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RESEARCH ARTICLE

PUNGENCY GENOTYPING OF COMMERCIALLY CULTIVATED CHILLI PEPPER *CAPSICUM* SPECIES USING *MAP1* AND *CSY-1* MOLECULAR MARKERS

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ABSTRACT

Pungency genotyping of commercially cultivated chilli peppers of *Capsicum* species seggregate in to pungent and non-pungent at seed level itself using *MAP1* and *Csy-1* molecular markers.

MAP1: A single DNA band admeasuring 494 bp was observed in 10 commercial chilli peppers, *C. annuum* Pragna, Teja, LCA-335, Special bullet, G-3, G-273, G-4, Badiga-1 and G-5 and *C.chinense* variety Kotpar. A single DNA band admeasuring 479 bp was observed in *C.annuum* varieties California wonder and Yolo wonder (positive control) using *Pun1* allel specfic primer *MAP1*.

***Csy 1*:** A single DNA band admeasuring 981 bp was observed in 10 pungent chilli peppers, *C. annuum* Pragna, Teja, LCA-335, Special bullet, G-3, G-273, G-4, Badiga-1 and G-5 and *C.chinense* variety Kotpar. The DNA band corresponding to 981 bp was absent in *C.annuum* varieties California wonder and Yolo wonder (positive control) using capsaicin synthase gene *Csy1* primer.

INTRODUCTION

Chilli pepper (*Capsicum* sp.) belonging to Solanaceae family, originated in Americas, then spread to various continents (Bosland *et al.,* 1996). Capsaicin is a secondary metabolite these component which adds pungency, aroma, taste, flavour and colour to food items. It has vast medicinal value, and with the discovery that alkaloid Capsaicin is endowed with anti-cancer properties, values of capsaicin in medicin has increased significantly. Chilli pepper cultivation is an important agribusiness for individual countries or localized geographic areas (Andrews, 1984) and thousands of farmers depend on a successful harvest. Export market has motivated commercial farming of highly / extremely pungent chilli pepper (Reifschnedier and Ribeiro, 2008) India export chilli pepper to Malaysia, Sri Lanka, Bangladesh, USA, UAE. During 2010-2011, India exported chilli pepper products like powders and flakes upto a amount of 2.40 lakh tonnes valued Rs.1,536 crores ($ 2,56,000) and earned precious foreign exchange. Chilli pepper cultivation in Andhra Pradesh and Telangana States in India is highly profitable. *C.annuum* varieties G4, G5, G-334, G-002, G-311, G3, G-3, LCA-334, LCA-353, LCA-335, LCA-331, G-273, Pragna, Teja, Agnirekha etc, are still ruling varieties in local area may be because of suitable agroclimatic conditions, active domestic, State, National markets and export market.

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The demand for India chilli pepper is expected to increase both at national and international market, with the discovery that worlds hottest chilli pepper is growing in Tejpur of Assam, India. Low to highly pungent Chilli pepper varieties are cultivated in various parts of Indian States; they belong to *C.annuum* species. For example *C.annuum* variety Teja and Special bullet is highly pungent. While *C. annuum* variety California wonder is zero pungent and extremely pungent chilli peppers is cultivated in North Eastern States viz., Nagaland, Mizoram and Assam etc; they belong to *C.chinense* and/or natural hybrids of *C.chinense* X *C.frutescens*. For example, the pungency of *C.chinense* cultivar Nagahari from Nagaland was about 855,000 SHU (Sanatombi *et al.,* 2010). Therefore, Indian chilli pepper, have good export potential provided they conform to the pungency level prescribed by importing countries, and follow the test guidelines for pepper pungency developed by International Union for the protection of new varieties of plants (UPOV). Application of DNA based molecular markers associated with pungency trait is an efficient strategy for discrimination of pungent chilli pepper varieties from non-pungent varieties. Moreover, it allows detection of pungency trait at early developmental stages (cotyledon, hypocotyls and leaf tissues) and *in vitro* cultures, even before fruit setting; it was not effect by environmental conditions (Rodriquez *et al.,* 2012). Molecular markers designed for pungency assessment in fruits using CAPS, CD35 and SCARs markers by (Blum *et al.,* 2002). Recently Prasad *et al.* (2006), purified and characterized Capsaicin Synthase, the key enzyme involved in capsaicin biosynthesis in fruit

tissue of chilli pepper (*Capsicum* sp.). They characterized Capsaicin Synthase (CS) enzyme and identified its gene *Csy1* for pungency factor, capsaicin. Using RT-PCR method, observed that the significant differences in *Cys1* gene transcript levels of pungency phenotypes, correlated with capsaicin content. Different types of molecular marker associated with pungency that is Pun1 locus have been developed. Ben-Chaim *et al.* (2001) reported a tomato RFLP marker, CD35 (Tanksley *et al.* 1988) linked to Pun1 locus. Blum *et al.* (2002) identified of 3 RFLPs linked to Pun 1 locus. A linkage map for F1 hybrid between *C.annuum* K9-11(non-pungent) and *C.chinense* ACC2258 (pungent) was constructed (Sugita *et al.,* 2005). Stewart *et al.,* (2005) reported that the development of 5 SCARs markers based on deletion in Pun1 locus in non-pungent chilli pepper genotypes. A SNP marker of a sequence tag of 307 bp related to pungency, from another locus other than Pun 1 was developed (Garces-Claver *et al.,* 2007). QTL analysis for pungency trait was conducted in chilli pepper. Blum *et al.* (2002) identified a major QTL termed CAP on chromosome 7 affecting capsaicinoids content. Ben-Chaim *et al.* (2001) reported 6 QTLs associated with pungency trait localized on 3,4 and 7 chromosomes. Paran *et al.* (2004) reported a new QTL regulating capsaicinoids, content localized on chromosome 7 and 8. Candidate genes pertaining to capsaicinoids biosynthesis have been localized on chilli pepper chromosomes (Mazourek *et al.,* 2009, Blum *et al.,* 2003; Steward *et al.,* 2005). Rodriquez *et al.* (1999) developed a single and universal versatile PCR marker called *MAP 1* associated with pungency in *Capsicum.* The present paper report on detection/segregation of pungent and non-pungent in 12 commercial chilli peppers, *C. annuum* varieties G-3, G-4, G-5, G-273, LCA-335 (G and LCA-series), Pragna and Teja (W-series), Badiga-1, Special bullet and *C. chinense* variety Kotpar at seed level using pungency associated molecular markers *MAP1* and *Csy1*.

MATERIAL AND METHODS

Seed of *Capsicum annuum* L. varieties G-3, G-4, G-5, G-273 and LCA-335 obtained from Agricultural Research Station (ARS), LAM, Guntur, Andhra Pradesh, India, *C. annuum* L. varieties Pragna and Teja obtained from ARS, Malyal, Warangal, Telangana State, India. *C. annuum* L. variety Badiga-1 obtained from Seed Stores, Karnataka, *C. annuum* L. variety Special bullet obtained from S.K. Nursery and Seed Company, Parganas (North), West Bengal, India, *C. annuum* L. California wonder and Yolo wonder (positive controls) obtained from Pocha Agro Science (P) Ltd, Bhalaswa, New Delhi, India and *Capsicum chinense* Jacq. variety Kotpar obtained from Tezpur, Assam, India, cultivated in local area and recently introduced were used in this study. Seeds of these varieties were used for genomic DNA and PCR based amplification with *MAP1, Csy-1* Primer.

DNA extraction

DNA was extracted from seeds of the above mentioned 11 *C. annuum* L. varieties and 1 variety of *C.chinense*, using Doyle and Doyle (1990) method. One day socked seeds (20 seeds from each variety) are used to isolated genomic DNA form above mentioned chilli pepper varieties. Seeds was grinded with a pre-cold treated pestle and mortar in 0.350 ml extraction solution (40 mM EDTA, 0.1 M NaCl and (pH 8.0), 50 mM Tris-HCl (pH 8.0) and 1. % mercaptoethanol). The sample was incubated at 45°C for 25 min in dry bath. After incubation, the sample was cooled to room temperature later chloroform and isoamyl alcohol (24:1) was added, centrifuged at 8000 rpm for 8 min. Collected supernatant was mixed with 2:2 ml of isopropyl alcohol and incubated for 8 min at -4 °C. After centrifugation, the pellet was dried, washed and dissolved in 1% TBE buffer and was used in *MAP1* and *Csy-1*-PCR amplification.

MAP1 and Csy1-PCR amplification

MAP1-PCR amplification was performed by the method of Rodriguez *et al.,* (2012). Allele specific primer *Pun1 MAP1F* (5' CCAGTCGTTCATTTTTGTTTG 3') and *MAP1R* (5' TCTGCCCTTGTTGGATTTTC3') were used, reaction mixture and working condition method fallowed by Prasad *et al.* (2013). *Csy1*-PCR amplification was performed by the method of Prasad *et al.* (2006). In this experiment, *Csy1* forward and *Csy1* revere primers given in Table 1. The PCR reactions were setup in a 20 µl given in table 2. Amplification was performed on a thermal cycler (Model No. TC-3000 Techne, USA) with a programme given in table 3 and finally the amplification produced was stored at -20°C.

Table 1. *Csy1* primer used in PCR amplification of genomic DNA in *C. annuum* L. 11 varieties *and C. chinense* Jacq. 01 variety

S. No	Primer Code	Base Sequence	Annealing temperature (°C)
1	*Csy1* F	5'ATGTTGCTGGAAATCAGTTGTCCG 3'	56
2	*Csy1* R	5' TTGACCGTAAACTTCCGTTG 3'	50

Table 2. PCR mixture used for amplification of *Csy*1 with genomic DNA of *C. annuum* L. 11 *varieties* & *C. chinense* Jacq. 01 variety.

S. No	Chemical	Quantity (µl)
1	35 ng of genomic DNA	2.0
2	10mM dNTPs	1.5
3	Taq buffer(100 mM Tris HCl , pH 8.3, 500 mM KCl and 0.1% gelatin)	4.0
4	25mM $Mgcl_2$	0.5
5	*Csy1* & *Csy2* primers (Table 15)	2.0
6	0.5 unit of Taq DNA polymerase	1.0
7	Autoclaved Milli Q water (PCR-grade water)	9.0
	Total	20.0

Table 3. The programming conditions of thermocycler for *Csy*1-PCR amplification of *C. annuum* L. 11 *varieties* and *C. chinense* Jacq. 01 variety.

Steps	Temp(^{O}C)	Duration(min.)	Cycles(No.)
Initial denaturation	94	4.0	1.0
Denaturation	94	1.0	38.0
Annealing	53	1.0	38.0
Extension	72	2.0	38.0
Final extension	72	10.0	1.0

PCR Mixture and Conditions

Preparation of PCR mixture

PCR mixture was prepared using the following chemicals (Table 2). PCR amplification was carried out in a thermocycler. The programming conditions are given in Table 3.

RESULTS

Genomic DNA was isolated from seeds of eleven chilli pepper *C. annuum* Pragna, Teja, LCA-335, Special bullet, G-3, G-273, G-4, Badiga-1, G-5, California wonder and Yolo wonder and one *C. chinense* variety Kotpar and PCR amplification was carried out using *Pun1* allelle specific primer *MAP1* and capsaicin synthase gene specific primer *Csy1*.

Pungency genotyping in fruit tissue with *MAP1*

A single DNA band admeasuring 494 bp was observed in 10 commercial chilli peppers, *C. annuum* Pragna, Teja, LCA-335, Special bullet, G-3, G-273, G-4, Badiga-1 and G-5 and *C.chinense* variety Kotpar. A single DNA band admeasuring 479 bp was observed in *C.annuum* varieties California wonder and Yolo wonder (positive control) (Fig.1 A).

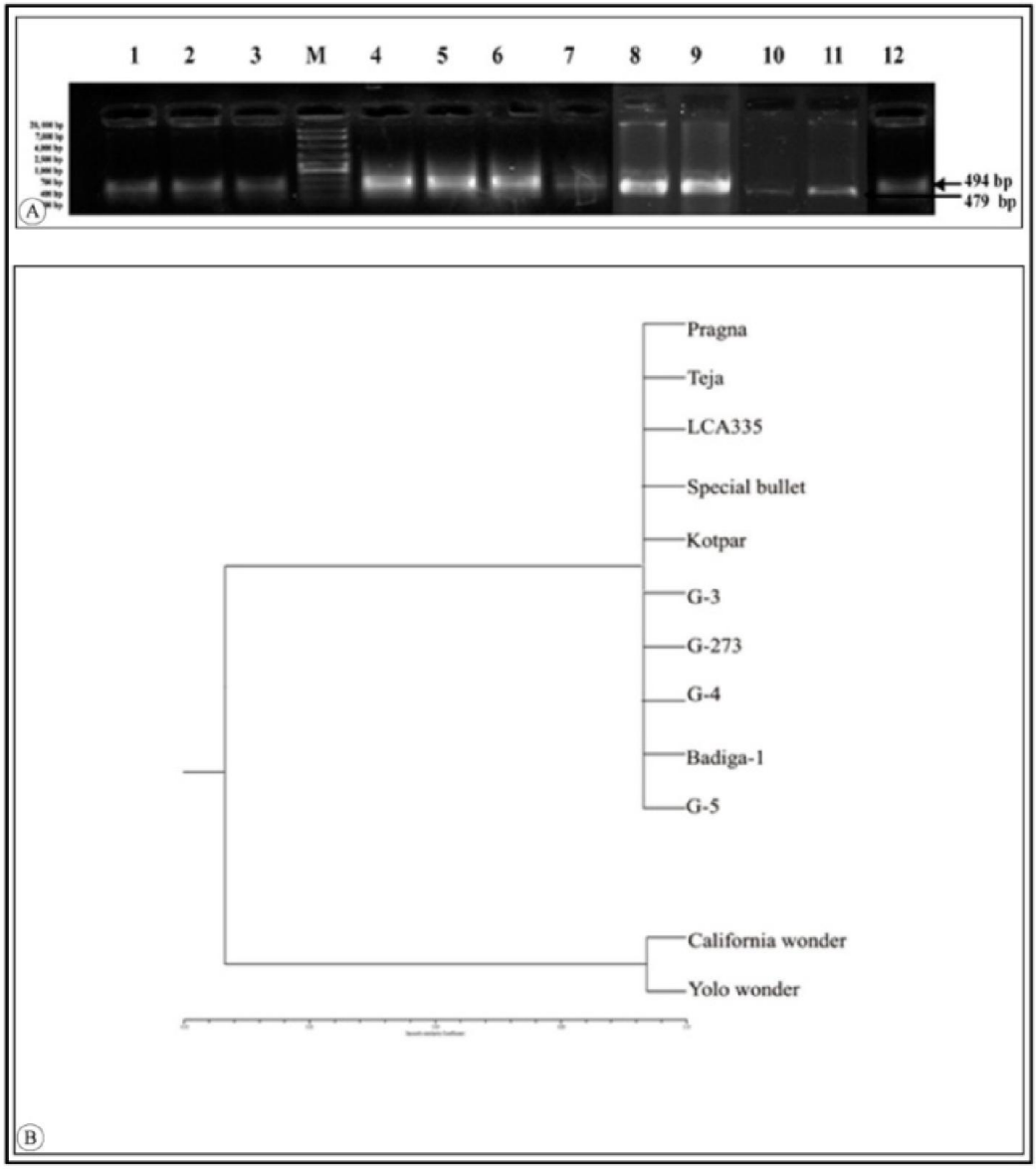

Fig.1. A. DNA profile in *C.annuum* L. 09 varieties, *C. chinense* Jacq 01 variety and *C. annuum* L. varieties California wonder and Yolo wonder (positive control), amplified with *Pun1* allele specific primer *MAP1*. Lane 1: Pragna, Lane 2: Teja, Lane 3: Badiga-1, Lane M: Marker, Lane 4: G-5, Lane 5: LCA-335, Lane 6: G-3, Lane 7: G-273, Lane 8: Special bullet, Lane 9: G-4, Lane 10: Kotpar, Lane 11: Yolo wonder, Lane 12: California wonder. B. Dendrogram constructed using *Pun1* allele specific primer *MAP1* in *C.annuum* L. 09 varieties, *C. chinense* Jacq. 01 variety and *C. annuum* L. varieties California wonder and Yolo wonder (positive control)

Dendrogram analysis of *MAP1*-PCR

Dendrogram results showed that all the 10 chilli peppers *C. annuum* Pragna, Teja, LCA-335, Special bullet, G-3, G-273, G-4, Badiga-1 and G-5 and *C. chinense* variety Kotpar grouped into a cluster. While *C. annuum* varieties Californa wonder and Yolo wonder which are postive controls separated into a cluster. (Fig.1 B).

Dendrogram analysis of *Csy-1*-PCR

Dendrogram results showed that all the 10 chilli peppers *C. annuum* Pragna, Teja, LCA-335, Special bullet, G-3, G-273, G-4, Badiga-1 and G-5 and *C. chinense* variety Kotpar grouped into a cluster. *C. annuum* varieties Californa wonder and Yolo wonder (postive control) separated into a cluster. (Fig.2 B).

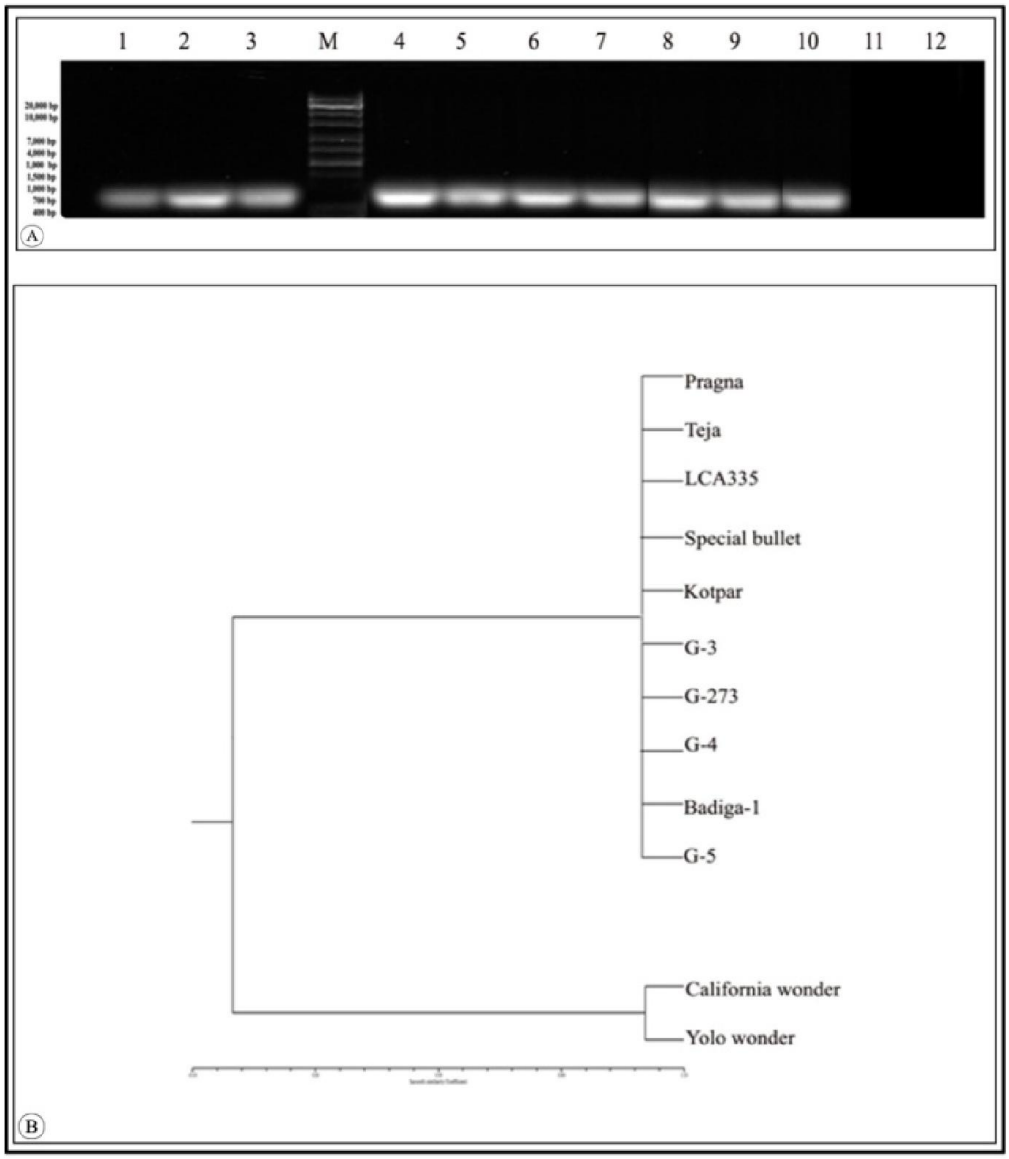

Fig.2. A: DNA profile in *C.annuum* L. 09 varieties, *C. chinense* Jacq 01 variety and *C. annuum* L. varieties California wonder and Yolo wonder (positive control), amplified with capsaicin synthase gene specific primer *Csy1*. Lane 1: Pragna, Lane 2: Teja, Lane 3: Badiga-1, Lane M: Marker, Lane 4: G-5, Lane 5: LCA-335, Lane 6: G-3, Lane 7: G-273, Lane 8: Special bullet, Lane 9: G-4, Lane 10: Kotpar, Lane 11: Yolo wonder, Lane 12: California wonder. B. Dendrogram constructed using Capsaicin synthase gene specific primer *Csy1* in *C.annuum* L. 09 varieties, *C. chinense* Jacq. 01 variety and *C. annuum* L. varieties California wonder andYolo wonder (positive control)

Pungency genotyping in fruit tissue with *Csy-1*

A single DNA band admeasuring 981 bp was observed in 10 pungent chilli peppers, *C. annuum* Pragna, Teja, LCA-335, Special bullet, G-3, G-273, G-4, Badiga-1 and G-5 and *C.chinense* variety Kotpar. The DNA band corresponding to 981 bp was absent in *C.annuum* varieties California wonder and Yolo wonder (positive control). (Fig.2 A).

DISCUSSION

In this study, we develop detection/segregation of pungent and non-pungent in 12 commercial chilli peppers,*C. annuum* varieties G-3, G-4, G-5, G-273, LCA-335 (G and LCA-series), Pragna and Teja (W-series), Badiga-1, Special bullet and *C. chinense* variety Kotpar, using pungency genotyping molecular markers viz., *Pun 1* allele specific primer *MAP1* and *Capsaicin synthase gene Csy1* primer. Pungency genotyping in chilli

pepper varieties cultivated in local area was achieved using *MAP1* and *Csy1* primers. When *MAP1* primer was used, a 494 bp DNA band was observed in pungent chilli peppers viz., *C. annuum* Pragna, Teja, LCA-335, Special bullet, G-3, G-273, G-4, Badiga-1 and G-5 and *C.chinense* variety Kotpar and a 479 bp DNA band was observed in non-pungent chilli peppers viz., *C.annuum* varieties California wonder and Yolo wonder. Similarly when *Csy 1* primer was used, a 981 bp DNA fragment was observed in pungent chilli peppers viz., *C. annuum* Pragna, Teja, LCA-335, Special bullet, G-3, G-273, G-4, Badiga-1 and G-5 and *C.chinense* variety Kotpar and the DNA fragment was absent in non-pungent chilli peppers viz., *C.annuum* varieties California wonder and Yolo wonder. Rodriguez *et al.,* (1999) developed a universal PCR marker, *MAP1* which precisely distinguished pungent chilli pepper accessions (494 bp fragment) from non-pungent chilli pepper accessions (479 bp fragment). Pickersgill, (2007) reported that in *Pun1* locus, polymorphism might have occurred within the intron and this variation ought to have to occurred early in evolution and is maintained through the species. *MAP1* is a robust, reliable, co-dominant and one step PCR marker for use in large scale breeding program with the objective of transfer of pungency traits in to chilli pepper. Prasad *et al.* (2006) identified the gene for capsaicin synthase gene 981 bp length in fruit tissue of *C. frutescens* and *C. annuum* genotypes. Kim *et al.* (2001) reported the development of C-DNA clones SB2-66 and *Pun1* the gene of capsaicin synthase that expressed in placental tissues.

Conclusion

We have utilized pungency associated markers *MAP1* and *Csy1* for detection/segregation of pungent and non-pungent in 12 commercial chilli peppers. This procedure is useful for early pungency genotyping in export quality chilli peppers and also used in plant breeding program.

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