Potato Transformation to Combine Natural and Engineered Resistance for Controlling Tuber Moth

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Abstract. **Potato tuber moth (*Phthorimaea operculella* Zeller) is a highly destructive pest of the cultivated potato (*Solanum tuberosum* L.) in the tropics and subtropics and causes significant damage to both leaves and tubers. Development of host plant resistance is a central component of an integrated pest management (IPM) program for potato tuber moth. The purpose of this research was to augment natural resistance by transforming potato with a codon-modified CryV-Bt gene using *Agrobacterium*-mediated techniques. 'Lemhi Russet' potato and two clones with different host plant resistance mechanisms, USDA8380-1 (leaf leptines) and L235-4 (glandular trichomes), were transformed with the CryV-Bt gene. Gene integration of regenerated plants was confirmed by polymerase chain reactions and Southern analyses; gene transcription was evaluated by northern analyses. Detached leaf bioassays showed that high levels of Bt expression occurred in the CryV-Bt transgenic lines ('Lemhi Russet' and L235-4), providing up to 96% control of potato tuber moth larvae, compared with 3% and 54% control in L235-4 and USDA8380-1, respectively. These transgenic lines can be used for breeding purposes to develop cultivars for (and eventual introduction into) IPM systems.**

One of the most important insect pests of the cultivated potato is the potato tuber moth, which causes damage to plants in the field and to tubers in storage in tropical and subtropical regions. Potato tuber moth larvae attack the potato by mining in the leaves and/or tubers (Trivedi and Rajagopal, 1992). Larvae tunnel and feed on the leaves, leaf veins, and stems of the plant, causing loss of leaf tissue, death of growing points, and weakening or breaking of the stems (Raman, 1980). The tubers are infested by larvae emerging from eggs deposited on the surface of the soil near the stem, in cracks in the soil, and near the eyes of stored tubers. The larvae mine into the tuber, causing irregular tunnels near the surface as well as inside the tuber. This mining introduces rot organisms and renders the tubers unfit for human consumption.

Host plant resistance is a potential component of an integrated pest management program to control the tuber moth. Two types of host plant resistance have been identified in potato: high levels of leptine glycoalkaloids (Sinden et al., 1986) and glandular trichomes (Plaisted et al., 1992). Introduced *Bacillus thuringiensis* (Bt) toxin genes offer an additional source of insect resistance. These Bt genes have been cloned, codon-modified, and inserted into various crop species (Barton and Miller, 1993). The efficacy of codon-modified Bt genes, such as Cry I and Cry III Bt, is greater than that of wild-type Bt genes when inserted into crop plants (Perlak et al., 1990; Wünn et al., 1996). The CryV-Bt gene we used in this study is effective against both Lepidoptera and Coleoptera and has also been codon-modified to increase its expression in plants (J. Tippet, personal communication).

The overall objective of this research was to use *Agrobacterium*-mediated transformation to combine the codon-modified CryV-Bt gene with natural mechanisms of resistance to the potato tuber moth. The specific research objectives were to: 1) obtain transgenic plants expressing the CryV-Bt toxin gene, and 2) document gene integration and expression via Southern analyses, northern analyses, and detached leaf bioassays.

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Materials and Methods

Vector construct. The codon-modified CryV-Bt gene (2200 bp) was obtained from Zeneca (Berkshire, U.K.) and was ligated into the *BamHI* site of pBI121 (Clontech, Palo Alto, Calif.) (Fig. 1). This vector construct (pBICryV) was transformed into *Agrobacterium tumefaciens* strain LBA4404 via tri-parental mating (Ditta et al., 1980).

Plant materials. The potato clones used for transformation experiments and potato tuber moth bioassays were USDA8380-1, a *Solanum chacoense* Bitt. (2n = 2x = 24) selection obtained from Steve Sinden, USDA/ARS, Beltsville, Md.; L235-4 (2n = 4x – 48), from R.L. Plaisted, Cornell Univ., Ithaca, N.Y.; and cv. Lemhi Russet (2n = 4x = 48). The first two genotypes were chosen because they possess natural host plant resistance factors; USDA8380-1 has a high concentration of leaf leptines (Sinden et al., 1986) and L235-4 has glandular trichomes (Plaisted et al., 1992). 'Lemhi Russet' is a potato tuber moth–susceptible commercial cultivar that also has high regeneration ability in tissue culture.

Transformation protocol. Stock plants of the three genotypes were micropropagated via shoot tips using MS inorganic salts plus thiamine and calcium pantothenic acid (Murashige and Skoog, 1962). Growth room conditions were 16 h of light under fluorescent tubes (30 $\mu E \cdot m^{-2} \cdot s^{-1}$) at 23 °C. The shoot regeneration protocol of Yadav and Sticklen (1995) was combined with *Agrobacterium*-mediated transformation as follows. Leaf sections were precultured on solidified M6 medium for 4 d, inoculated with *Agrobacterium tumefaciens* (LBA4404 with pBICryV vector) in log phase growth for 3 to 10 min, blotted, and returned to M6. Following 2 d of co-cultivation on M6, leaf sections were rinsed in liquid R4 medium and placed onto solidified R4 medium containing 200 $mg \cdot L^{-1}$ Timentin (ticarcillin disodium and clavulanic acid) (SmithKline Beecham, Philadelphia) and 50 $mg \cdot L^{-1}$ kanamycin. The leaf explants were transferred to fresh solidified R4 medium every 2 weeks. Forty leaf explants were used in the transformation experiments with 'Lemhi Russet' and USDA8380-1 and 80 leaf explants with L235-4. Healthy shoots were excised when 3 to 5 mm in length and transferred to a solidified medium containing MS inorganic salts, thiamine, and calcium pantothenic acid (Murashige and Skoog, 1962), plus 50 $mg \cdot L^{-1}$ kanamycin (15-mL per 25 × 100-mm culture tube). Individual shoots that rooted readily in selection medium were given a code and further propagated for molecular characterization and tuber moth bioassays.

Potato tuber moth bioassay. Potato tuber moth rearing and bioassays were carried out according to Westedt et al. (1998). First instar larvae were placed on detached leaves (with stems in vials of water). Mortality was determined after 48 h.

Molecular characterization of plants. Two 9-mm-diameter leaf disks were collected from each plant for genomic DNA isolation (Edwards et al., 1991). The DNA was quanti-

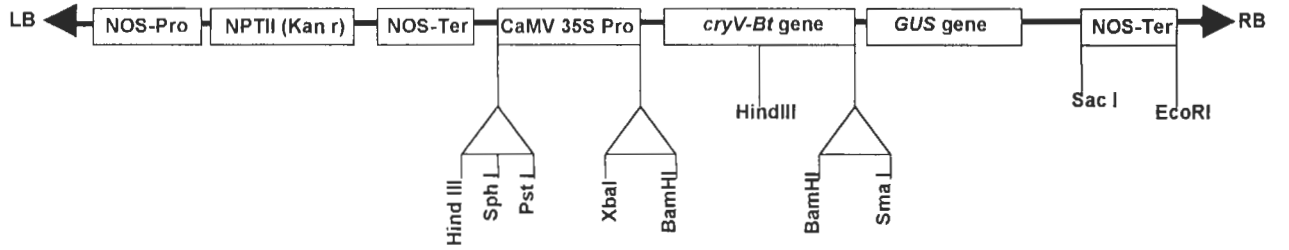

Fig. 1. Diagram of the tDNA region of the plasmid pBICryV. This gene construct was made by ligating the 2.2 kb CryV-Bt gene into the *BamHI* site adjacent to the CaMV 35S promoter within pBI121 (Clontech, Palo Alto, Calif.).

fied using a mini-fluorometer model TKO100 (Hoeffer Scientific, San Fernando, Calif.). DNA was amplified according to Gibco BRL (Bethesda, Md.) with the CryV-Bt (forward 5′AAC TGG AGG TCG GTG GTG CTG GCG T3′; reverse 5′GGA CCA TCG GCG GCA CCC TCA ACA T3′) and NPTII (forward 5′CGC AGG TTC TCC GGC CCG CTT GGG TG3′, reverse 5′AGC AGC CAG TCC CTT CCC GCT TCA G3′) primers producing 684 bp and 255 bp fragments, respectively. The amplification conditions were: 1 cycle = (4 min. at 94 °C); 40 cycles = (1 min at 94 °C, 1 min at 58 °C, 1.5 min at 72 °C). Once completed, a 30-µL aliquot of the polymerase chain reaction (PCR) reaction mix was separated on a 1% agarose gel and stained with ethidium bromide (0.5 µg·mL^{-1}).

Plants that had >80% potato tuber moth mortality in bioassays (Westedt et al., 1998) were also analyzed by Southern blot for gene integration and copy number. Total DNA was extracted according to the method of Saghai-Maroof et al. (1984), modified by using 2% beta-mercaptoethanol in the extraction buffer. The DNA (10 µg) from each line was digested with *BamHI* to cut out the CryV gene, and the copy number was analyzed by digesting with *XbaI*. The fragments were separated by electrophoresis through a 1.0% agarose gel, eluted onto a nylon membrane (Hybond N, Amersham, England) using a capillary transfer procedure (Sambrook et al., 1989). Nucleic acids were fixed to the membrane by autocrosslinking (Stratagene, LaJolla, Calif.). Prehybridization was conducted for 2 h at 42 °C in a solution containing 5× SSC (sodium salt citrate), 1% skim milk, 0.1% N-lauroylsarcosine, 0.02% SDS (sodium dodecyl sulfate), 50% formamide, and 125 µg·mL^{-1} sheared salmon sperm DNA. Hybridization was performed at 42 °C overnight in fresh solution with a nonradioactive random primed DIG-labeled probe [2.2 kb *BamHI* fragment containing the CryV gene was inserted into a Bluescript SK+ vector (Stratagene) amplified by PCR using the T7\T3 primers] according to manufacturer's instructions (Genius 2 Kit, BMB, Indianapolis). Following hybridization, the membrane was washed twice in 2× SSC plus 0.1% SDS for 15 min at room temperature, and twice in 0.5× SSC plus 0.1% SDS for 20 min at 65 °C. To detect chemiluminescence 0.25 mM disodium 3-{4-methoxyspiro[1,2-dioxetane-3,2′-(5′-chloro)tricyclo (3.3.1.3^{3,7}) decan]-4-yl} phenyl phosphate (CSPD, Tropix Inc., Bedford, Mass.) was added according to the manufacturer's instructions (Boehringer Mannheim, 1995). The membrane was then exposed to X-ray film (Kodak X-OMAT, Rochester, N.Y.) for 15 to 30 min.

Total RNA from a young leaf was isolated by using the Qiagen RNeasy Plant Total RNA Kit (Qiagen, Chatsworth, Calif.). Total RNA (20 µg) was fractionated by formaldehyde gel electrophoresis through a 1.0% agarose gel in MOPS buffer [20 mM MOPS, 5 mM sodium acetate, 1 mM EDTA (ethylenedinitrilotetra acetic acid), pH 7], followed by blotting onto a Hybond N nylon membrane. Prehybridization, hybridization, and detection conditions were as described above for DNA analysis, except that the blots were hybridized at 52 °C and a CryV RNA probe was used by synthesizing an in vitro transcript of the CryV DNA in a Bluescript SK+ vector (Stratagene) according to manufacturer's instructions (Genius 4 Kit, BMB).

Results

Plant transformation with CryV-Bt. Putative transgenic shoots were first produced on 'Lemhi Russet' explants after 4 weeks and shoot production continued for 2 months. A total of 60 individual shoots were removed and transferred to kanamycin rooting medium (50 mg·L^{-1}). The first 33 shoots to root in this medium were then assayed to determine the presence of the CryV-Bt gene. A total of 27 of these 33 lines were PCR-positive for the CryV-Bt gene. Nontransgenic plants often developed roots, but these roots only grew on the medium surface and up the side of the culture tube. In contrast, transgenic lines produced roots quickly and the roots spread throughout the medium.

Clone USDA8380-1 was slower to regenerate shoots, which emerged from 8 to 12 weeks after transformation. However, once emerged, they grew rapidly in the kanamycin rooting medium. Initially, 60 shoots were selected, of which 30 rooted. All but one of these rooted plants were PCR-positive for CryV-Bt.

Clone L235-4 was the most difficult genotype from which to obtain transgenic plants. Using twice as many leaf disks for L235-4 as for 'Lemhi Russet', 45 healthy shoots were obtained for the rooting assay. Shoot regeneration occurred after 8 to 12 weeks. Fifteen shoots rooted in the kanamycin rooting medium and 14 of these were PCR-positive for the CryV-Bt gene.

Potato tuber moth bioassays. Eighteen PCR-positive 'Lemhi Russet' CryV-Bt and nontransgenic 'Lemhi Russet' lines were used in potato tuber moth bioassays. Mortality of the moths ranged from 20% to 95% among the transgenic 'Lemhi Russet' lines, with all transgenic lines producing higher mortality than the nontransgenic lines (data not shown). Mortality on the eight most resistant 'Lemhi Russet' lines was 83% to 93%, significantly higher than on the control leaves (Table 1).

Twenty-five transgenic lines of USDA8380-1 CryV-Bt were tested. The nontransgenic USDA8380-1 caused 54% mortality in larvae. A wide range of potato tuber moth mortalities were observed among larvae on the USDA8380-1 CryV-Bt lines (40% to 100%) (data not shown). Larvae on the seven most resistant USDA8380-1 CryV-Bt lines

Table 1. Mortality[z] of potato tuber moth larvae on foliage of three cultivars of potato and their CryV-Bt transgenic lines in detached leaf bioassays.

Line	Mortality (%)	SD[y]	Line	Mortality (%)	SD[y]	Line	Mortality (%)	SD[w]
'Lemhi Russet'	3	4.7	USDA 8380-1	54	9.1	L235-4	4	8.0
Lemhi-1	90	2.7	8380-1.1	75	9.3	L235-4.3	96	4.6
Lemhi-7	93	2.7	8380-1.5	78	9.3	L235-4.5	90	4.6
Lemhi-12	83	2.7	8380-1.9	88	9.3	L235-4.8	96	4.5
Lemhi-14	93	2.7	8380-1.16	80	9.4	L235-4.11	90	4.6
Lemhi-15	83	2.7	8380-1.18	78	8.5	L235-4.12	90	4.6
Lemhi-17	88	3.9	8380-1.19	69	9.2	L235-4.13	96	4.5
Lemhi-21	86	2.7	8380-1.25	87	9.3	L235-4.14	88	4.6
Lemhi-22	88	3.9	8380-1.26	78	9.3	L235-4.16	96	4.5

[z]All values significantly different from the respective nontransformed lines at $P < 0.05$, Wilcoxon test.
[y]Three replications per treatment.
[x]Eight replications per treatment.
[w]Five replications per treatment.

exhibited 69% to 88% mortality, significantly higher than for larvae on the control leaves (Table 1).

Eight L235-4 CryV-Bt lines were tested in moth feeding bioassays. Mortality of larvae ranged from 88% to 96% on leaves from the L235-4 CryV-Bt lines, compared with 4% on nontransformed L235-4 leaves (Table 1).

Molecular characterization of CryV-Bt-transgenic plants. The CryV-Bt transgenic lines of 'Lemhi Russet' (8), L235-4 (8), and USDA8380-1 (7) that induced high levels of potato tuber moth mortality were further analyzed in Southern and northern blot analyses. The Southern blot analyses confirmed the presence of the CryV-Bt gene in all these lines except USDA8380-1.18 (lane 13) (Fig. 2A). CryV-Bt gene copy number ranged from one to three (Fig. 2B). This analysis also identified duplicate lines (Lemhi-6 and Lemhi-14; L235-4.8 and L235-4.13) (lanes 3, 6, 7, and 8, Fig. 2B). Northern blot analysis of leaf total RNA showed that CryV-Bt transcription levels were similar in all but one line. Transgenic line USDA8380-1.19 (lane 11) caused the lowest level of moth mortality in the leaf bioassay and also had a lower level of CryV-Bt RNA (Fig. 3). The line USDA8380-1.18, which was negative in the Southern analysis, was also negative in the northern analysis (lane 12) (Fig. 3). Tuber CryV-Bt transcription levels were not examined in this study.

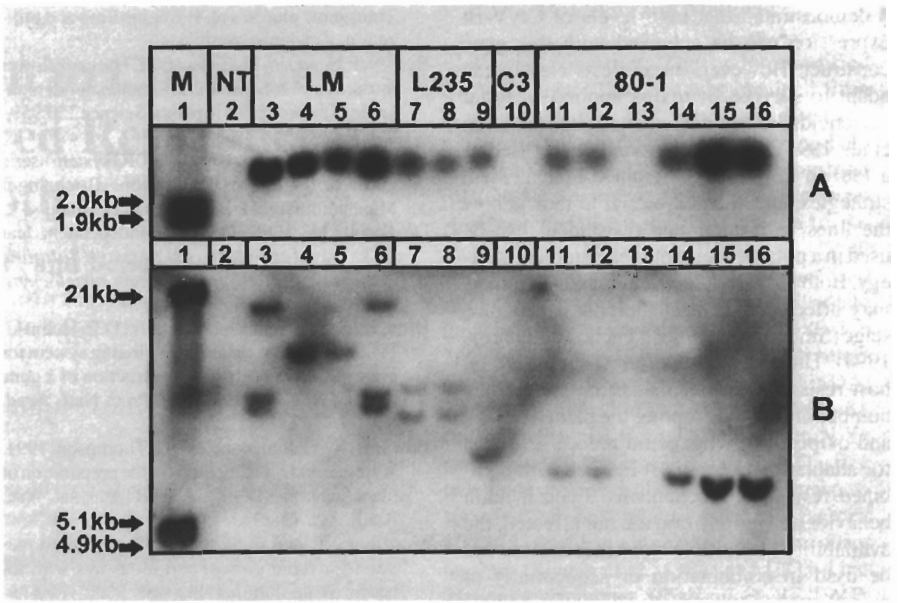

Fig. 2. (**A**) Southern blot of *BamHI* digested DNA (10 µg) from various CryV-Bt lines. Lane 1 is lambda DNA digested with HindIII/EcoRI (M). DNA of nontransformed 'Atlantic' is in lane 2 as a negative control (NT). The CryV-Bt gene (2.2 kb) was used as the probe. Lane 10 contains DNA of a CryIII-transgenic potato line (C3). LM, L235, and 80-1 refer to CryV-Bt-transgenic lines of 'Lemhi Russet', L235-4, and USDA8380-1, respectively. Hybridized fragments of 2.2 kb were observed for all transgenic lines except USDA8380-1.18 (lane 18). (**B**) A Southern blot of EcoRI digested DNA of same samples above shows copy number of CryV-Bt gene. Copy number of the CryV-Bt gene ranged from 1 to 3.

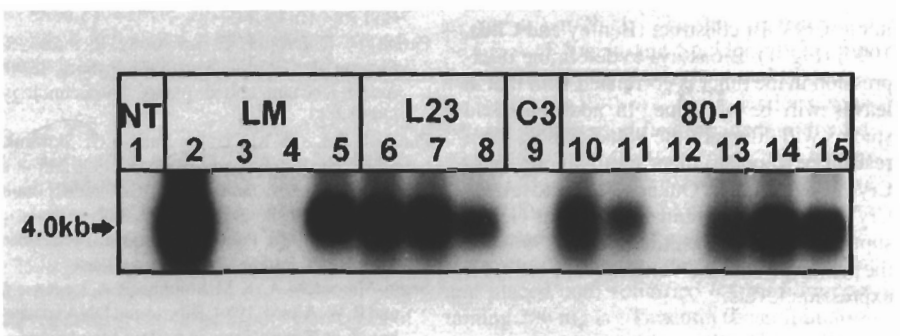

Fig. 3. Northern analysis of total RNA from various CryV-Bt lines probed with CryV-Bt gene. CryV-Bt/GUS fusion mRNA transcripts (5.0 kb) were observed for all lines except Lemhi-7, Lemhi-13, and USDA8380-1.18 in lanes 3, 4, and 12, respectively. Lane 1 contains RNA of nontransgenic 'Atlantic' (NT) and lane 9 RNA of a CryIII-Bt transgenic line (C3). A lower hybridization was observed for USDA8380-1.18 (lane 11). LM, L235, and 80-1 refer to CryV-Bt-transgenic lines of 'Lemhi Russet', L235-4, and USDA8380-1, respectively.

Discussion

Our purpose was to combine natural host plant resistance mechanisms with the CryV-Bt gene via transformation, thus pyramiding host plant resistance mechanisms against potato tuber moth. The *Agrobacterium*-based transformation/shoot regeneration protocol proved highly efficient with 165 total shoots recovered from three genotypes. Rooted regenerates (75) were tested by PCR and 67 were confirmed PCR-positive. Among the PCR-positive lines, a total of 16 CryV-Bt transgenic lines with 80% or greater potato tuber moth mortality were obtained from both 'Lemhi Russet' and L235-4.

The adventitious shoot regeneration protocol of Yadav and Sticklen (1995) used in this research was combined with *Agrobacterium tumefaciens*–mediated transformation procedures to obtain transgenic plants. This regeneration protocol produced transgenic lines for all three potato genotypes (two with wild *Solanum* spp. background), suggesting broad genotypic adaptability of this protocol. In comparison, Sheerman and Beven (1988) experienced highly genotype-dependent responses to their regeneration protocol and did not recover any transgenic lines for some potato genotypes. The De Block (1988) transformation/shoot regeneration protocol gave mixed results in our laboratory in previous studies, producing many regenerates and transgenic plants from some cultivars and few to none for others (data not shown). Stiekema et al. (1988) used tuber disks as the explant source for *Agrobacterium*-mediated transformation and only 1% of recovered shoots were transgenic. Newell et al. (1991) proposed a transformation technique using potato stem sections. Transgenic plant recovery was 2% to 5%. Our transgenic plant recovery was at the 30% to 50% level; however, these numbers may be biased since duplicate shoots were observed in the Southern analysis (Fig. 2b).

Some somaclonal variation was observed among the regenerated plants. Minor distortion of leaf morphology was observed in two of the low-expressing 'Lemhi Russet' transgenic lines. These lines were not selected for further investigation. Two shoots, one each from 'Lemhi Russet' and L235-4, produced plants which lacked apical dominance. Somaclonal variation from the regeneration process may also affect the naturally bred resistance factors, leptines, and glandular trichomes. Further investigation into CryV-Bt expression and leptine levels among the transgenic lines would resolve this dilemma. Western blot analysis and HPLC of CryV-Bt protein levels and leptine levels (Sinden, 1987), respectively, would apply.

The vector construct used in this study had the ß-glucuronidase (GUS) gene fused to the CryV-Bt gene, with the CaMV 35S promoter controlling both genes (Fig. 1). The results of the tuber moth bioassays demonstrated that high levels of expression of the CryV-Bt can be obtained. Simultaneously, northern blot analysis showed that the GUS gene was also transcribed in these CryV-Bt transgenic lines. Comparison of the expression of CryV-Bt constructs in potato with and without the GUS gene would be of interest.

The bioassay results from the CryV-Bt transgenic lines of 'Lemhi Russet' and L235-

4 demonstrated that high levels of CryV-Bt expression can be achieved with this gene construct. However, insect pests clearly can adapt to survive Bt toxins applied as foliar insecticides or engineered into plants (Whalon et al., 1993). If Bt expression is to be used as a tool in host plant resistance management strategies, then the next step is to look at how the lines with combined resistances can be used in a potato tuber moth management strategy. Both the glandular trichomes and leptines may affect the moth at the host acceptance stage (Sinden et al., 1986; Yencho and Tingey, 1994). Under field conditions, these natural host resistance mechanisms may reduce the number of moths accepting the plant as a host and ovipositing. This could reduce selection for adaptation to CryV-Bt in lines with combined resistance mechanisms. Even if adult behavior and oviposition are not affected, the availability of multiple resistance factors can be used in combination or sequentially to manage potato tuber moth adaptation to host plant resistance factors.

The leaf bioassay used in these studies demonstrated high levels of Bt expression in the leaf; however, the tuber is the other economically damaged organ of the potato plant. The next key step is to determine whether potato tuber moth larvae are controlled by CryV-Bt in the tuber. The constitutive expression promoter, CaMV 35S promoter, was used in our CryV-Bt construct (Benfey and Chua, 1990) (Fig. 1). Bioassays to determine if expression in the tuber is correlated with that in leaves will be of value. In addition, field studies will compare the value of host plant resistance to potato tuber moth among these CryV-Bt genotypes. Our next step is to test the CryV-Bt gene in combination with the Gelvin super promoter (Narasimhulu et al., 1996) and the patatin promoter (Wenzler et al., 1989) for expression levels.

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