecular marker, Lambda DNA / EcoRI + HindIII Marker.

DNA extraction

DNA was extracted using Dellaporta *et al*. (1983) method.

APD-PCR reaction

PCR was performed in a volume of 25 µl containing 1.5 µl of DNA template, 2 µl of 2.5 mM each of dNTP, 1.8 µl of 25 mM $MgCl_2$, 1 µl of 30 ng/µl of each forward and reverse primer, 2.5 µl of 10X PCR buffer and 0.2 µl SmarTaq Polymerase. The cycle parameters in the PCR program were as follows: 94°C for 5 min, 40 cycles of: 34°C for 40 sec, 72°C for 80 sec, followed by a final extension at 72°C for 10 min. PCR product was analyzed by agarose gel electrophoresis on a 2.1% agarose gel with 100 voltages and were stained with ethidium bromide and finally gel image was taken. Genotypes banding pattern was observed on gel.

Statistical analyses

To calculate the distance between marker and *Rz1* locus in repulsion phase, Barzen *et al*. (1997) method was used:

Repulsion marker distance = number of susceptible plants without band / total number of plants

Following relation was used for agreement percentage of ELISA results with molecular data:

Agreement percentage of ELISA results with molecular data = Number of samples in which molecular results had agreement with ELISA test × 100 / Total number of samples

For determination of *Rz1* gene dosage in resistant genotypes, unbalanced completely randomized design was used based on comparison of ELISA and molecular results. Because of the abnormality of ELISA results, before statistical analysis, logarithmic conversion was performed. Data were analysed by NC, Cary, and SAS software and mean values were compared. After analysis of variance and comparison of treatments based on converted data, treatments mean was returned to its original scale.

RESULTS AND DISCUSSION

ELISA results

After running ELISA test, the sample which had higher virus concentration in root (susceptible) indicated dark yellow colour in ELISA's plate and also a higher value. With the measurement of 2X and X+3sd, the above and below ruler for sample resistance evaluation was obtained. Based on Amiri *et al*. (2003) method, samples with OD higher than 2X and lower than X+3sd were considered as susceptible (S) and resistant (R), respectively. In bulks with determined ELISA results, the agreement percentage between ELISA test and RAPD was estimated and in rest of the bulks, only gene presence was determined. For selected markers confirmation in breeding bulks, the agreement percentage of marker with ELISA, and in commercial cultivars, the presence or absence of the marker is a very important issue which is brought in related tables. Results of examined markers are as follows:

Marker PN_3

Band observed in the genotypes used for this marker was about 800 bp in repulsion phase (Fig. 1). This marker was examined on breeding bulks S2, S1, FC bulks, pollinator HM1990, commercial susceptible and resistant cultivars (Table 1). The agreement percentage of the above marker with ELISA test in three breeding bulks of S1, S2 was 86 to 100%. Repulsion marker was not found in resistant commercial cultivar Flores and possible reason was the presence of dominant homozygous gene *Rz1* in this cultivar.

Table 1. Repulsion marker PN_3 in different sugar beet genotypes

Row	Genotype	Number of samples	Agreement with ELISA (%)	Marker presence (%)
1	Breeding bulks S1-A	120	92	…
2	Breeding bulks S2-A	73	100	…
3	Breeding bulks S1-B	116	86	…
4	Breeding bulks FC	691	…	78
5	Pollinator HM1990	167	…	79
6	Flores cultivar	15	…	0
7	External resistant commercial cultivar*	101	…	75
8	Commercial cultivars Zarghan and Jaam	22	…	91
9	Susceptible commercial cultivar**	75	…	87

*commercial resistant cultivar: Flores, Dorouti, Brigita, and Latitia
**Susceptible commercial cultivar: Rasoul, Shirin, and Regina

Table 2. Repeatability results of repulsion marker PN7-1 in different sugar beet genotypes

Row	Genotype	Number of plant samples	Agreement with ELISA (%)	Marker presence (%)
1	Breeding bulks S1-A	60	95	…
2	Breeding bulks S2-A	41	83	…
3	Breeding bulks S1-B	81	99	…
4	Breeding bulks FC	16	…	69
5	External resistant commercial cultivar*	38	…	40
6	Commercial cultivars Zarghan and Jaam	13	…	46
7	Susceptible commercial cultivar**	38	…	76

*commercial resistant cultivar: Flores, Dorouti, Brigita, and Latitia
**Susceptible commercial cultivar: Rasoul, Shirin, and Regina

Table 3. Repeatability results of repulsion marker PN7-2 in different sugar beet genotypes

Row	Genotype	Number of samples	Agreement with ELISA (%)	Marker presence (%)
1	Breeding bulks S1-A	90	98	…
2	Breeding bulks S2-A	41	100	…
3	Breeding bulks S1-B	98	97	…
4	Breeding bulks FC	16	…	100
5	External resistant commercial cultivar*	65	…	97
6	Commercial cultivars Zarghan and Jaam	13	…	85
7	Susceptible commercial cultivar**	48	…	90

*commercial resistant cultivar: Flores, Dorouti, Brigita, and Latitia
**Susceptible commercial cultivar: Rasoul, Shirin, and Regina

Table 4. Repeatability results of repulsion marker PN10 in different sugar beet genotypes

Row	Genotype	Number of samples	Agreement with ELISA (%)	Marker presence (%)
1	Breeding bulks S1-A	30	90	…
2	Breeding bulks S2-A	10	90	…
3	Resistant cultivar Brigita	6	…	83
4	Susceptible cultivar Rasoul	5	…	60

PN7 marker

1280 base pair repulsion (PN7-1 marker) and 1350 base pair repulsion (PN7-2) bands were detected for PN7 marker (Table 2 and 3) and Figs. 2 and 3 show the banding pattern.

Marker PN10

900 base pair repulsion band was observed. This marker was used in breeding bulks S1, S2, commercial susceptible cultivar Rasoul, and resistant Brigita (Table 4).

Marker PN11-2

For this marker 500 bp repulsion band was found in breeding bulks S1, S2, susceptible commercial cultivar Rasoul, and resistant Brigita (Table 5).

Marker PN13-3

830 base pair repulsion band was observed. This marker was used in breeding bulks S1, S2, FC bulk, susceptible and resistant cultivars (Table 6).

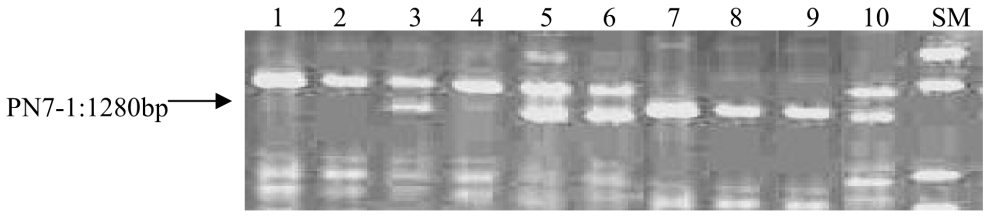

Fig. 2. Electrophoresis pattern of repulsion marker PN7-1 in 10 samples of S1-A, SM = molecular marker, Lambda DNA / EcoRI + HindIII Marker.

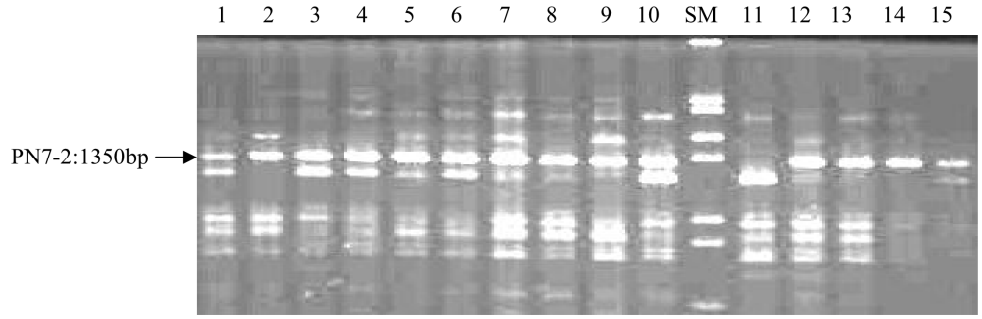

Fig 3. Electrophoresis pattern of repulsion marker PN7-2 in 15 samples of S1-A, SM = molecular marker, Lambda DNA / EcoRI + HindIII Marker.

Table 5. Repeatability results of repulsion marker PN11-2 in different sugar beet genotypes

Row	Genotype	Number of samples	Agreement with ELISA (%)	Marker presence (%)
1	Breeding bulks S1-A	17	76	…
2	Breeding bulks S2-A	10	80	…
3	Resistant cultivar Brigita	13	…	100
4	Susceptible cultivar Rasoul	8	…	100

Table 6. Repeatability results of repulsion marker PN13-3 in different sugar beet genotypes

Row	Genotype	Number of samples	Agreement with ELISA (%)	Marker presence (%)
1	Breeding bulks S1-A	16	88	…
2	Breeding bulks S2-A	33	97	…
3	Breeding bulks S1-B	4	100	…
4	Breeding bulks FC	15	…	73
5	Resistant cultivar Brigita	47	…	85
6	Susceptible cultivar Rasoul	44	…	59

*commercial resistant cultivar: Flores, Dorouti, Brigita, and Latitia
**Susceptible commercial cultivar: Rasoul, Shirin, and Regina

Summary of tested repulsion markers

The overall results of the markers tested are summarized in Table 7. For each marker, the total agreement of marker with ELISA results and the percentage of marker presence in resistant and susceptible commercial cultivars are indicated.

Using RAPD markers and BSA technique, Amiri (2003) identified molecular markers linked with rhizomania resistance genes. He identified a paired marker with low linkage for resistant source Holly and a repulsion marker with high linkage for resistant source WB42 with 3.6 cM distance from *Rz2* locus. In similar method applied by Nouhi *et al*. (2008), marker OF-09 with 1150 bp in paired condition and 27 cM distance from *Rz1* and marker OP-AN9 with 600 bp in repulsion phase and 13.7 cM distance from *Rz1* gene were identified which were similar to Mesbah (2007) results. Norouzi and Feghhi (2009) identified markers R1 and R2 in 2.32 and 8.3 cM distance from *Rz1* gene in repulsion phase and markers C4 and C1 in 21.4 and 27.5 cM distance from *Rz1* gene, respectively in paired phase. However, none of these researchers have applied the above markers for high number of samples. Therefore, in this study, main markers linked with *Rz1* locus were evaluated and their repeatability in several breeding bulks and resistant and susceptible cultivars to rhizomania was studied. Some of them were confirmed and most of them produced other markers than the primary ones. In study by Giorio *et al*. (1997), they

Table 7. Summary of the repulsion molecular markers confirmation linked with *Rz1* gene

Row	Marker	Marker size (bp)	Total agreement with ELISA (%)	Marker presence (%)	
				Susceptible cultivar	Resistant cultivar
1	PN3	800	92	87	75
2	PN7-1	1280	94	76	40
3	PN7-2	1350	98	90	97
4	PN10	900	90	60	83
5	PN11-2	500	78	100	100
6	PN13-3	830	94	59	85

Table 8. Average ELISA OD result in resistant homozygous and heterozygous plants to rhizomania for selected repulsion markers.

Marker	Average ELISA absorption of *Rz1Rz1* plants	Average ELISA absorption of *Rz1rz1* plants
PN3	0.097[a]	0.142[b]
PN7-1	0.089[a]	0.135[b]
PN7-2	0.093[a]	0.136[b]
PN13-3	0.125[a]	0.225[b]

Cultivars with same alphabet had no significant difference ($P < 0.05$)

used 10 RAPD markers linked with resistant gene Holly (Barzen *et al*. 1997) in a separating bulk and confirmed that only 6 of 10 markers were confirmed in their bulk with repeatability. Using similar materials, Grimmer *et al*. (2007) reported that only one marker had repeatability in their bulk which was confirmed and other markers did not confirm previous reported distances. The reason for this disapproval was low repeatability of RAPD bands and also difference in the genetic background of different studies which can affect the banding pattern of RAPD. However, this study identified a large number of repulsion markers linked with *Rz1* gene which can be used in breeding bulks and commercial cultivars in Sugar Beet Seed Institute. Confidence coefficient obtained by each of the markers can be considered as a total agreement of marker with ELISA test. However, it is needless to say that the confirmation of these markers was based on several breeding bulks average and resistant and susceptible commercial cultivars of sugar beet and in fact, for all markers in all genotypes, similar agreement results or presence was not achieved. Thus, it is suggested that in future studies, the linkage of selected molecular markers in this study with number of genotypes that have pollution score in field be evaluated in order to identify the best molecular markers linked with resistant gene to rhizomania. Secondly, for the selection of each breeding bulks, markers having the highest percentage of correlation with resistance results (based on ELISA test or pollution score in field) should be used. Thirdly, the markers introduced in the future have to be converted to SCAR markers which have more specific annealing sites, high repeatability, and simplicity for use in molecular markers labs. Therefore, in this way, markers with higher practical value can be identified to reduce the time and cost in production process of rhizomania resistant cultivars and increase the single plant selection efficiency.

The effect of rhizomania resistant gene dosage

In repulsion markers, resistant homozygous plant (*Rz1Rz1*) is identified without band, and resistant heterozygous (*Rz1rz1*) and susceptible (*rz1rz1*) with band. With comparison of ELISA test and molecular test, susceptible plants (*rz1rz1*) can be identified from resistant heterozygous (*Rz1rz1*) plants. In this study average ELISA OD was estimated for each (*Rz1rz1*) and (*Rz1Rz1*) genotypes which have been tested for molecular marker RAPD with selected repulsion markers PN3, PN7-1, PN7-2, and PN13-3. Average ELISA OD of the plants with heterozygous genotype (*Rz1rz1*) was higher than plants with dominant homozygous genotype (*Rz1Rz1*) in all repulsion markers. Therefore, it is likely that the higher resistance of dominant homozygous plants (*Rz1Rz1*) compared with heterozygous plants (*Rz1rz1*) is owing to *Rz1* allele dosage which demands more studies in higher polluted condition.

Scholten *et al*. (1996) reported the effect of Holly gene as complete dominance and showed that in F_1 plants derived from a cross between a completely susceptible plant with resistant homozygous plant, heterozygous and dominant homozygous plants had similar virus absorption and thus did not confirm gene dosage effect. Wisler *et al*. (1999) reported that diploid *Rzrz* had lower ELISA absorption compared with *Rzrzrz*. They also

reported that *Rz* allele dosage (number of *Rz* alleles in a genotype or individual) and its frequency (*Rz*/*rz* ratio) are the main factors influencing total yield of sugar beet under rhizomania pollution. Ghanbari *et al*. (2007) did not confirm *Rz2* gene dosage in field and reported low experimental field pollution and also one diploid level usage in their study as the main reasons. They suggested for gene dosage determination, more studies needed in heavily polluted field and usage of different ploidy such as triploid and tetraploid is recommended. Nouhi *et al*. (2009) differentiated dominant homozygous genotypes from heterozygous via applying a repulsion marker data and ELISA absorption in F_2 bulk and obtained similar ELISA absorption for these genotypes. They concluded that *Rz1* gene dosage had no effect on resistance to rhizomania. However, it seems that due to higher number of dominant homozygous compared with heterozygous (two times) this result achieved. According to Wisler *et al*. (1999), resistance to rhizomania in most commercial cultivars is owing to dominant allele *Rz* and a number of factors that affect *Rz* gene expression. Therefore, the disagreement about *Rz* gene dosage effect in different studies is due to the above fact.

CONCLUSION

In this study, markers from previous studies were evaluated on some breeding bulks and susceptible and resistant commercial cultivars to rhizomania from Holly source. Markers were confirmed with different degrees in which repulsion markers PN3 and PN7-2 with agreement percentage of 92 and 98% with ELISA results, and 87 and 90% presence in susceptible commercial cultivars and 75 and 97% presence in resistant commercial cultivars are the appropriate markers for identification of *rz1* susceptible allele. Thus with absence of those repulsion markers in sugar beet genotypes, most likely dominant homozygous can be identified.

ACKNOWLEDGEMENT

The management of Sugar Beet Seed Institute are highly appreciated for providing the facilities of this study.

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