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GRAIN SIZE, SUCROSE LEVEL AND STARCH ACCUMULATION IN DEVELOPING RICE GRAIN

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Key Word Index—*Oryza sativa*; Gramineae; rice; starch accumulation; ADP/UDP glucose pyrophosphorylase; detached rice panicles; liquid culture.

Abstract—Five rices (*Oryza sativa* L.) differing in final grain size were studied at the midmilky stage to determine if any factor could be identified which might limit rate of starch accumulation. Only UDP glucose pyrophosphorylase activity increased with increasing grain size. Detached rice panicles incubated in liquid medium containing 1% sucrose and 0.1% glutamine, in addition to minerals and vitamins, produced grains similar to those on intact plants. Sucrose level (0–1.5%) in the medium determined the extent of dry matter and starch accumulation and influenced physiological development of the ripening grains. Chemical and enzymic composition of the grain were similar to previously reported levels in grains of intact panicles analysed at regular intervals after anthesis. Addition of 3-P glycerate or K^+ to the medium did not improve dry matter accumulation in the developing grain.

INTRODUCTION

Starch is the major (> 90%) constituent of rice endosperm and first appears as compound granules 4 days after flowering [1]. These granules increase in size, not number, during grain development [2]. Maximum rate of starch accumulation has been observed at the midmilky stage at 7–9 days after flowering [3, 4] and a linear increase in starch content and dry matter per grain occurs during the milky stage from 5 to 9 days after flowering [1–6].

Studies of factors affecting starch accumulation in developing rice grain by analysing grains at various weights and stages of physiological development showed that the level of sucrose [4] and UDP glucose pyrophosphorylase [3] was highest also at the midmilky stage. In addition, sucrose level in the grain did not limit starch accumulation [4]. To verify the results of the natural system in which physiological development and starch accumulation are closely linked, two systems were studied and compared: (a) rices differing in grain size and (b) detached panicles in liquid medium of varying sucrose levels.

Rice varieties differ in caryopsis size and weight (10–40 mg) and the grain-filling period was 12 days for the small-grain varieties and 15–21 days for the medium- and large-grain varieties [6]. In addition, the large-grain varieties have higher grain growth rates and more efficient starch accumulation than the small-grain varieties [6]. We measured various grain components and enzymes at the milky stage in 5 rices differing in grain size and have attempted to relate these to the rate of starch accumulation.

Recent studies in detached wheat ears indicated that sucrose concentration in the liquid medium can affect the rate of starch accumulation in developing grain [7–9]. As a second means of controlling starch accumulation, we developed a procedure for culturing detached rice panicles which allowed differing rates of grain growth and starch accumulation for a single variety. Incubation periods were selected to coincide with the period of linear accumulation during the milky stage of developing rice grain. The relationship between grain components and enzymes, and increase in grain weight in the detached rice panicle system at various sucrose levels were also studied.

RESULTS

Rices differing in grain size

The 5 rices differed in the weight of both milky and mature grains, which was related closely to starch and protein contents of the grain (Table 1). Starch constituted from 74 to 87% of grain dry wt and thus was the major constituent in dehulled grain. The developing grain of the large-grain variety Khao Lo had the highest content of all constituents. The levels of ATP and 3-P glycerate did not show any relationship to grain size for the 4 other rices. With the two sister $IR747B_2$ lines, the medium-grain $IR747B_2$-6-3 had higher levels of all constituents than the small-grain $IR747B_2$-6-3-1, but the differences were not significant for reducing sugars, 3-P glycerate and ATP.

Among the 4 enzymes assayed, only UDP glucose pyrophosphorylase activity paralleled weight and starch content of the grain (Table 1). Sucrose-ADP and sucrose-UDP glucosyltransferases (sucrose synthetase) and ADP glucose pyrophosphorylase did not show any parallel trend to either grain weight or starch content. Khao Lo had the highest levels of sucrose-UDP glucosyltransferase and UDP glucose pyrophosphorylase among the 5 samples. Except for ADP glucose pyrophosphorylase, the two $IR747B_2$ sister lines had higher levels of all enzymes in the medium-grain line, $IR747B_2$-6-3.

Detached panicle in liquid media

Preliminary experiments using only sucrose solution

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Table 1. Properties of dehulled milky stage grains of five rices differing in grain weight

Property per grain	Kalajira	IR747B$_2$-6-3-1	IR747B$_2$-6-3	IR8	Khao Lo	LSD (5%)
Fresh weight of mature grain (mg)	10.9	10.6	15.3	20.8	41.5	
Fr. wt of grain (mg)	9.6	9.8	14.4	19.7	41.4	1.2
Dry wt of grain (mg)	3.5	3.5	7.8	12.2	20.3	
Crude protein (μg)	416	472	1104	1257	2690	66.8
Soluble protein (μg)	129	134	202	154	452	38.6
Free sugars (μg glucose)	103	74	114	123	260	2.6
Reducing sugars (μg glucose)	15	5.6	19	25	83	36.0
Starch (mg)	2.6	2.8	6.5	9.9	17.8	1.7
Pi (μmol)	0.09	0.09	0.22	0.13	0.31	0.02
3-P glycerate (μmol)	2.54	1.58	3.10	1.96	15.3	2.57
ATP (nmol)	9.91	3.30	4.80	8.93	42.4	2.08
K (μmol)	0.66	0.49	0.84	1.40	2.86	
Sucrose-ADP glucosyltransferase (nmol/min)	0.05	0.15	2.14	0.23	0.59	1.02
Sucrose-UDP glucosyltransferase (nmol/min)	6.8	3.6	22.5	11.2	68.8	6.17
ADP glucose pyrophosphorylase (nmol/min)	11.5	15.4	10.3	72.5	8.1	23.6
UDP glucose pyrophosphorylase (nmol/min)	67	65	155	236	466	15.6

(0, 2, 4, 6, 8 and 10%) at room temperature as liquid medium after Jenner [7–9] were unsuccessful. Net panicle weight increases were noted only during the first 4–5 hr of incubation after which panicle weights decreased. Desiccation of the panicles occurred at sucrose concentrations above 2%. Optimum age of panicles for the experiment was 1–2 days after anthesis. Addition of 10 mM EDTA to 2% sucrose had no appreciable effect on dry matter accumulation by the detached panicle. Microbial contamination of the medium became a problem after 24 hr of incubation.

The improved wheat system of Donovan and Lee [10, 11] provided better results than the system of Jenner for detached rice panicles. The modifications included a N source, vitamins and minerals in the liquid medium. The medium was also kept at 2–3° to minimize microbial growth. A sucrose concentration of 1% gave grain growth closer to field samples than 0 and 2% sucrose. Absorption of the liquid medium during incubation did not affect the sucrose concentration of the medium and the 40 ml were completely absorbed by the panicle within 8 days. Optimum age of panicles for culturing was also 1–2 days after anthesis as older grains suffered from tip desiccation.

Dry matter and starch accumulation were manipulated in developing rice grains by varying the sucrose concentration in the medium while keeping the sucrose to glutamine ratio constant. Daily visual observation indicated that the rate of grain development and dry matter accumulation was directly affected by sucrose level in the medium. Dry matter accumulation of IR42 developing grains showed a lag phase of 1 day, after which essentially linear dry matter accumulation occurs (Fig. 1). Very little dry matter accumulation is noted during the first 3 days after anthesis [6]. Absorption of liquid medium was faster in grains containing 1% sucrose–0.1% glutamine than the water control and lower sucrose–glutamine levels. Dry matter accumulation in 1% sucrose–0.1% glutamine was similar to that of field-grown grains. Donovan and Lee [11] reported linear weight increases in developing wheat grains from detached heads in liquid culture, with or without sucrose in the medium, and in the complete medium, grain development closely followed that observed under natural conditions. The poor grain growth of panicles incubated in water confirms the low contribution of photosynthesis of the green hull to dry matter accumulation in the developing rice grain [12].

The optimum concentration of glutamine to be added to 1% sucrose to provide similar level of free sugars in developing grain as field samples was 0.1% (Table 2). However, at this glutamine concentration, levels of free amino acids and protein in the grain were higher than that of field-grown samples. By contrast, Donovan and Lee [10, 11] found 2% sucrose and 0.4% glutamine or amino acid mixture as optimum for growth of detached wheat heads.

Content of starch, crude and insoluble protein, and free amino acids tended to increase with increasing IR22 grain weight and sucrose level in the medium, but the level of soluble protein and free sugars did not have any trend with sucrose level in the medium (Table 3). Except for the sample incubated in sucrose-free medium in which little growth occurred, starch accounted for 57–58% of dry wt. The starch content of the sample in sucrose-free medium was in the same order as free sugars, i.e. 19% of dry wt. Activity of sucrose-UDP glucosyltransferase and bound ADP glucose starch synthetase per grain tended

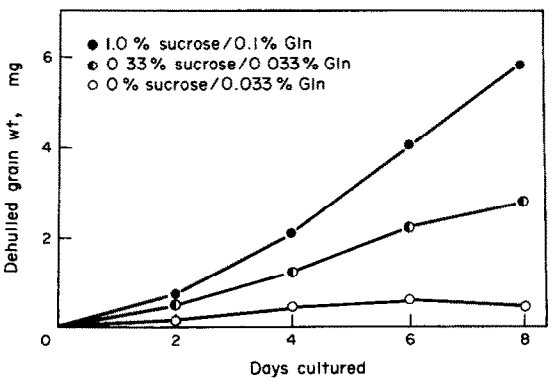

Fig. 1. Dry matter accumulation of IR42 developing grain from detached panicles in liquid culture of varying sucrose and glutamine concentrations. Initial age of grains was 1–2 days after flowering.

Table 2. Mean chemical composition of developing grains of IR32 detached rice panicles cultured for 192 hr at 25–27° in a complete liquid medium containing 1 % (w/v) sucrose and different glutamine levels

Glutamine level in medium (% w/v)	Protein (% dry basis)	Free amino acids (% leucine)	Starch (% anhydroglucose)	Free sugars (% glucose)
0.05	8.8	1.3	58.8	11.5
0.075	11.4	1.2	60.0	8.0
0.1	14.0	1.5	54.7	4.4
0.2	19.8	1.8	51.8	3.2
0.4	22.2	2.8	53.5	1.5
Water control	16.4	2.8	—	—
Field grown	9.8	1.1	61.6	5.0

Table 3. Effect of sucrose and glutamine concentrations in the liquid medium on chemical and enzymic levels in developing grains of IR22 panicles incubated for 160 hr

Property per grain	Sucrose/glutamine levels in medium (% w/v) 0.00/0.05	0.50/0.05	0.75/0.075	1.0/0.1	1.5/0.15	LSD (5%)
Grain wt, fresh (mg)	10.44	11.92	13.38	14.59	15.08	1.15
Grain wt, dry (mg)	1.02	6.00	7.42	8.56	10.68	1.10
Free sugars (mg glucose)	0.20	0.20	0.26	0.24	0.24	0.01
Starch (mg)	0.20	3.44	4.25	5.00	6.22	0.07
Free amino acids (mg leucine)	0.04	0.05	0.06	0.06	0.07	0.009
Crude protein (mg)	0.30	0.80	0.94	1.09	1.47	0.02
Soluble protein (μg)	83	76	100	92	94	NS
Insoluble protein (μg)	322	382	406	513	645	89.9
Enzyme levels (nmol/min)						
Soluble invertase	2.80	2.91	5.51	4.86	3.96	NS
Bound invertase	1.61	1.57	3.79	4.44	3.12	0.56
Sucrose-UDP glucosyltransferase	12.2	13.5	16.4	16.0	18.4	3.34
Sucrose-ADP glucosyltransferase	1.8	1.0	1.4	1.0	1.6	0.51
UDP glucose pyrophosphorylase	190	178	225	167	150	NS
Bound ADP glucose starch synthetase	1.3	4.8	5.1	7.0	8.2	0.77

to increase with an increase in grain weight but activities of soluble invertase, sucrose-ADP glucosyltransferase, and UDP glucose pyrophosphorylase did not. ADP glucose pyrophosphorylase could not be detected. UDP glucose pyrophosphorylase tended to have a maximum level in the grain cultured in 0.75% sucrose–0.075% glutamine where free sugars were highest, whereas sucrose-ADP glucosyltransferase did not show any trend.

A verification of this IR22 experiment using IR36 detached panicles again showed a correspondence of grain weight to sucrose level in the medium (Table 4). Amount of insoluble protein, but not that of soluble protein, tended to increase with grain weight. Very little grain development occurred in the sucrose-free medium. The activity of soluble invertase per grain was the same in the 3 samples incubated in the presence of sucrose but the activity of bound invertase was higher in the two heaviest grain samples. The activity of sucrose-UDP glucosyltransferase and primed soluble ADP glucose starch synthetase was highest in the heaviest grain than in the others incubated in sucrose-containing media. UDP glucose pyrophosphorylase activity was higher in the two heaviest grain samples but was again numerically highest at other than the heaviest grain weight—at 1.0%

Table 4. Effect of sucrose and glutamine concentrations in the liquid medium on protein and enzymic levels in developing grains of IR36 panicles incubated for 160 hr

Property per grain	Sucrose/glutamine levels in medium (% w/v) 0.00/0.05	0.5/0.05	1.0/0.10	1.5/0.15	LSD (5%)
Grain wt, fresh (mg)	7.72	12.48	14.78	16.91	0.09
Grain wt, dry (mg)	0.75	6.28	8.68	12.00	
Soluble protein (μg)	70.3	113	95.6	112	NS
Insoluble protein (μg)	156	274	316	520	85.1
Enzyme levels (nmol/min)					
Soluble invertase	2.00	5.34	5.20	4.73	2.06
Bound invertase	0.63	2.85	5.03	4.05	0.19
Sucrose-UDP glucosyltransferase	16.6	32.8	30.5	45.2	4.99
UDP glucose pyrophosphorylase	11.8	12.4	21.6	19.7	3.89
Soluble ADP glucose starch synthetase (primed)	0.51	0.22	0.28	1.18	0.13
Bound ADP glucose starch synthetase	0.08	0.60	1.16	2.78	0.30

sucrose–0.1% glutamine. However, the activity of UDP glucose pyrophosphorylase was lower than that of IR22. Bound ADP-glucose starch synthetase activity followed closely the grain weight of samples.

With both IR22 and IR36, difficulty was encountered in detecting ADP glucose pyrophosphorylase, which was always present in lower activity than UDP glucose pyrophosphorylase as previously observed by Perez *et al.* [3]. Sucrose-ADP glucosyltransferase activity was also a fraction of sucrose-UDP glucosyltransferase activity.

SDS-polyacrylamide gel electrophoresis of protein extracted by 0.5% SDS–0.6% β-mercaptoethanol from developing grains containing 8.8–22.2% protein on a dry wt basis (from Table 2) revealed little effect of protein content on the MW distribution of the protein subunits, as previously observed for milled rices differing in protein content [13]. The 3 major subunits corresponded to MW 38 000, 25 000 and 16 000, the 3 subunits of glutelin [13]. Glutelin constitutes at least 80% of rice endosperm protein and the increase in protein is mainly due to an increase in glutelin level in the endosperm [13, 14].

In developing corn endosperm, 3-P glycerate is an effector of ADP glucose pyrophosphorylase and 10 mM 3-P glycerate enhanced the *in vitro* activity of the pyrophosphorylase *ca* 3-fold [15]. The physiological level of 3-P glycerate in the moisture of IR26 grain at the midmilky stage was found to be *ca* 2.2 mM [16]. In this study, the addition of 0.5 or 1 mM 3-P glycerate in the culture medium resulted in lower IR22 grain weight after 7 days incubation (Table 5). Short-term incubation in U-sucrose-[U^{14}C] showed a decrease in the extent of ^{14}C-incorporation into starch in the presence of 4 mM 3-P glycerate but no effect on ADP glucose pyrophosphorylase activity. Possibly the level of 3-P glycerate in the developing grain was adequate and the additional 3-P glycerate inhibited starch synthesis. In the absence of actual grain analysis, the extent to which 3-P glycerate was metabolized prior to uptake into the developing grains is not known.

Potassium (0.1 M) was reported by Murata [17, 18] to have a 36% stimulative effect on the *in vitro* activity of granule-bound rice ADP glucose starch synthetase. Optimum K^+ level was reported at 10–20 mM. In our study, increasing the K^+ level in the nutrient medium from 0 to 22 mM did not result in higher grain weight after 6–7 days incubation (Table 6). Actual K level in the dehulled grain showed an increase both in terms of percent of dry wt and in concentration in the grain moisture with an increase in K^+ level in the medium. Developing grains at the midmilky stage in Table 1 had 0.3% K dry basis. Since minerals including K are concentrated in the surface non-endosperm tissues of the rice grain [19], the level of K in the endosperm of developing grain incubated in K^+-free medium was probably already optimum for granule-bound ADP glucose starch synthetase activity.

DISCUSSION

Regulation of starch synthesis in cereal endosperm has been suggested to be at the ADP(UDP) glucose pyrophosphorylase step [15] or the sucrose-UDP(ADP) glucosyltransferase step [17]. The study of grains at the midmilky stage of development from 5 rices differing in grain size showed that only the activity of UDP glucose pyrophosphorylase tended to parallel the amount of accumulated starch whereas that of sucrose–UDP glucosyltransferase did not (Table 1).

In detached panicles grown in liquid medium, different sucrose concentrations in the medium also resulted in differences in dry matter and starch accumulation in developing grains after 6–7 days of incubation. A lag period of not more than 1 day was observed in all treat-

Table 5. Effect of concentration of 3-P glycerate in the liquid medium on weight of dehulled developing grains of IR22 panicles incubated for 7 days (1% sucrose, 0.1% glutamine), and on starch accumulation and ADP glucose pyrophosphorylase activity incubating in sucrose-[^{14}C]

3-P glycerate level in medium (mM)	7-days freeze-dried grain wt (mg)	4-hr incubation in 2 μCi sucrose-[^{14}C] Radioactivity in starch (cpm/grain)	ADP glucose pyrophosphorylase (cpm/min/grain)
0.0	8.59	2422	672
0.5	7.32	—	—
0.1	6.55	—	—
4.0	—	795	769
LSD (5%)	1.00	471	NS

Table 6. Effect of K^+ level in liquid medium on weight and K^+ level of dehulled developing grain of IR36 panicles incubated for 6 or 7 days

K^+ in medium (mM)	Mean dehulled grain dry wt (mg) 6 days	7 days	Grain K level (% dry basis)	(mM in grain moisture)
0*	5.58	5.85	0.68	123
5.65†	5.19	—	—	—
11.0	4.10	5.64	1.35	213
22.0‡	4.49	4.82	1.36	168
LSD (5%)	NS	NS	0.54	77.3

* Replacement of 400 mg KCl by 298 mg $CaCl_2$, 300 mg K_2SO_4 by 424 mg $MgSO_4.7H_2O$, and 300 mg KH_2PO_4 with 265 mg NaH_2PO_4/l. of medium [21].

† K^+ level was reduced from 11.0 mM by replacing 400 mg/l. KCl with 298 mg/1. $CaCl_2$ in incubation medium [21].

‡ K^+ level was increased by adjusting KCl level from 400 mg/l. to 1.22 g/l. in the medium [21].

ments. However, physiological development of the grains also followed closely the grain weight, as in the intact panicles. UDP glucose pyrophosphorylase activity was highest in grains incubated in the medium with 0.75–1.0% sucrose, in which dry wt is closest to field grown samples at the midmilky stage 9–10 days after anthesis. Activities of sucrose-UDP and sucrose-ADP glucosyltransferases and ADP glucose pyrophosphorylase per grain are maximum at this stage of grain development and there is maximum rate of starch accumulation [3]. The results from both systems suggest that the ADP(UDP) glucose pyrophosphorylase step may be the rate-limiting step in starch accumulation in developing rice grain. However, projection of *in vitro* assays to the natural system has to be done with caution, as can be seen in the case of addition of 3-P glycerate and K^+ in the liquid medium for detached panicles.

Although the use of detached rice panicles in liquid medium containing different sucrose levels resulted in grains differing both in grain weight and physiological development, it has the advantage over the natural system in permitting sampling of all the panicles at the same time. It is expected that with greater capacity containers (> 40 ml) for the liquid medium, the detached panicle can be incubated successfully for periods longer than 7 days, as has been reported for detached wheat heads [10, 11]. In addition, the system has potential value for labelling of polymers, such as proteins, lipids and hemicelluloses in the developing rice grain. Protein content of the grain followed closely the glutamine level in the liquid medium (Table 2).

EXPERIMENTAL

Pot experiments. 5 rices differing in grain wt were grown in pots as described earlier [20]. Grains at milky stage (*ca* 5–10 days after anthesis) were harvested from at least 6 plants for chemical and enzyme assay as described below.

Panicles in liquid medium. Panicles with 13 cm long stems were cut under H_2O from rice plants in the field 1–2 days after anthesis. The stem was surface-sterilized with 0.5% (w/v) NaOCl, then further cut to 11 cm under H_2O and washed with sterilized H_2O. The stem of each panicle was then pushed through a sterile cotton plug into a sterile 3 × 11 cm test tube containing 40 ml liquid medium. All operations were done under UV light. The culture tubes were immediately transferred to a 2–3° H_2O bath in a fluorescent lamp illuminated platform with a light intensity of 8 klx at the top of the panicles, a day length of 16 hr and air-conditioned room temp. of 25–27°. A minimum of 4 panicles were used per treatment.

The quantities of major salts in the liquid medium were the same as those of ref. [21], except that 0.05–0.4% glutamine replaced the amino acid mixture as the N source [10, 11]. The maximum sucrose level was 1.5% and no supplementary Pi soln was used. The composition of the liquid medium with respect to minor salts, thiamine, *myo*-inositol, and Fe was the same as that of ref. [22]. The pH of the mixture of stock soln was adjusted to 6.1 with NaOH before diluting to vol. with H_2O. The culture medium was then sterilized by filtration through Diaflo PM-10 membrane (Amicon Corp.).

After incubation periods for as long as 7 days, the developing grains were harvested, pooled and portions analysed directly; the rest were freeze-dried for chemical analysis after hand dehulling. Freeze-dried samples were ground in a cyclone sample mill (UD Corp.) with 60-mesh sieve.

Chemical analysis. Protein and amino acids of freeze-dried, dehulled grain powder were extracted according to ref. [20] except that the extractant used was 0.1 N NaOH. TCA was added to the extract to 10% (w/v) to precipitate the protein. Free amino acid analysis was done on the supernate by the method of ref. [23] using L-leucine as standard. The protein ppt. was redissolved in 0.1 N NaOH and protein determined colorimetrically by the method of ref. [24] using BSA as a standard. Crude protein N was analysed by the micro Kjeldahl method and converted to crude protein by multiplying by the factor 5.95 [13].

Free sugars and starch were determined by the methods described in ref. [14], except that hot 80% EtOH was used instead of hot H_2O to extract the free sugars. Dehulled grain samples were dryashed; P was determined colorimetrically [5] and K by atomic absorption spectrophotometry. ATP was measured by the luciferase assay [25] and 3-P glycerate content by the method of ref. [14].

Whole protein of dehulled grain flour was extracted with 0.5 SDS–0.6% β-mercaptoethanol in 0.1 M Pi buffer (pH 7) and subjected to SDS–polyacrylamide disc gel (12%) electrophoresis according to ref. [13].

Enzyme assays. Preliminary assays were done on all enzymes to determine conditions where linear rates with respect to time and substrate concns were obtained. Duplicate samples of a known number of dehulled grains were weighed and homogenized at 0° in a mortar and pestle with 10 mM Tris–maleate buffer, pH 7, containing 1 mM dithiothreitol [3]. The homogenate was centrifuged at 30 000 *g* for 30 min at 0° and the supernate was decanted. The residue was washed × 3 with the extracting buffer and centrifuged as before. The combined extract plus washings were dialysed against H_2O at 4° and used as the enzyme prepn. The washed residue was used for the assay of bound enzymes.

The enzyme prepns were assayed for soluble invertase, sucrose-UDP and sucrose-ADP glucosyltransferase, ADP glucose and UDP glucose pyrophosphorylase and soluble ADP(UDP) glucose starch synthetase [3,5]. However, ADP(UDP) glucose from the ADP(UDP) glucose pyrophosphorylase incubation was adsorbed on Whatman DE 81 DEAE cellulose paper instead of being isolated by PC. The washed residue after enzyme extraction was assayed for activity of bound invertase and bound ADP-glucose starch synthetase. All assays were done at 35°, except that of UDP(ADP) glucose starch synthetase, which was incubated at 37°.

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