研究紹介

リンゴ小球形潜在ウイルスベクターを用いたサクラ属果樹のウイルス誘導性ジーンサイレンシングに関する研究

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Virus-induced gene silencing in *Prunus* fruit tree species with the *Apple latent spherical virus* vector

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Virus-induced gene silencing (VIGS) has been used as a rapid and effective tool for functional analysis of genes in various plants, including woody fruit tree species. In this study, we attempted to develop a VIGS-based gene evaluation system for seven *Prunus* species, including apricot (*P. armeniaca* L.), sweet cherry (*P. avium* L.), almond [*P. dulcis* (Mill.) D. A. Webb.], peach (*P. persica* Batsch), Japanese apricot (*P. mume* Siebold & Zucc.), Japanese plum (*P. salicina* Lindl.), and European plum (*P. domestica* L.), with the *Apple latent spherical virus* (ALSV) vectors. ALSV vectors carrying part of the apricot *PHYTOENE DESATURASE* (*PDS*) gene sequence were amplified in *Nicotiana benthamiana*, and inoculated into the cotyledons of *Prunus* seedlings by particle bombardment. Typical *PDS*-silenced phenotypes, characterized by uniform discoloration of the upper leaves, were observed in sweet cherry and some cultivars of apricot and almond several weeks after inoculation. In contrast, attempted ALSV infections of Japanese apricot, Japanese plum, European plum, and the other cultivars of apricot and almond were unsuccessful. Furthermore, although the infection rate of ALSV in peach was high, severe viral infection symptoms were observed in the infected leaves. These results collectively suggested that the efficiency of ALSV infection and VIGS could vary depending on species and/or cultivar in *Prunus*. The possible use of the ALSV-mediated VIGS system for functional analysis of genes in *Prunus* is discussed.

Key words : gene evaluation system, post-transcriptional gene silencing, virus vector

Introduction

VIGS is a useful reverse genetics tool for functional analysis of plant genes. This approach is based on plant endogenous defense responses against invading foreign agents such as viruses or viroids, and used to induce the knock-down of target gene expression through a post-transcriptional gene silencing (PTGS) mechanism. VIGS is triggered by the infection of recombinant virus vectors carrying partial sequences of the target genes to be silenced. Plant mRNA corresponding to the partial sequence carried by the viral vectors is degraded in a homology-dependent manner[1-3]. So far, more than 37 different plant viruses have been developed as VIGS-vectors[4], and are increasingly used for functional analysis of genes in various plant species because they can induce rapid knock-down phenotypes of target genes without the lengthy transformation step using *Agrobacterium*[5-7]. Although most virus vectors were initially established for herbaceous model plants, recent studies have shown the successful application of some virus vectors for the silencing of endogenous genes in woody fruit tree species[8-10].

Among the various virus vectors reported, ALSV vectors are particularly promising because they have been used to effectively induce VIGS in a broad range of plant species including rosaceous fruit tree species[11,12]. ALSV consists of isometric virus particles ca. 25 nm in diameter, and contains two ssRNA species (RNA1 and RNA2) and three capsid proteins (Vp25, Vp20, and Vp24)[13]. It can induce systemic VIGS including in the meristematic region without causing any viral symptoms in most host plants[11,12]. Because of these advantages, ALSV vectors have been used not only for VIGS-based functional analyses of plant genes but also for various practical studies[14-16]. Although the ALSV vector system has been applied to a limited number of fruit tree species at present, it has the potential to be used for the evaluation of gene functions or genetic improvement in a broader range of fruit tree species considering the wide host range of ALSV.

Prunus, which belongs to the family Rosaceae, includes many economically important fruit and nut tree species such as apricot, sweet cherry, almond, peach, Japanese apricot, Japanese plum, and European plum. Because of

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their relatively small genome size and short juvenile phase, some *Prunus* species have been used as genetic and morphological experimental models of woody fruit and nut plants to identify genes associated with agriculturally important traits[17-21]. So far, many candidate genes that could be used as potential molecular markers in breeding programs have been identified. However, functional verification of these genes has lagged behind because of the lack of an efficient transformation system in *Prunus*. The development of an efficient gene evaluation system in *Prunus* is therefore very important for rapid progress in *Prunus* genetics and breeding.

As a first step toward the establishment of an efficient gene evaluation system in *Prunus*, we previously showed that ALSV vectors could successfully induce VIGS of the endogenous *PDS* gene in apricot[22]. In this study, we assessed the VIGS efficiency of ALSV vectors in a wide range of *Prunus* species and cultivars. Based on the results, possible use of the ALSV-mediated VIGS system for gene function analysis in *Prunus* species is discussed.

Materials and Methods

Plant materials

As shown in Table 1, sixteen cultivars of 7 fruit tree species of *Prunus* were used in this study. Seeds of these cultivars were germinated under 4 ℃, dark conditions, and the seedlings just after germination were used for viral inoculation by particle bombardment. *N. benthamiana* plants were used to produce and amplify recombinant ALSV particles for use as an inoculum in particle bombardment. They were grown under 16 / 8 h LD conditions at 23℃. Young plants at the 3 - 4 leaf stage were used for *Agrobacterium*-mediated viral inoculation.

Table 1　Infection rates of ParPDS-ALSV in *Prunus* species one month post inoculation

Plant species	Cultivars	Number of infected/ inoculated seedlings	Infection rate (%)
Apricot (*P. armeniaca*)	'Shinyo'	2/18	11.1
	'Shingetsu'	3/24	12.5
	'Shinshuomi'	2/21	9.5
	'Nanbuhachisuke'	0/22	0
	'Niigataomi'	0/15	0
Sweet cherry (*P. avium*)	'Satonishiki'	6/30	20
Almond (*P. dulsis*)	'Nonpareil'	12/40	30
	'Carmel'	5/17	29.4
	'Marcona'	0/10	0
Peach (*P. persica*)	'Ohatsumomo'	12/14	85.7
Japanese apricot (*P. mume*)	'Ryukyokoume'	0/9	0
	'Benisashi'	0/19	0
	'Koshinoume'	0/17	0
	'Hachiro'	0/31	0
Japanese plum (*P. salicina*)	'Sordum'	0/20	0
European plum (*P. domestica*)	'Sanctus Hubertus'	0/27	0

Amplification of ALSV vectors in *N. benthamiana*

To assess the VIGS efficiency of ALSV vectors in various *Prunus* species, we selected the *PDS* gene, which encodes a key enzyme in carotenoid biosynthesis, as a target of silencing[23]. To express recombinant ALSV vectors targeting the *PDS* gene of each *Prunus* species, the binary plasmids pBICAL1 and pBICAL2-ParPDS described previously[22] were used in this study. Briefly, pBICAL1 contains an expression cassette of ALSV RNA1 between CaMV35S promoter and nos terminator sequences. pBICAL2-ParPDS contains a 108 bp fragment of the apricot *PDS* (*ParPDS*) gene in-frame with an expression cassette of ALSV RNA2 located between CaMV35S promoter and nos terminator sequences. These constructs were separately introduced into a disarmed *Agrobacterium* strain, EHA105, and inoculated into *N. benthamiana* using a toothpick as described previously[22]. The resultant recombinant virus was designated ParPDS-ALSV. To confirm successful infection and amplification of ParPDS-ALSV, microtissue direct RT-PCR was performed 2 - 3 weeks post inoculation (wpi) according to Kawai et al. (2014)[22].

Viral inoculation of *Prunus* seedlings

Total RNA containing ParPDS-ALSV was extracted from infected *N. benthamiana* using TRIzol reagent (Life Technologies, Carlsbad, CA, USA), purified by phenol/chloroform extraction, and used for viral inoculation by particle bombardment essentially as described previously[22,24]. The seed coats were gently removed from *Prunus* seedlings just after germination, and their cotyledons were bombarded with gold particles coated with total RNA using a Helios Gene Gun system (BIO-RAD, Hercules, CA, USA). After acclimatization, the inoculated seedlings were planted in soil and grown in a growth chamber under 16 / 8 h LD conditions at 22 ℃.

RT-PCR and tissue blot analyses

Total RNA was extracted from the upper leaves of inoculated *Prunus* seedlings about 1 month post inoculation (mpi) by a small scale CTAB method according to Sasaki et al. (2011)[12], but with slight modifications. After cDNA synthesis, RT-PCR for detection of ALSV

and real-time RT-PCR analysis of the mRNA levels of the *PDS* genes were carried out as described previously[22]. For inoculated peach seedlings, lower and upper leaves were collected about 2.5 mpi, and used for RT-PCR and tissue blot analyses to confirm the distribution of ALSV in these leaves as described previously[25].

Results and discussion

Infection of *Prunus* seedlings with ALSV vectors

ParPDS-ALSV was detected in the inoculated seedlings of apricot 'Shinyo' (11.1%), 'Shingetsu' (12.5%), and 'Shinshuomi' (9.5%), sweet cherry 'Satonishiki' (20%), almond 'Nonpareil' (30%) and 'Carmel' (29.4%), and peach 'Ohatsumomo' (85.7%) at about 1 mpi (Table 1). The infection rates of ParPDS-ALSV in apricot cultivars were consistent with that in apricot 'Heiwa' (11.1%) as reported previously[22]. Apricot, sweet cherry, and almond seedlings infected with ALSV exhibited no obvious viral symptoms. This indicates that ALSV can be used effectively for gene functional evaluation in these species because viral symptoms often disturb the correct observation of knock-down phenotypes of VIGS-target genes. Although the infection rate of ParPDS-ALSV in 'Ohatsumomo' peach was highest among the inoculated *Prunus* species and cultivars, the infected seedlings showed severe viral symptoms (pale spots) in the upper leaves (Fig. 4 A and B ; see details later). We could not detect ParPDS-ALSV from the inoculated seedlings of apricot 'Nanbuhachisuke' or 'Niigataomi', almond 'Marcona', Japanese apricot 'Ryukyokoume', 'Benisashi', 'Koshinoume', or 'Hachiro', Japanese plum 'Sordum', or European plum 'Sanctus Hubertus' (Table 1). These results indicated that ALSV susceptibility could vary depending on species and/or cultivar in *Prunus*.

VIGS of *PDS* genes in apricot, sweet cherry, and almond

In apricot, 2 of the 18 inoculated seedlings of 'Shinyo', 3 of the 24 inoculated seedlings of 'Shingetsu', and 2 of the 21 inoculated seedlings of 'Shinshuomi'were successfully infected with ParPDS-ALSV (Table 1). Uniform discoloration of the upper leaves, a characteristic phenotype of *PDS* downregulation, was observed in all infected seedlings 3－5 wpi (Fig. 1 A–C). Real-time RT-PCR indicated that the amounts of *PDS* mRNA in the photo-bleached leaves were significantly decreased compared with those in healthy leaves with no viral inoculation (Fig. 1 D). These results suggested that

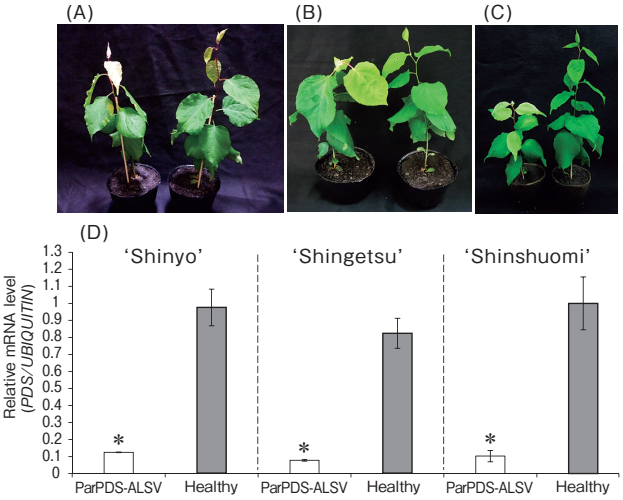

Fig. 1　VIGS of the *PDS* gene in apricot seedlings infected with ALSV vectors.
(A) Seedlings of 'Shinyo', (B) 'Shingetsu', and (C) 'Shinshuomi' infected with ParPDS-ALSV (left) and healthy plants with no viral inoculation (right) (42, 41, 36 dpi, respectively). (D) Real-time RT-PCR analysis of *PDS* mRNA levels in the leaves of apricot seedlings infected with ALSV vectors. *PDS* mRNA levels are shown as mean values ± SE (n = 2–3 ; biological replicates) normalized against the *UBIQUITIN* gene. ParPDS-ALSV, photo-bleached leaves infected with ParPDS-ALSV ; Healthy, healthy leaves with no viral inoculation. A single asterisk indicates significant difference ($P < 0.05$) using Student's *t*-test.

ALSV could successfully induce VIGS of *PDS* genes in these apricot cultivars.

In sweet cherry, 6 of the 30 inoculated seedlings of 'Satonishiki' were successfully infected with ParPDS-ALSV (Table 1). The upper leaves of all infected seedlings exhibited uniform photo-bleached phenotypes 2－3 wpi (Fig. 2 A). Real-time RT-PCR showed that the amounts of *PDS* transcript in the photo-bleached leaves were significantly reduced compared with those in uninoculated leaves (Fig. 2 B). These results indicated that VIGS of *PDS* genes was effectively induced by ALSV vectors in sweet cherry 'Satonishiki'.

In almond, 12 of the 40 inoculated seedlings of 'Nonpareil' and 5 of the 17 inoculated seedlings of 'Carmel' were successfully infected with ParPDS-ALSV (Table 1). Uniform discoloration of newly generated leaves and stipules was observed in all infected almond seedlings 2－4 wpi (Fig. 3 A and B). The *PDS* mRNA levels were distinctly decreased in the photo-bleached leaves compared with those in the control leaves (Fig. 3 C). These results suggested that ALSV infection could trigger efficient knock-down of the *PDS* genes in these almond cultivars.

The above results collectively suggested that ALSV vectors could induce effective VIGS of *PDS* genes in apricot, sweet cherry, and almond, if viral infection was successfully established. However, this study highlighted several issues with regard to the stability of VIGS ; new yellow or light green upper leaves were generated in some infected seedlings of apricot and almond later in their growth. In these plants, the VIGS efficiency seemed to decrease, probably because of a lower virus concentration or environmental factors. In fact, it has been reported in herbaceous plants that the efficiency or stability of VIGS, or amounts of virus or virus-derived siRNAs, can vary depending on growth conditions such as temperature or humidity[26–28]. To establish an efficient and stable VIGS system that can be used for long-term functional evaluation of diverse target genes, we need to optimize growth conditions, considering their integrative effects on VIGS efficiency in *Prunus* species.

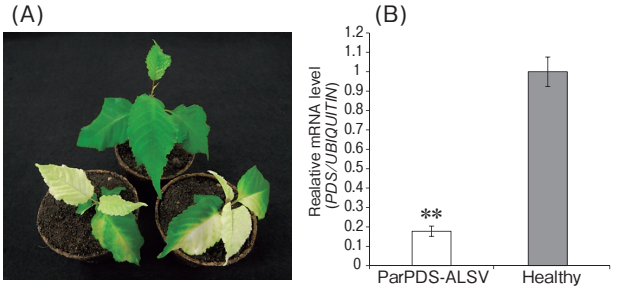

Fig. 2　VIGS of the *PDS* gene in sweet cherry seedlings infected with ALSV vectors.
(A) Seedlings of 'Satonishiki' infected with ParPDS-ALSV (below) and a healthy plant with no viral inoculation (above) (36 dpi). (B) Real-time RT-PCR analysis of *PDS* mRNA levels in the leaves of sweet cherry seedlings infected with ALSV vectors. For details of real-time RT-PCR analysis, see the legend of Fig. 1. Double asterisks indicate significant difference ($P < 0.01$) using Student's *t*-test.

Viral symptoms in infected peach seedlings

In peach, 12 of the 14 inoculated seedlings of 'Ohatsumomo' were infected with ParPDS-ALSV (Table 1). Although a high infection efficiency was obtained, leaves with severe pale spot symptoms were observed in infected peach seedlings about 2 wpi (Fig. 4 A and B). Unlike apricot, sweet cherry, and almond, neither discoloration of the upper leaves nor a significant reduction in the amount of *PDS* mRNA was observed in infected peach seedlings (Fig. 4 A–D). This result suggested that ALSV vectors could not effectively induce VIGS of the *PDS* gene in peach 'Ohatsumomo'. About 50 days after bombardment, normal leaves formed on the branches from axillary buds, while the main branch stopped growing because of severe distortion of leaves and the shoot tip (Fig. 4 C). We investigated whether ALSV was present in the newly generated asymptomatic upper leaves, by RT-PCR and tissue blot analyses. In both analyses, ALSV was detected only from lower leaves with symptoms, not from the upper leaves generated after the symptoms disappeared (Fig. 4 E ; data not shown). These results indicated that the application of ALSV-based VIGS for functional analysis of genes may be difficult in peach. It is still unclear whether other cultivars or genotypes of peach respond in the same way as 'Ohatsumomo'. As peach is one of the most economically and experimentally important fruit trees in *Prunus*, it is worth studying ALSV infectivity and VIGS efficiency using various peach genotypes to assess whether a VIGS-based gene evaluation system can be used in

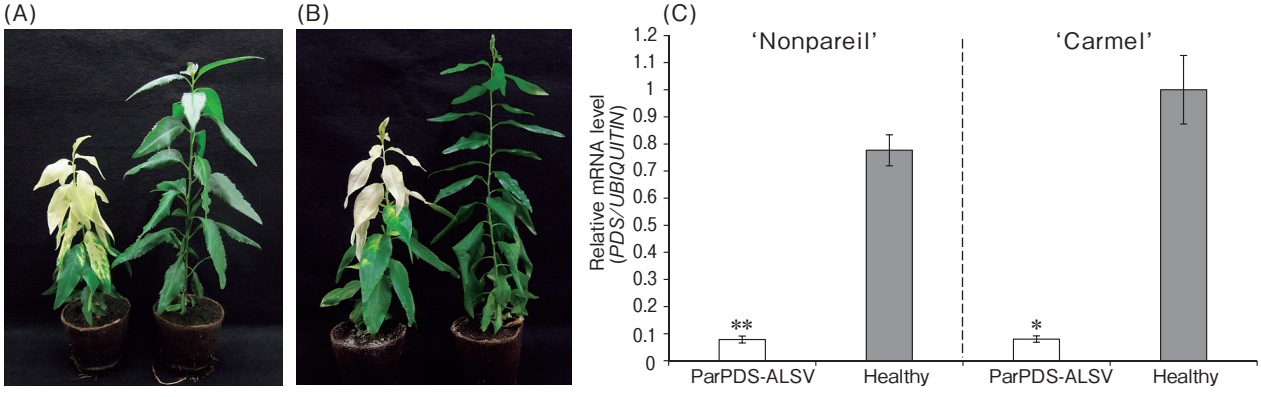

Fig. 3　VIGS of the *PDS* gene in almond seedlings infected with ALSV vectors.
(A) Seedlings of 'Nonpareil' and (B) 'Carmel' infected with ParPDS-ALSV (left) and healthy plants with no viral inoculation (right) (44 and 43 dpi, respectively). (C) Real-time RT-PCR analysis of *PDS* mRNA levels in the leaves of almond seedlings infected with ALSV vectors. For details of real-time RT-PCR analysis, see the legend of Fig. 1. Single and double asterisks indicate significant difference ($P < 0.05$ and 0.01, respectively) using Student's *t*-test.

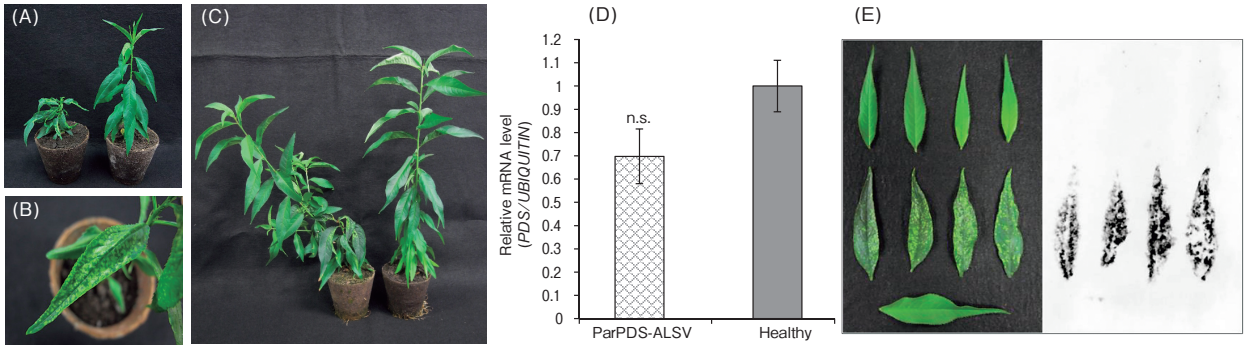

Fig. 4　Viral symptoms in peach seedlings infected with ALSV vectors.
(A) A seedling of 'Ohatsumomo' infected with ParPDS-ALSV (left) and a healthy plant with no viral inoculation (right) at 30 dpi. (B) A leaf showing pale spot symptoms. (C) A peach seedling infected with ParPDS-ALSV (left) and a healthy plant (right) at 87 dpi. (D) Real-time RT-PCR analysis of *PDS* mRNA levels in the leaves of peach seedlings infected with ALSV vectors. For details of real-time RT-PCR analysis, see the legend of Fig. 1. ParPDS-ALSV, leaves showing symptoms after infection with ParPDS-ALSV ; Healthy, healthy leaves with no viral inoculation. n.s. indicates non-significant ($P < 0.05$) using Student's *t*-test. (E) Tissue blot analysis of lower and upper leaves of 'Ohatsumomo' seedlings infected with ALSV vectors. Upper leaves with no symptoms (above), lower leaves with symptoms (middle), and a healthy leaf with no viral inoculation (below) are shown. The left and right pictures are of the same leaves.

peach.

Conclusion

ALSV-mediated VIGS could be a useful reverse genetic tool for the functional study of genes associated with agriculturally important traits in *Prunus*. The present study demonstrated that ALSV vectors could effectively induce VIGS of endogenous *PDS* genes in some cultivars of apricot, sweet cherry, and almond, although ALSV infectivity and VIGS efficiency seemed to vary depending on species and/or cultivar in *Prunus*. Further optimization of viral inoculation procedures and growth conditions for infected plants will lead to the full use of ALSV vectors for evaluation of gene functions in various *Prunus* species. Nonetheless, the ALSV-mediated VIGS system reported here has the potential to enable high-throughput functional genomics that can facilitate the application of molecular biological and genetic information in *Prunus* breeding programs.

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リンゴ小球形潜在ウイルスベクターを用いたサクラ属果樹のウイルス誘導性ジーンサイレンシングに関する研究

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ウイルス誘導性ジーンサイレンシング（virus-induced gene silencing；VIGS）は，迅速かつ効率的な遺伝子機能評価の手法として，木本性の果樹種を含む様々な植物で利用されている．本研究では，アンズ（*Prunus armeniaca* L.），カンカオウトウ（*P. avium* L.），アーモンド［*P. dulcis*（Mill.）D. A. Webb.］，モモ（*P. persica* Batsch），ウメ（*P. mume* Siebold & Zucc.），ニホンスモモ（*P. salicina* Lindl.）およびヨーロッパスモモ（*P. domestica* L.）を含むサクラ属果樹 7 種を対象に，リンゴ小球形潜在ウイルス（*Apple latent spherical virus*；ALSV）ベクターを用いた VIGS による遺伝子機能評価系の開発を試みた．アンズのフィトエン不飽和化酵素（*PHYTOENE DESATURASE；PDS*）遺伝子の部分配列を有する ALSV ベクターを *Nicotiana benthamiana* で増幅し，遺伝子銃を用いてサクラ属果樹の子葉に接種した．接種数週間後，カンカオウトウ，およびアンズ，アーモンドの数品種の上位葉において *PDS* のサイレンシングによる白化が観察された．一方，ウメ，ニホンスモモ，ヨーロッパスモモ，およびアンズ，アーモンドのそれ以外の品種では ALSV の感染は確認できなかった．さらに，モモは高い ALSV 感染率を示したが，感染葉において激しい病徴が観察された．これらの結果は，サクラ属果樹の種や品種によって ALSV 感染や VIGS の効率が異なることを示唆している．サクラ属果樹の遺伝子機能評価への利用を見据え，ALSV ベクターを用いた VIGS の有効性について議論した．

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