A Putative Gene sbe3-rs for Resistant Starch Mutated from SBE3 for Starch Branching Enzyme in Rice (Oryza sativa L.)

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Abstract
Foods high in resistant starch (RS) are beneficial to prevent various diseases including diabetes, colon cancers, diarrhea and chronic renal or hepatic diseases. Elevated RS in rice is important for public health since rice is a staple food for half of the world population. A japonica mutant ‘Jiangtangdao 1’ (RS = 11.67%) was crossed with an indica cultivar ‘Miyang 23’ (RS = 0.41%). The mutant sbe3-rs that explained 60.4% of RS variation was mapped between RM6611 and RM13366 on chromosome 2 (LOD = 36) using 178 F2 plants genotyped with 106 genome-wide polymorphic SSR markers. Using 656 plants from four F3:4 families, sbe3-rs was fine mapped to a 573.3 Kb region between InDel 2 and InDel 6 using one STS, five SSRs and seven InDel markers. SBE3 which codes for starch branching enzyme was identified as a candidate gene within the putative region. Nine pairs of primers covering 22 exons were designed to sequence genomic DNA of the wild type for SBE3 and the mutant for sbe3-rs comparatively. Sequence analysis identified a missense mutation site where Leu-599 of the wild was changed to Pro-599 of the mutant in the SBE3 coding region. Because the point mutation resulted in the loss of a restriction enzyme site, sbe3-rs was not digested by a CAPS marker for Spel site while SBE3 was. Co-segregation of the digestion pattern with RS content among 178 F2 plants further supported sbe3-rs responsible for RS in rice. As a result, the CAPS marker could be used in marker-assisted breeding to develop rice cultivars with elevated RS which is otherwise difficult to accurately assess in crops. Transgenic technology should be employed for a definitive conclusion of the sbe3-rs.

Introduction
Starch is the major dietary source of carbohydrates which is composed of two types of molecules, amylase (Am) and amylopectin (Ap) [1,2]. Ap is essentially a linear molecule composed of α(1,4)-linked glucosidic chains, whereas Am is a highly branched glucan with α(1,6) glucosidic bonds for a connection of linear chains [3]. Based on the characteristics of enzymatic digestion, starch can be classified into rapidly digestible starch (RDS), slowly digestible starch (SDS) and resistant starch (RS) [4,5]. RS is a small fraction of starch resistant to hydrolysis by exhaustive α-amylase and pullulanase treatment in vitro [5], and is defined as “the sum of starch and products of starch degradation not absorbed in the small intestine of healthy individuals” [6,7].

RS has similar physiological functions to those of dietary fiber, and is completely resistant to enzymatic digestion in the human small intestine. As a result, RS reaches the large intestine where it acts as substrate for fermentation by microflora [8]. Short-chain fatty acids (SCFA) are major end products of the fermentation, and these acids are able to promote optimal function of the viscera [9–11]. Increasing RS consumption is becoming an effective means to improve nutrition for public health. Foods high in RS have the potential to prevent pathogen infections and diarrhea with benefits in various aspects, such as inflammatory bowel disease [12], colon cancer risk [13], insulin resistance and diabetes [14], and chronic renal or hepatic disease [15]. Diet-related noninfectious chronic diseases including coronary heart disease, certain cancers (especially of the colon and rectum), and diabetes are major causes of morbidity and mortality world wide [10]. These unique physical functions of RS have received increasing attention from plant researchers in recent years.

As the primary dietary source of carbohydrates in the world, rice (Oryza sativa L.) plays an important role in meeting energy requirements and nutrient intake in cereal crops. However, RS content is generally under 3% in hot cooked rice cultivars, which is not enough to confer the associated health benefits [16,17]. Therefore, many studies have focused on an elevation of RS content in rice cultivars using mutation breeding and bioengi-

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neering. Many mutants with elevated RS content have been identified in rice, including Goami 2 [18], RS111 [19], and ‘Jiangtangdao 1’ [20]. ‘Teqing Resistant Starch’ (TRS) is another high amylose and RS transgenic line developed by modifying antisense RNA inhibition for starch branching enzymes (SBE) in rice [21]. Similarly, high RS mutants have been reported in wheat (Triticum durum L.) [22], maize (Zea mays L.) [11] and barley (Hordeum vulgare L.) [23].

Determination of starch digestibility is difficult, which has greatly hindered breeding progress for improving RS in crops. Ideally, the amount of RS is determined in vivo using techniques such as the human ileostomy model or intubation [24]. However, these approaches are problematic for various reasons, notably because they are laborious or invasive and expensive. Sometimes, the humans involved in the experiments may be at risk. In fact, such in vivo techniques are neither feasible nor practical in many laboratories. Therefore, most analysis of RS is conducted in vitro to spectrophotometrically determine the RS after enzymatic reaction. However, the physiological relevance of in vitro determined RS to in vivo determined RS is questionable [24,25]. There are some evidences to show that in vitro assays do not quantitatively measure RS as the in vivo assays do [26,27].

Analytical difficulties for determining RS could be overcome with the use of marker-assisted selection in breeding programs. Breeding materials in the early generations can be screened with the appropriate RS markers, and in vitro analysis for RS using enzymatic reactions can be applied in the later generations. Before a high RS cultivar is released, in vivo experiments should be carried out for verification. However, limited information is available for RS related genes or QTLs in crops with only one report in rice [28]. Subsequently, a F2 population was used for primary mapping of putative quantitative trait loci (QTL) for RS. In this study, seven polymorphic InDel markers were identified from Jiangtangdao 1 and Miyang 23. The identified markers were employed for primary mapping using the F2 population.

Materials and Methods

Plant materials

The mutant Jiangtangdao 1, which is high in RS content, was identified from a doubled haploid (DH) population derived from ‘Huaqingdao’, an early-maturing japonica cultivar [20]. Jiangtangdao means reducing-sugar rice in Chinese. The mutagenesis using 0.015% of N-methyl-nitrosourea (NMU) solution was performed on young panicles of Huaqingdao eight hours after fertilization for 45 min. Following the mutagenesis, anther culture was carried out for regeneration [30]. From each F2 individual, leaf tissue was harvested for genotyping and seeds were harvested for phenotyping of RS. Both genotyping and phenotyping of the F2 population were used for primary mapping of putative quantitative trait loci (QTL) for RS. Subsequently, a F2 plant heterozygous in the target region identified in the primary mapping was selected to generate four F3.4 family populations for fine mapping.

Measurement of amylose and resistant starch

Apparent amylose content (AAC) was determined using a previously described colorimetric method with potassium iodide [31]. RS was measured according to the method of Megazyme RS assay kit (Megazyme, Co. Wicklow, Ireland), which has been widely used for RS determination in crops [32]. The grain sample was treated with 10 mg/ml pancreatic α-amylase and 3 U/ml amyloglucosidase (AMG) enzymes for hydrolysis and solubilization of non-resistant starch. After the enzymatic reaction was terminated by adding a 99% ethanol solution, RS was recovered as a pellet by centrifugation (approx. 3,000 rpm, 10 min). RS in the pellet was dissolved in 2 M KOH before the reacted solution was repeatedly washed and decanted. Then, starch in the solution was quantitatively hydrolyzed to glucose with AMG. D-glucose was measured with glucose oxidase/peroxidase (GOPOD) reagent at 510 nm wavelength against the reagent blank. All analyses were repeated three times for error control.

DNA extraction, SSR primers selection and PCR

Total genomic DNA was extracted from fresh leaves of each offspring plant using the cetyltrimethylammonium bromide (CTAB) method with minor modifications [33]. A total of 541 SSR markers distributed across 12 chromosomes were selected from Gramene (http://www.gramene.org/markers/) to determine polymorphism between the parents Jiangtangdao 1 and Miyang 23. The identified markers were employed for primary mapping using the F2 population.

The volume of PCR reaction was 20 μl, containing 2 μl 10×PCR buffer (100 mM Tris-HCl pH 8.0, 15 mM MgCl2, 500 mM KCl, 1% TritonX-100), 0.2 mM dNTPs, 0.2 μM forward and reverse primer, 50–100 ng genomic DNA and 0.625 U Taq polymerase. The PCR products were separated on 8% polyacrylamide gel. Bands were detected using a rapid silver staining method [34].

Marker development

For high-resolution genetic mapping, sequence tagged site (STS) and insertion-deletion (InDel) markers were developed based on DNA sequence difference between 9311(O. sativa indica) and Nipponbare (O. sativa japonica). Genome sequences for indica 9311 and japonica Nipponbare were downloaded from BGI (http://rice2.genomics.org.cn/page/rice/index.jsp) and NCBI (http://www.ncbi.nlm.nih.gov/), respectively. The downloaded sequences were aligned using the SeqMan program (A component of DNASTar Lasergene 8.0) for identification of InDel markers. Primers flanking the identified InDels were designed using the online software Primer 3 (http://fokker.wi.mit.edu/primer3/input.htm). In this study, seven polymorphic InDel markers were identified (Table 1).

Analysis of DNA markers and QTL mapping

Genetic linkage maps were constructed using data from the F2 and F3 populations, respectively. The RS values along with either parental allele pattern of each individual in the populations were applied to Joinmap 4.0 software [35] to calculate marker distances and assign the linked markers to appropriate linkage groups. QTL parameters (location, effect and test statistics) of all putative QTL were estimated using the multiple QTL model mapping (MQM) with MapQTL 6.0 (http://www.kyazma.nl/index.php/mc.
The reaction mixtures contained a marker for SpeIw e r eF : TACCGTTCTTA. The PCR products were digested by a CAPS restriction digestion assay was designed to verify the mutation site. Isolation of the candidate gene and identification of the mutation site

Total genomic DNA was extracted from leaf tissue of both wild type Huaqingdao and mutant Jiangtangdao 1 using the CTAB method. The annotated sequences in the identified candidate gene were targeted for DNA sequencing using the genomic DNA. The primer pairs for the DNA sequencing were designed using the exon coverage according to the sequence of the genomic DNA. The primer sequences for PCR-gene. A 571-bp fragment was amplified by PCR using genomic DNA as a template. The primer sequences for PCR-flanking markers were screened using the Rice Genome Annotation Project–MSU Release 7 based on the defined physical locations. All the ORFs and their functional products in the region were analyzed to predict the candidate gene based on the structures and functions of known starch synthesis genes.

| Marker name | Forward primer (5‘-3’) | Reverse primer (5‘-3’) | Product size (bp) in Nipponbare/9311 |
|-------------|------------------------|------------------------|--------------------------------------|
| InDel 1     | CCATCTCCCCGGTCGGATTGAT | GTCAGCCCAAGGACACTC     | 250/227                              |
| InDel 2     | CCATGATGCAAGCCTTCTTT   | GACAATGCAATGAGGAGGT    | 270/258                              |
| InDel 3     | TCTGACCCCACTGGTCTGG    | TGAACCACAAGGGTGAGTC    | 210/190                              |
| InDel 4     | CATGCCAATTTTCTGCTTG    | GGCACTGAAATTTGGGTA     | 245/267                              |
| InDel 5     | AGCAAGGAGAGACTGGAGG    | GGGACATCTGCTTTGTGTT    | 222/240                              |
| InDel 6     | CCCATGGCATACAGCAGG     | TGGTTTCTACTCTATGGGAAA  | 244/267                              |
| InDel 7     | CCGGCAATGACACATATTGA   | GGCAACCTGGAGATGAAA     | 203/180                              |

After the mutation site was confirmed by comparative analysis of the genomic DNA between Jiangtangdao 1 and Miyang 23. D, Brown rice of Miyang 23. A, Milled rice of Jiangtangdao 1. B, Brown rice of Jiangtangdao 1. C, Milled rice of Miyang 23. D, Brown rice of Miyang 23.

Results

Variation of resistant starch (RS) content

Parent Jiangtangdao 1 is high in both contents of RS (11.67±0.43%) and apparent amylose content (AAC) (31.10±0.15%). Its RS was 28 times that of the other parent Miyang 23 (0.41±0.16%), and its AAC was two times that of Miyang 23 (15.13±0.05%). Jiangtangdao 1 had an almost-completely opaque endosperm due to increased chalkiness, while the grain of Miyang 23 was quite transparent (Figure 1A, B). In the F2 populations, the majority of individuals were in the low RS region of 0.4–1.0%, which shaped a long and flat tail extending from 4.0 to 13.67% of RS (Figure 2). This distribution indicated that the responsible QTL were few for RS inheritance, and the mutated high RS locus was recessive to regular-low RS. Few QTL traits usually are less affected by the environment than many QTL.
traits, evidenced by small standard deviation (0.43%) of RS in
Jiangtangdao 1 [36].

Primary mapping
In total, 106 out of 541 SSR markers were polymorphic
between Jiangtangdao 1 and Miyang 23. These markers covered
1204.1 cM on the rice genome. Average marker coverage was
approximately one marker every 11.4 cM. Based on RS data from
178 F2 individuals with the 106 SSRs, only one QTL on
chromosome (chr) 2 was identified (Figure 3). This QTL saturated
by 18 markers, appeared to be a major QTL as indicated by a
high LOD value of 36. The major QTL explained 60.4% of total
variation in the F2 population of Jiangtangdao 1 and Miyang 23
for RS with additive effect of 2 \(3.2611\) and dominant effect of
2 \(3.1314\). Thus, this locus was designated \(sbe3-rs\) according to
QTL nomenclature [37]. The \(sbe3-rs\) locus was flanked by
RM6611 and RM13366 with a genetic distance of 11.7 cM and
a physical distance of 2.2 Mb, approximately.

Fine mapping of \(sbe3-rs\)
Four F3 plants derived from a recombinant F2 plant were
identified as heterozygous in the region flanked by RM6611 and
RM13366 (Table 2). Selfed seeds of these recombinant F3 plants
generated 656 F4 individuals (210 plants of F3 family 156, 86 plants
of family 157, 208 plants of family 159, and 152 plants of family
160) for fine mapping. Screening all the available SSR markers in
Gramene (http://www.gramene.org/) within the flanking region
of RM6611 and RM13366 identified five SSRs polymorphic
between the parents, RM13256, RM13295, RM13313, RM13345
and RM8254, plus a STS marker. Fine mapping with these
markers and RS data of 656 F4 individuals narrowed the region
harboring \(sbe3-rs\) to a physical distance of 809.4 kb between
RM13313 and RM8254 (Figure 4A).

To further validate the mapping result, seven polymorphic
InDel markers, InDel 1–7, were developed for fine mapping \(sbe3-rs\)
(Table 2). We analyzed RS variation and the seven InDel markers
among 360 F4 individuals derived from two F3 families, family 159

| F4 No | RM5699 | RM6611 | RM13366 | RM475 | RM262 | RM3512 |
|-------|--------|--------|---------|-------|-------|--------|
| 156   | A      | H      | H       | H     | H     | A      |
| 157   | A      | H      | H       | H     | A     | A      |
| 159   | A      | B      | H       | H     | H     | A      |
| 160   | A      | A      | H       | H     | A     | A      |

Table 2. Genotypes of four recombinant F4 individuals for
putative resistant starch \(sbe3-rs\) flanked by RM6611 and
RM13366: A – ‘Miyang 23’, B – ‘Jiangtangdao 1’ and H –
heterozygous.

Figure 2. Variation of resistant starch content in the F2 population derived from Jiangangdao 1 and Miyang 23.
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Figure 3. Genetic linkage map of rice resistant starch on
chromosome 2 for \(sbe3-rs\) genetic distance (Kosambi, centi-
Morgan) and SSR marker name on the left and right of the
chromosome, respectively and highlighted region for the
estimated position of \(sbe3-rs\).
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and 160. RS segregated in F3:4 family 159, which genotypes for markers between RM13313 and InDel2 were same to the high RS parent Jiangtangdao 1 (Figure 4B). Thus, sbe3-rs was determined not to be between RM13313 and InDel2. On the other hand, RS did not segregate in F3:4 family 160, which genotypes for markers between RM13313 and InDel5 matched the low RS parent Miyang 23. Thus, the region between Indel6 and RM13366 was rejected as the location for sbe3-rs. Both rejections on each end suggested that sbe3-rs was between InDel2 and InDel6, which physical distance was narrowed to 573 kb.

Verification and mutation identification using sequencing the candidate gene

From Rice Genome Annotation Project (http://rice.plantbiology.msu.edu/index.shtml), 86 genes (Table S1) were predicted in the region where sbe3-rs was flanked by InDel2 and InDel6.
Table 3. Primers used for sequencing the putative gene SBE3 (LOC_Os02g32660).

| Name     | Sequence (5’-3’) | Exon coverage | Product size (bp) |
|----------|------------------|---------------|------------------|
| SBE3-P1F | GTGAGGGAGGTTAGTGGTGAAG | Exon 1–3 | 1201 |
| SBE3-P1R | TGGTGAAGACTGCAAGATGGA | | |
| SBE3-P2F | TAAAGGGTTCGACCTGTCGA | Exon 4–6 | 1209 |
| SBE3-P2R | CCTACTAAGTCACAAAAACTGC | | |
| SBE3-P3F | TCGCTGTGATGCTAGTGTAGT | Exon 7–9 | 1174 |
| SBE3-P3R | CAATAGGGTCGACGGTTTAT | | |
| SBE3-P4F | TTTGCACATCGTCAACATAG | Exon 10–11 | 1345 |
| SBE3-P4R | GGAACCCAAATTCATACACAAA | | |
| SBE3-P5F | GTGACATCTTTTGTGATTTCT | Exon 12–14 | 1070 |
| SBE3-P5R | AATTTCTTGACCCACCTAAAA | | |
| SBE3-P6F | ACAGGGAAGAAGGAGAAATTG | Exon 15 | 789 |
| SBE3-P6R | CATGCTAAAGGACCAATGAT | | |
| SBE3-P7F | ATGTTGACCTGGCAAGCTTACAT | Exon 16–18 | 1301 |
| SBE3-P7R | GAATTTCAATTTGGAACCTCA | | |
| SBE3-P8F | AACCTTTCTCTGTCTGACCTC | Exon 19–20 | 1192 |
| SBE3-P8R | AGATCTAGCACTAGCAACTG | | |
| SBE3-P9F | TACCTACATCTAGTCTGGCATC | Exon 21–22 | 551 |
| SBE3-P9R | TGGTCTAGTCTATCCGGCATTCAT | Exon 23–24 | 1201 |

InDel6. Among the 86 genes, starch branching enzyme (SBE3, LOC_Os02g32660) was the only candidate gene known to be associated with starch synthesis and could be a top candidate for the resistant starch locus in rice (Figure 5A). The SBE3 candidate gene, which has 3 protein domains, encodes a branching enzyme to introduce α-1,6 bonds that is essential for the formation of amylopectin (Figure 5B). The genomic DNA sequence of SBE3 includes 11,380 nucleotides with 22 exons. The SBE3 gene consisted of 2,478 bp encoding a predicted protein with 825 amino acid residues. We designed nine pairs of primers to sequence genomic DNA of both mutant Jiangtangdao 1 and its wild type. Huaqingdao for the exons of SBE3 gene (Table 3). Comparison of the sequencing results displayed that a single nucleotide substitution (T to C) occurred within the coding region of SBE3 in the wild type (Figure 6A). This nucleotide substitution caused a missense base change from a CTA (Leu) to CCA (Pro) at codon 599 in exon 16 (Figure 6B). Analysis of the predicted products from gene SBE3 demonstrated that the mutated amino acid was on protein domain clo7093, an Alpha amylase catalytic domain family.

The sequence result was also verified by restriction enzyme SpeI digestion (TakaRa, Dalian, China). The mutant Jiangtangdao 1 was not digested by the CAPS marker for SpeI (Figure 6C, lane 4), while the wild type was (Figure 6C, lane 2). Among 178 F2 plants, 70 had Miyang 23 genotype which RS contents ranged from 0.25 to 0.73%, 17 had Jiangtangdao 1 genotype which RS contents ranged from 4.56 to 12.73%, and the remaining 91 had heterozygote genotype which RS contents ranged from 0.35 to 2.19% (Figure 7). Obviously, plant genotype revealed by the CAPS marker co-segregated with RS phenotype, and the cosegregation further supported sbe3-rs responsible for RS in rice.

Discussion

Cultivar improvement for resistant starch

Mapping genes or QTL and development of markers are essential for marker-assisted breeding (MAB), especially for RS for which assessment is extremely difficult in vitro [24, 25]. We 1) mapped sbe3-rs, a recessive gene for high RS in Jiangtangdao 1 mutated from SBE3 using a F2 population, 2) finely mapped sbe3-rs using F3:4 populations, 3) conducted a verification by comparatively sequencing the putative region for sbe3-rs and SBE3 in the mutant and wild type, respectively, and 4) further verified the mapped gene using the products from sbe3-rs and SBE3 restricted by a CAPS marker. The CAPS marker was developed based on the nucleotide difference in coding region between sbe3-rs and SBE3. Because the point mutation resulted in the loss of a restriction enzyme site SpeI, sbe3-rs was not digested by the CAPS marker for SpeI while SBE3 was. Co-segregation of the sbe3-rs genotype with high RS phenotype demonstrated that the CAPS marker could be used for MAB to screen for elevated RS in rice reliably and accurately.

Because of its effective control of glycemic index (GI) for diabetic patients, Jiangtangdao 1 with high RS content has been commercially developed. However, its commercialization has been largely restricted by its productivity since Jiangtangdao 1 is prone to lodging and diseases in addition to low milling yield. The low productivity along with high demands in the market has resulted in a high price of Jiangtangdao 1. Employing marker-assisted breeding techniques with the developed CAPS marker should...
improve the productivity of Jiangtangdao 1 by improving the efficiency of breeding with a reliable and accurate selection, and increase its availability to serve more diabetic patients subsequently.

**SBE3 and sbe3-rs in starch synthesis**

Starch branching enzymes, such as the one encoded by SBE3, introduce α-1,6 linkages into starch that are critical for the formation of amylpectin [11]. There are two families of starch branching enzymes in rice based on primary structure and functional analysis. SBE3 belongs to family A, which is responsible for a unique aspect of amylpectin biosynthesis and structure in rice development [38]. Lack of SBE I combined with a lack of SBE IIb has been reported to produce a much more branched starch without any change in the property of amylose [39]. It has been reported that rice deficient in SBE3 produces apparent amylose up to 29%–35% [40].

Genetic modification of SBE has demonstrated to successfully increase resistant starch and amylose in cereals. Using genetic transformation technology, wheat produces up to 80% amylose along with substantially high resistant starch when SBEIIa is knocked out [10]. Similarly, maize produces up to 80% amylose with high resistant starch when SBEIIb is knocked out [41]. The differences between wheat and maize are due to the fact that SBEIIa is the predominant isoform in wheat grain, while SBEIIb is the predominant isoform in maize [10]. A high-amylose transgenic rice line (TR5) modified by antisense RNA inhibition of SBEI and SBE3 yields substantially high resistant starch [21], which effectively improves large bowel health in rats [42].

By comparing the nucleotide sequences of SBE3 and sbe3-rs, we located the recessive point mutation in exon 16 where the nucleotide T in the wild type was substituted by C in mutant Jiangtangdao 1. This substitution resulted in a missense in SBE3 coding region for 599 amino acid from Leu-599 of the wild to Pro-599 of Jiangtangdao 1. The mutation occurred in the protein domain clo7893 as Alpha amylase catalytic domain family. It has been reported that the proline residues may cause a bend in the helix [43], evidenced by their presence on the solvent exposed face of each helix [44]. As a result, these residues play an important role in determination of local conformation for protein three-dimensional structure [45]. Therefore, the change of missense base, in our case from Leu-599 coded by SBE3 to Pro-599 coded by sbe3-rs may impact the protein conformation of SBE3. However, a functional complementation of the sbe3-rs gene in Jiangtangdao 1 with the wild type copy of the SBE3 gene using transgenic technology should be conducted for an absolute conclusion on resistant starch in rice.

**Supporting Information**

Table S1 The 86 genes and their putative functions in the region between InDel2 and InDel6 where sbe3-rs was located for rice resistant starch, predicted in Rice Genome Annotation Project (http://rice.plantbiology. msu.edu/index.shtml), (DOC)

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**Author Contributions**

Conceived and designed the experiments: WY ZP. Performed the experiments: RY JB ZL. Analyzed the data: RY CS. Contributed reagents/materials/analysis tools: BS JZ ZL. Wrote the paper: RY WY ZP.

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