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Isolation of Partial Housekeeping Genes on Tuntun Angin (*Elaeocarpus floribundus*)

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Abstract

Some genes like *18S rRNA*, *26S rRNA*, elongation factor 1-alpha (*EF1α*), and beta-tubulin (*TUB*) are members of housekeeping genes group that are commonly used as internal control in gene expression study. This study aimed to isolate those four housekeeping genes of tuntun angin (*Elaeocarpus floribundus*). The research material included fresh leaves of *E. floribundus* that were picked up from Kajuik Lake in Riau Province and four primer pairs. The procedures consisted of total DNA isolation using Genomic DNA Mini Kit Plant (Geneaid), polymerase chain reaction (PCR), electrophoresis on 1% agarose gel, sequencing, and bioinformatic analysis. This study has been isolated *18S rRNA*, *26S rRNA*, *EF1α*, and *TUB* genes with the size of 422 bp, 922 bp, 856 bp, and 877 bp, respectively. The *EF1α* and *TUB* genes has never been reported in Elaeocarpaceae family. Thus, those partial DNA sequences are the first sequences reported from this species and can be used as reference genes in this plant after validation.

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INTRODUCTION

Housekeeping genes are a group of genes which are coding proteins responsible for basic celluer functions in organisms – such as cell structural components, translation, ubiquitination, and glycolisis – and their expressions were abundant and relatively stable at any developmental stages and tissues (Sinha et al., 2015). Some of housekeeping genes are the genes encoding actin (*ACT*), tubulin (*TUB*), ubiquitin (*UBQ*), ribosomal RNA (*rRNA*), elongation factor-1-alpha (*EF1*α), glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), serine/threonine-protein phosphatase catalytic subunit (PP2A), aquaporin tonoplast intrinsic protein (TIP2), YT521-B-like protein family protein (*YT521-B*), and histone 3 (*HIS3*) (Basa et al., 2009; Lee et al., 2010; Ray & Johnson, 2014; Pabuayon et al., 2016).

Actin and tubulin proteins are components of the eukaryotic cell cytoskeleton. In plants, the genes encoding actin and tubulin proteins have many variants and are called gene family. They participate in movement of chromosomes during mitosis and meiosis, movement of organells, and intracellular transport (McDowell et al**.**, 1996; Dominguez & Holmes, 2011; Kandasamy et al., 2012; Rebouças et al., 2013). *Arabidopsis thaliana* has ten actin genes (McDowell et al**.**, 1996) while *Pinus taeda* (Schwarzerova et al., 2010) and *Physcomitrella patens* usually have seven to eleven actin variants (Zhang et al., 2010). Meanwhile, there are up to eight isotypes of beta-tubulin in plants, for example, six beta-tubulin isotypes in *Physcomitrella patens* (Jost et al., 2004), twenty beta-tubulin genes in *Salix arbutifolia* (Rao et al., 2016), twenty in *Populus* (Oakley et al., 2007), nine in *Arabidopsis thaliana* (Cheng et al., 2001), and eight in *Oryza sativa* (Yoshikawa et al., 2003).

Ubiquitin protein is involved in recycle of damage proteins or other cell components and the process is named ubiquitination (Pickart & Eddins, 2004). Ubiquitin gene in plant also consists of many isotypes. Ubiquitin gene familiy in *Arabidopsis thaliana* comprises 14 members that are grouped into three types such as polyubiquitin, ubiquitin-like, and ubiquitin extension genes. The *UBQ3, UBQ4*, *UBQ10*, *UBQ11*, and *UBQ14* genes are included as polyubiquitin genes and *UBQ7, UBQ8*, *UBQ9*, and *UBQ12* genes are included as ubiquitin like genes (Callis et al., 1995).

Ribosomal RNA and elongation factor-1-alpha are involved in translation (Sasikumar et al., 2012; Gantasala et al., 2013). Ribosome is composed of ribosomal RNA (rRNA) molecules and ribosomal proteins. In eukaryotic cell, the ribosome is 80S in size and consists of large subunit measuring 60S and small subunit measuring 40S. In large subunit, the rRNA molecule consists of three types, namely 26S/28S, 5.8S, and 5S. In small subunit, there is only one type namely 18S. Genes of 28S, 5.8S, and 18S are in one-unit transcript separated by internal transcribed spacer 1 and 2 (ITS1 and ITS2). The 18S rRNA molecule has an important role in the accuracy of the start codon reading during translating initiation. The 18S DNA sequence is more conserved than 26S/28S (Gillespie et al., 2006; Porter & Golding, 2012). Validation of 26S/28S and 18S genes has been done and they have already been used as reference genes in plants (Bao et al., 2016; Hou et al., 2017; Singh et al., 2018).

The elongation factor-1-alpha (EF1α) is a GTPase enzyme that catalyzes the eficiency and accuracy of the installation of certain tRNA into ribosome and the elongation of polypeptide chains during translation elongation stage (Negrutskii & El'skaya, 1998; Talapatra et al., 2002). The *EF1*α, β*TUB*, and *UBI* genes are accurate enough to normalize expression data in jute (*Corchorus capsularis*) (Niu et al., 2015).

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) protein is a key enzyme in glycolysis in all organisms. The GAPDH enzyme catalyzes the catabolism of glyceraldehyde-3-phosphate (G3P) to 1,3-biphosphoglycerate with the help of NAD and inorganic phosphate. Twenty-two GAPDH genes have been identified in wheat plants (*Triticum aestivum*) and they are grouped into four types namely *gapA/gapB*, *gapC*, *gapCp*, and *gapN* (Zeng et al., 2016). The GAPDH genes are reference genes that are often used the most as an internal control in plants because its expression is stable compared to other housekeeping genes (Sirover 2011; Kozera & Rapacz, 2013).

Tuntun angin (*Elaeocarpus floribundus* BI) is a medicinal plant but not widely known yet. This plant has some fruits with the shape and size resemble to olive fruit (*Olea europaea*) (Bhowmick, 2011; Sircar & Mandal, 2017). In Indonesia, *E. floribundus* can be found at Ruteng Park of natural tourism, in East Nusa Tenggara (Setiadi, 2004) and Kajuik Lake in Riau. Riau local people use its fruit for cooking (Roslim et al., 2016).

Part of the plant's stem will submerge in rainy season for a few months but this plant still survives. This situation indicates that this plant has adapted to flooding stress and thus has some tolerant genes related to the flooding stress and also other stresses. Gene expression study may be performed to investigate the genes. The study requires some genes as internal control for target gene expression data normalization and the genes are called reference genes (Gantasala et al. 2013; Wang *et al.*,

2017). The reference genes come from houkeeping genes groups because their expressions are abundant and stable at any developmental stages, tissues, and conditions. Previously, a housekeeping gene was isolated from *E. floribundus* such as partial actin gene (Roslim & Herman, 2017). However, for the purposes of gene expression studies, more than one reference gene are needed as internal control (Rebouças et al., 2013; Wang et al., 2017; Bao et al., 2016). Moreover, the most widely used housekeeping genes as internal control are 18S rRNA, 26S rRNA, *EF1*α, *GAPDH*, actin, and beta tubulin (β*TUB*) in plants like tea (*Camellia sinensis*), jute (*Corchorus capsularis*), and olive (*Olea europaea*) (Kozera & Rapacz, 2013; Ray & Johnson, 2014; Niu et al., 2015; Wang et al., 2017). Therefore, the objective of this research was to isolate the partial housekeeping genes in *E. floribundus* such as genes encoding 18S rRNA, 26S rRNA, elongation factor 1 alpha (*EF1*α), and beta tubulin (β*TUB*). This research aims to provide information about some housekeeping genes of *E. floribundus* that have not been reported in the previous studies. The genes can be evaluated for possible using as internal control in gene expression study of this plant.

METHODS

Fresh leaves of tuntun angin (*Elaeocarpus floribundus* BI) for DNA extraction were collected from Kajuik Lake in Riau Province, Indonesia. The primer pairs used in this study are presented in Table 1.

Procedures in this study were performed based on Roslim et al. (2018). The fresh leaves were used to extract the total DNA by using Genomic DNA Mini Kit (Plant, Geneaid GP100). The total DNA obtained was then checked for quantity and quality using agarose gel electrophoresis. After that, polymerase chain reaction (PCR) was performed for amplification of partial genes of 18S rRNA, 26S rRNA, *EF1*α, and β*TUB*. The agarose gel electrophoresis was also done for checking of the existence of PCR products. Fourty five microliters of the PCR products were sent to PT Genetika Science for purification and sequencing at 1st Base in Malaysia.

The DNA sequences were then analyzed using BioEdit version 7. Aligment using BLASTn was also conducted to determine the similarity to other sequences available in GenBank database. Dendrogram was then constructed using MEGA6 software. These sequences analysis were conducted following a procedure suggested by Roslim et al. (2018).

RESULTS AND DISCUSSION

Profile of PCR Products

The PCR products of 18S rRNA, 26S rRNA, *EF1*α, and β*TUB* genes were obtained with the size approximately of 400 bp, 1000 bp, 900 bp, and 900 bp, respectively (Figure 1). Those bands were thick and suitable for sequencing requirements.

Analysis of Partial Housekeeping Gene Sequences

The partial DNA sequences obtained in this study were 422 bp for 18S rRNA, 922 bp for 26S rRNA, 856 bp for *EF1*α, and 877 bp for β*TUB*.

Table 1. Primer pairs for amplification of partial genes of *18S rRNA*, *26S rRNA*, *EF1*α, and β*TUB* on tuntun angin (*Elaeocarpus floribundus* BI).

Primer	5'----------3'	Annealing Temperature (°C)	Region	References
18S_F	CGCGCAAATTACCCAATCCTGACA	55.0	18S ribosomal RNA	Gantasala et al. (2013)
18S_R	TCCCGAAGGCCAACGTAAATAGGA			
26S_NL1_F	GCATATCAATAAGCGGAGGAAAAG	56.1	26S ribosomal RNA	Porter & Golding (2012)
26S_LR5_R	ATCCTGAGGGAAACTTC			
EF1α_F	GACTCTGGAAAGTCGACCA	54.7	elongation factor 1-alpha	Roslim et al. (2018)
EF1α_R	TGGTGCATCTCCACAGACTT			
TUB3-F	TGGGCCAAGGGICACTAYAC	58.4	tubulin	Einax & Voigt (2003)
TUB4-R	GCCTCRGTGAACTCCATCTCGTCCAT			

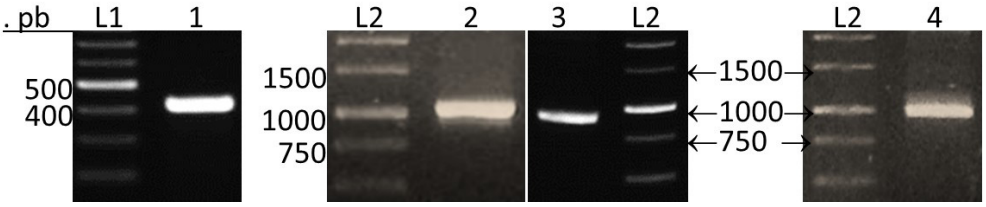

Figure 1. The PCR products of (1) 18S rRNA, (2) 26S rRNA, (3) *EF1*α, and (4) β*TUB* genes on *Elaeocarpus floribundus.* (L1) 100 bp DNA Ladder and (L2) 1 kb DNA Ladder (Thermo Scientific).

These DNA sequences have already been registered to GenBank database (Figure 2). Based on data in GenBank, the complete coding sequence of those genes were approximately 1754 bp for 18S rRNA, 2801 bp for 26S rRNA, 1350 bp for *EF1*α, and 1745 bp for β*TUB* (Emrich et al., 2007)

> MK580498 | Elaeocarpus floribundus 18S ribosomal RNA gene, partial sequence
TTTCGCGCAAATTACCCAATCCTGACACGGGGAGGTAGTGACAATAAATA
ACAATACCGGGCTCTTCGAGTCTGGTAATTGGAATGAGTACAATCTAAAT
CCCTTAACGAGGATCCATTGGAGGGCAAGTCTGGTGCCAGCAGCCGCGGT
AATTCCAGCTCCAATAGCGTATATTTAAGTTGTTGCAGTTAAAAAGCTCG
TAGTTGGACCTTGGGTTGGGTCGGCCGGTCCGCCTCAGGTGTGCACCGGC
CGGCTCGTCCCTTCTGCCGGCGATGCGCTCCTGGCCTTAGCTGGCCGGGT
CGTGCCTCCGGCGCTGTTACTTTGAAGAAATTAGAGTGCTCAAAGCAAGC
CTACGCTCTGGATACATTAGCATGGGATAACATCATAGGATTCCGGTCCT
ATTTACGTTGGCCTTCGGGAAA

> MK580499 | Elaeocarpus floribundus 26S ribosomal RNA gene, partial sequence
TTTGCATATCCAAATAGCGGAGGAAAAGATACTTACCAGGATTCCCCTAG
TAACGGCGAGCGAACCGGGAAGAGCCCAGCTTGAGAATCGGGCGCCCTCG
GCGTCCGAATTGTAGTCTGGAGAAGCGTCCTCAGCGGCGGACCGGGCCCA
AGTCCCCTGGAAGGGGGCGCCGGAGAGGGTGAGAGCCCCGTCGTGCCCGG
ACCCTGCCGCACCACGAGGCGCTGTCGGCGAGTCGGGTTGTTTGGGAATG
CAGCCCAAATCGGGCGGTAAATTCCGTCCAAGGCTAAATACGGGCGAGAG
ACCGATAGCAAACAAGTACCGCGAGGGAAAGATGAAAAGGACTTTGAAAA
GAGAGTCAAAGAGTGCTTGAAATTGCCGGGAGGGAAGCGGATGGGGGCCG
GCGATGCGCCCCGGTCGGATGTGGAACGGCGGTGAGCCGGTCCGCCTATC
GACTCGGGGCGTGGACCGATGCGGATTGCGGCGGCGGCCCAAGCCCGGGC
CTCAGAAACGCCCGCGGAGACGCCGTCGCCGCGATCGTGGCTGGCAGCGC
GCGCCGTCCCGGCGTGCTTCGGCACCTGCGCGCTCCCGGCATCGGCCTGC
GGGCTCCCCATTCGGCCCGTCTTGAAACACGGACCAAGGAGTCTGACATG
TGTGCGAGTCAACGGGCCATTAAACCCGTAAGGCGCAAGGAAGCTGATTG
GCGGGATCCCCAACGGGTGCACCGCCGACCGACCTTGATCTTCTGAGAAG
GGTTCGAGTGAGAGCATGCCTGTCGGGACCCGAAAGATGGTGAACTATGC
CTGAGCGGGGCGAAGCCAGAGGAAACTCTGGTGGAGGCCCGCAGCGATAC
TGACGTGCAAATCGTTCGTCTGACTTGGGTATAGGGGCGAAAGACTAATC
GAACCGTCTAGTAGCTGGTTCA

> MK580501 | Elaeocarpus floribundus elongation factor 1 alpha gene, partial sequence
GTGCATCTCCACAGACTTGACTTCAGTGGTCAGTCCAGTGGGGCCAAAGG
TCACAACCATACCAGGCTTGAGGACACCAGTCTCAACACGACCAACTGGT
ACAGTACCAATACCACCGATCTTGTAGACGTCCTGAAGTGGGAGACGGAG
AGGCTTGTCTGAGGGCCTCTTGGGCTCATTGATCATGTCAAGAGCTTCAA
GAAGGGTAGGGCCCTTGTACCAGTCGAGGTTTGTGGACCTCTCAATCATG
TTATCACCCTCAAAACCAGAGATGGGGACGAAGGGAATCTTGTCAGGGTT
GTAACCAACCTTCTTCAAGTAGGAAGACACTTCCTTCACAATTTCATCAT
ACCTAGCCTTGGAATACTTGGGGGTGGTGGCATCCATCTATAAAGTAAGT
GCAACAAATAACATCAGTATATGGCACTTAAGAAACAACAAACAGCAAAT
TATTCAAGTGATAAAAGAGAGATTGCAATAATACCTTGTTACAACAGCAA
ATCATTTGCTTGACACCAAGGGTGAAAGCAAGCAGAGCATGCTCACGGGT
CTGGCCATCCTTGGAGATACCAGCTTCAAAACCACCAGTGGTGGAGTCAA
TAATGAGGACGGCACAGTCAGCCTGTGAGGTACCGGTAATCATGTTCTTG
ATAAAGTCACGATGTCCAGGGGCATCAATGACAGTGCAGTAGTACTTGGT
AGTCTCAAACTTCCACAAAGCAATATCAATGGTAATACCACGTTCACGCT
CAGCCTTGAGCTTGTCCAACACCCAAGCATACTTGAAAGACCTCTTGTTC
ATCTCAGCAGCCTCCTTCTCGAACCTCTCAATGACACGCTTGTCAATACC
ACCAAG

> MK580500 | Elaeocarpus floribundus beta tubulin gene, partial sequence
TCGACCAGGTCCTCGATGTCGTTCGACGTGAGGCTGAAGGCTGTGACTGC
CTCCAGGGTTTCCAGATCACCCACTCTCTTGGTGGTGGTACTGGTGCTGG
TATGGGTACGCTCCTCATCAGCAAGATCCGTGAGGAGTTCCCAGACCGAA
TGATGGCCACCTTCTCAGTCATGCCATCTCCAAAGGTCTCTGACACTGTC
GTCGAGCCATACAACGCCACTCTCTCTGTCCACCAGCTGGTCGAGAACTC
TGACGAGACCTTCTGTATCGACAACGAGGCTCTCTACGACATCTGCATGC
GCACACTCAAGCTTCCAAACCCAAGCTACGGCGACCTCAACCACCTGGTC
TCTGCCGTCATGTCTGGCATCACCACCTGCCTGCGATTCCCAGGTCAGCT
CAACAGCGATCTTCGCAAGCTTGCTGTCAACATGGTGCCATTCCCACGTC
TGCACTTCTTCATGGTCGGCTTCGCACCACTCACCTCCCGCCACTCTCAC
AGCTTCCGCGCACTCAGCGTGCCAGAGCTCACCCAGCAGATGTTCGACCC
AAAGAACATGATGGCTGCCTCAGACTTCCGCAACGGACGATACCTCACCT
GCTCTGCCATCTACCGTGGTAAGGTCTCCATGAAGGAGGTTGAGGACCAG
ATCCGCAACGTCCAGAACAAGAACACCGCCTACTTCGTTGAGTGGATTCC
AAACAACATCCAGACCGCCCTCTGCTCAATCCCACCACGTGGCCTCAAGA
TGTCATCCACCTTCGTCGGCAACAGCACTGCCATCCAGGAGCTGTTCAAG
CGTGTCGGCGACCAGTTCTCTGCCATGTTCCGACGCAAGGCTTTCTTGCA
CTGGTACACTGGTGAGGGTATGGACGA

Figure 2. The partial sequences of 18S rRNA, 26S rRNA, *EF1*α, and β*TUB* genes on *Elaeocarpus floribundus*.

BLASTn analysis shows that the four sequences of *E. floribundus* have approximately 75.39%-99.04% similarity to the sequences deposited in GenBank database. The *E. floribundus* 18S rRNA and 26S rRNA genes have a higher similarity than *EF1*α and β*TUB* genes (Table 2). The result shows that the four genes are relatively conserved between species. The conserved genes are genes that have the same function in all organisms and the genes which involved in any biological processes, cellular localization, and molecular functions (Jayaswal et al., 2017). The high level conserved genes are actin, ubiquitin, tubulin, PP2A, translation elongation factor, and small subunit ribosomal RNA (Hug et al., 2016; Jayaswal et al., 2017).

The *EF1*α obtained in this study comprises two exons flanking one intron. Exon is a part of eukaryotic gene encoding protein. In other words, the exon will be transcribed and translated into protein. While intron is a part of eukaryotic gene which does not encode protein in other words, intron will be transcribed but not be translated. The intron region will be removed after transcription through splicing process. Thus, DNA molecule contains exon and intron while mRNA or cDNA molecule consists only

Table 2. BLASTn result of the four housekeeping genes of tuntun angin (*Elaeocarpus floribundus*).

Species	Family	Identity (%)	Accession
18S rRNA			
Elaeocarpus sphaericus	Elaeocarpaceae	99.04	GU476421.1
Sloanea latifolia	Elaeocarpaceae	99.04	U42826.1
Aristotelia serrata	Elaeocarpaceae	98.80	GU476422.1
Elaeocarpus hookerianus	Elaeocarpaceae	98.80	GU476420.1
Brunellia acutangula	Brunelliaceae	98.80	FJ669718.1
26S rRNA			
Elaeocarpus sphaericus	Elaeocarpaceae	98.68	AY177422.1
Crinodendron patagua	Elaeocarpaceae	98.23	AY935811.1
Sloanea berteroana	Elaeocarpaceae	98.12	AF479126.1
Eucryphia lucida	Cunoniaceae	97.25	AF036494.1
Perrottetia longistylis	Dipentodontaceae	96.47	AY935805.1
Elongation Factor-1-alpha **(***EF1α)*			
Hedera helix	Araliaceae	95.16	KU942512.1
Aralia elata	Araliaceae	94.89	JX067859.1
Betula luminifera	Betulaceae	94.09	KP245811.1
Panax notoginseng	Araliaceae	93.82	KF815708.1
Citrus maxima	Aurantioideae	93.82	JQ353767.1
Beta-Tubulin *(TUB)*			
Hordeum vulgare subsp. vulgare	Triticeae	77.10	AM502856.1
Zea mays	Andropogoneae	76.46	NM_001348079.1
Triticum aestivum	Triticeae	75.95	U76744.1
Alopecurus aequalis	Poeae	75.87	MH450177.1
Oryza sativa	Oryzae	75.39	AB104732.1

exon (Rogozin et al*.,* 2005; Zhu et al*.,* 2009). Such conditions cause the *EF1*α to be able to be used as a marker to determine DNA contamination in total cDNA in addition to its potential as internal control in gene expression studies. For this purpose, PCR using DNA or cDNA molecule as template was performed separately and after that the sizes of the PCR products from the two templates were compared (Hannum et al., 2010). In this case, the PCR product from DNA template around 850 bp in length and cDNA template will produce a shorter PCR product which is about 750 bp in length.

Previously, one housekeeping gene of *E. floribundus* was isolated, namely actin gene. The actin gene also contains two exons and one intron intron (Roslim & Herman, 2017) like the *EF1*α obtained in this study. Hence, in addition to the *EF1*α, the actin gene may also be used as DNA contaminant marker in total cDNA of *E. floribundus*.

Housekeeping genes isolated from *E. floribundus* must be evaluated further to determine which one is appropriate as the reference gene. One of the validation processes can be done using the quantitative real time PCR (qRT-PCR) technique and the statistical analysis using geNorm, NormFinder, Best Keeper, and Rank Aggreg (Andersen et al., 2004; Pfaffl et al., 2004; Pihur et al., 2009; Bao et al., 2016). Some housekeeping genes have been validated using qRT-PCR technique in plants such as poplar (Basa et al., 2009), eggplant (Gantasala et al., 2013), rice (Pabuayon et al., 2016) and pigeonpea (Sinha et al., 2015). It is very crucial to select and validate the housekeeping gene for each treatment because it can thus reduce possible errors and contamination during extraction and manipulation of mRNA or cDNA (Rebouças et al., 2013).

The four housekeeping genes such as 18S rRNA, 26S rRNA, elongation factor 1 alpha (*EF1*α), and beta tubulin (β*TUB*) obtained in this study are the first reported from *E. floribundus*. After selection and validation, they can be used as internal controls in gene expression analysis in *E. floribundus.* For example, the study of gene expression related to flooding stress uses these genes as internal controls

so that the expression level can be trusted. Finally, the mechanism underlying this plant's tolerance to flooding stress can be understood.

CONCLUSION

Four housekeeping genes such as 18S rRNA, 26S rRNA, elongation factor 1 alpha (*EF1*α), and beta tubulin (β*TUB*) were isolated from *tuntun angin* (*E. floribundus*) and these are the first genes reported from *E. floribundus*. The sizes of these genes are 422 bp for 18S rRNA, 922 bp for 26S rRNA, 856 bp for *EF1*α, and 877 bp for β*TUB*. Then, the four genes should further be validated so they can be used as reference genes. One gene, the *EF1*, can be used as a marker of DNA contamination in total cDNA in gene expression studies of *E. floribundus*.

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