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1 **Enhanced production of antiviral dsRNA in the *Chlamydomonas reinhardtii* chloroplast via**
2 **a novel convergent promoter expression system**

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1 **Abstract**

2 The present work presents an improvement of microalgal antiviral dsRNA production for controlling disease
3 in shrimp aquaculture. 307 bp of sequence targeting the VP28 gene of white spot syndrome virus (WSSV) was inserted
4 between two convergent rrnS promoters in the novel vector p2XTRBL, which was then subcloned into the
5 transformation vector pSS116 using Golden Gate assembly. The recombinant plasmid was transformed into the
6 *Chlamydomonas reinhardtii* chloroplast, and transformants selected by the restoration of photosynthesis. The presence
7 of the cassette and homoplasmy of the algal transformants was confirmed by PCR analysis. Transcribed sense and
8 antisense VP28-RNA were hypothesised to form an RNA duplex in the chloroplast stroma, and quantitative RT-PCR
9 indicated that ~100 µg dsRNA was obtained per litre of transgenic microalgae culture. This accumulation of dsRNA
10 represents a 10,000-fold increase relative to previous reports using convergent *psaA* promoters. Recombinant *C.*
11 *reinhardtii* was assessed for its ability to prevent WSSV infection in shrimp larvae by direct feeding. After WSSV
12 challenge, the survival of shrimp treated with dsRNA-expressing *C. reinhardtii* was significantly enhanced (95.2%)
13 relative to the negative control without dsRNA treatment. The study suggests that this new algal production platform
14 for dsRNA is significantly more efficient than the previous report, and it merits further scale-up and downstream
15 processing studies.

16 **Keywords**: *Chlamydomonas reinhardtii*; double-stranded RNA; shrimp diseases; white spot syndrome virus;
17 chloroplast transformation

18

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9 **Authors' contributions:** SP and HT designed and constructed the p2xTRBL and pSS116 vectors for chloroplast
10 transformation, designed the golden gate strategy used for plasmid assembly, and created the TN72 recipient *C.*
11 *reinhardtii* strain. PC and CW constructed transformation plasmids and performed chloroplast transformation and
12 transformant selection. PC determined dsRNA production yield, performed the shrimp trial experiment, analysed and
13 interpreted the data. PL performed dsRNA confirmation and harvesting time experiment. PC, SP, HT, VS, and CR
14 drafted the manuscript. VS, CR, and SP supervised the project. The authors declare no conflict of interest.

15 **Additional declarations for articles in life science journals that report the results of studies involving humans**
16 **and/or animals:** Experiments related to shrimps used in this study were approved by BT-IACUC, protocol no. BT
17 Animal 8/2562.

18 **Ethics approval:** All procedures performed in studies involving animal were in accordance with the guidelines of the
19 Australian, New South Wales state government for the humane harvesting of fish and crustaceans.

20 **Consent to participate:** All authors have consented to participate in this manuscript.

21 **Consent for publication:** All authors have consented to the submission of the case report to the journal.

22

23

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1 1. **Introduction**

2 White Spot Syndrome Virus (WSSV) is the causative agent of White Spot Syndrome Disease (WSSD) in penaeid
3 shrimp, including commercially relevant species such as Black Tiger prawns, King Prawns, and Atlantic White
4 Shrimp. WSSD is widely regarded as the most detrimental disease seen in commercial aquaculture of these organisms,
5 causing up to 100% mortality within 3-5 days (Oakey et al. 2019). The virus was first identified during a 1992 outbreak
6 in mainland China and Taiwan, where it is still endemic in shrimp cultivation.

7 The detrimental commercial impact of WSSV has led to considerable research efforts towards developing
8 mitigation strategies, with several biotechnological measures reported to decrease and delay losses in shrimp
9 populations under lab-scale conditions (Flegel et al., 2008). Suitable antiviral agents are significantly limited
10 compared to the wide range of antibiotics that target bacterial pathogens. However, RNA interference (RNAi)
11 strategies have been reported to give a high degree of protection against WSSV infection. Delivery of double-stranded
12 (ds) RNA specific to WSSV sequences into the shrimp cell triggers the RNAi mechanism, which consequently
13 degrades invading viral RNA, thus halting the infection (Krishnan et al. 2009). A key mediator of WSSV entry into
14 the host cell is the major envelope protein VP28, making it an attractive target for RNAi. Specific silencing of the
15 *vp28* gene has been shown to reduce viral copy number in infected shrimp, resulting in a reduction in shrimp mortality
16 (Xu et al. 2007), and the feeding of *Escherichia coli* expressing *vp28* dsRNA to shrimp yielded survival rates up to
17 80% relative to untreated shrimp (Thammasorn et al. 2015). Although promising, this strategy does not lend itself to
18 commercial application, not lease due to concern over the dietary effects of *E. coli* on the shrimp, and the potential for
19 the release of antibiotic resistance genes into the environment.

20 Microalgae are a broad class of photosynthetic unicellular eukaryotes, and are found in fresh, salt, and brackish
21 waters. There has been a significant rise in interest in microalgae as feed and food ingredients over the past two
22 decades, largely due to their sustainable nature and rich nutritional profile. Examples of commercially available
23 microalgae for human consumption include *Chlorella vulgaris*, *Dunaliella salina*, *Haematococcus pluvialis*, and
24 *Euglena gracilis*, with many other species forming an important component of animal feeds, including for shrimp and
25 fish larvae (Muller-Feuga 2000). Genetic manipulation of the nuclear genome is possible for a considerable number
26 of microalgae species, with a smaller subset also amenable to chloroplast genome modification (Spicer and Purton
27 2016). One application of these technologies is the production of functional biomolecules for oral deliver to aquatic
28 animals (Fajardo et al. 2020).

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1 *Chlamydomonas reinhardtii*, a freshwater microalga, is arguably the most studied microalgal species, and as such
2 has a considerable set of molecular tools (Mussgnug 2015). These include well annotated nuclear and chloroplast
3 genomes, established transformation protocols, and substantial parts libraries for gene expression. *C. reinhardtii* is
4 able to grow photosynthetically, and also heterotrophically if provided with a suitable fixed carbon source. The ability
5 to dispense completely with photosynthesis has made it a model organism for studying photosynthesis, but also allows
6 the restoration of light-driven growth to be used as a transformation selection marker, side-stepping traditional markers
7 based on antibiotic resistance genes (Economou et al. 2014). Although *C. reinhardtii* does not provide specific
8 nutritional benefits such as the long chain PUFAs seen in many marine microalgae, its GRAS (Generally Recognised
9 As Safe), status and ease of genetic manipulation makes it an attractive chassis for production and oral delivery of
10 biomolecules (Rosales-Mendoza et al. 2020). We previously reported the first case of *C. reinhardtii* as an expression
11 platform for dsRNA targeting yellow head virus, and demonstrated partial protection against the virus in *Penaeus*
12 *vannamei* shrimp (Charoonnart et al. 2019). The lack of full protection is thought to be due to the relatively low yield
13 of antiviral dsRNA (16 ng/L of transgenic *C. reinhardtii* culture). The present study set out to remedy this shortfall
14 by increasing the level of dsRNA produced in the *C. reinhardtii* chloroplast using a novel expression cassette, and to
15 demonstrate the efficacy of the improved platform both by direct quantification of dsRNA, and by *in vivo* challenge
16 of treated shrimp with WSSV.
17
18 **2. Materials and Methods**

19 ***2.1 Algal strain and maintenance***

20 The *C. reinhardtii* strain CC-5168, also known as TN72, (*cw15*, **Δ***psbH*) was used as a recipient line
21 (Wannathong et al. 2016). The strain was maintained on Tris Acetate Phosphate (TAP) medium (Kropat et al. 2011)
22 solidified with 2% agar, and grown under continuous low-intensity light (<10 μmol photon m^{-2} s^{-1}) at 25 °C. Sub-
23 culturing was performed monthly. Two-full loops of healthy *C. reinhardtii* from solid medium were used to inoculate
24 400 ml TAP for liquid cultivation.
25
26 ***2.2 Plasmid design and construction***

27 The p2xTRBL dsRNA expressing cassette was constructed using a Golden Gate approach modeled on the STEP
28 platform (Taunt et al. manuscript in press). Three level 0 plasmids were built containing the 5' atpB_T/rrnS_P element,

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1 the mRFP cassette, and the 3' rrns_P/rbcL_T element. The *C. reinhardtii* expression elements were amplified from
2 genomic chloroplast DNA, with the addition of adaptor sequences to allow scar-free fusion of the promoter/ terminator
3 pair, and insertion into the Level 0_Empty recipient vector. Also included were additional adapter sequences to allow
4 the three Level 0 constructs to be assembled into the final Level 1 vector, p2xTRBL. The mRFP cassette was
5 synthesized *de novo*, incorporating both adapter sequences for assembly, and additional sequences to allow dsRNA
6 target sequences to be cloned into the final vector. Construction of this vector is summarized in supplementary figure
7 1.

8 A 307 bp fragment of the WSSV VP28 gene was amplified by PCR from a crude virus preparation with the
9 primers designed to include inwards facing Esp3I sites and specific fusion sites for cloning into p2xTRBL (Table 1).
10 The GoldenGate reaction was conducted with 1x CutSmart buffer, 1 mM ATP, 400 units T4 ligase, and 10 units Esp3I
11 at plasmid to fragment molar ratio of 1:8. The GoldenGate reactions were incubated at 37°C for 15 min, followed by
12 30 cycles of 16/37°C (2 min each), with a final digestion step at 37°C for 15 min, and heat inactivation at 65°C for
13 20 min. The resultant products transformed into DH5α and plated onto LB+Tet^{10} agar. White colonies were picked
14 and the correct insertion of the WSSV fragment confirmed by PCR. The resulting plasmid was named
15 p2xTRBL_VP28. The dsRNA expression cassette from this plasmid was then sub-cloned into the *C. reinhardtii*
16 chloroplast transformation vector pSS116 following a similar protocol but using the dual outward facing BsaI sites,
17 and with transformants plated on LB+Amp^{100} (Figure 1). White colonies were again confirmed by PCR, with the final
18 construct named pSS116_VP28. Additional confirmation of correct cloning was achieved by diagnostic restriction
19 endonuclease digestion using XhoI and BstXI. This plasmid was further amplified in *E. coli* DH5α for *C. reinhardtii*
20 transformation.
21

22 ***2.3 Confirmation of ds RNA expression in E. coli***

23 pSS116-VP28 was transformed into the RNase III deficient *E. coli* strain HT115(DE3) (Papić et al. 2018)
24 and colonies selected on LB agar containing 100 µg/ml ampicillin. A single colony was inoculated into liquid
25 LB+Amp^{100} broth and continuously shaken at 37°C for 18 hours. Re-inoculation was performed at 1:100 into fresh
26 LB+Amp^{100} broth and continuously shaken for 8 hours following by cell collection by centrifugation. Total RNA was
27 extracted using the phenol-chloroform (1:1) extraction method, and dsRNA purified according to Saksmerprome et

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al. (2009). Purified dsRNA was visualized by agarose gel electrophoresis and kept at -20 °C for further use as a standard for detection of dsRNA from transformant microalgae.

2.4 Chloroplast transformation and transformant selection

Chloroplast transformation of the cell wall-deficient strain TN72 was performed using the glass bead transformation method (Economou et al. 2014). 10 ml of starter culture was inoculated into 400 ml TAP medium and cultured with continuous shaking under low-intensity light until late log-phase (OD_{750} at 1.0–1.2). Then, cells were concentrated 100-fold to obtain a final cell density at approximately $2x10^8$ cells/ml. 300 µl of concentrated cells were aliquoted into 5 ml tubes containing 300 mg pre-sterilized 400-625 µm glass beads along with 5 µg of pSS116-VP2, and vortexed at maximum speed for 15s. High Salt Minimal (HSM) medium supplemented with 0.5% agar was melted and cooled to 42 °C prior to addition to the mixture followed by immediate pouring onto HSM agar plates. The transformation plates were incubated overnight in the dark cultured under 50 µmol photon m^{-2} s^{-1} white light at 25 °C. Successful transformation colonies where photosynthesis had been restored were obtained after 4–6 weeks.

Single colonies were individually picked and streaked on fresh HSM plates and continuously cultured for 3-4 weeks before next passaging. Homoplasmy was monitored by PCR following extraction via a modified CTAB method from Juntadech et al. (2012). To assure chloroplast DNA extraction, Rubisco-Large Subunit primers (RbcL_F and RbcL_R) were used as an internal control for PCR analysis (Charoonnart et al. 2019) (Table 1). The VP28 fragment was detected using specific primers, VP28_ESP_F and VP28_ESP_R, to confirm insertion of the convergent *rrnS* promoter cassette. The primers TN72_F and TN72_R were used to confirm homoplasmy of the transformed chloroplast genome. Once confirmed to be homoplasmic, the transformant line was named TN72-dsVP28, and was further cultured for dsRNA quantification and viral protection studies. The pSS116 plasmid was also transformed using the same method to generate a photosynthesis-positive TN72 strain, called TN72-SS, that was used as a negative control for further experiments.

2.5 Double-stranded RNA detection and quantification

A full loop of TN72-dsVP28 was inoculated into 400 ml TAP medium and cultured by shaking at 100 rpm under continuous light (50 µmol photon m^{-2} s^{-1}) at 25 °C. The culture was harvested (centrifugation at 3,000 x *g* for 5 min), at late log phase (day 4) and the 1st day of stationary phase (day 5) (growth analysis data not shown) and re-suspended

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1 with Trizol solution (Ambion, USA) for RNA extraction to obtain total RNA. Undesired DNA and single-stranded
2 RNA (ssRNA) were removed by DNase I and RNase A treatment as per the manufacturer's instructions to obtain
3 isolated dsRNA. VP28 specific dsRNA was detected by RT-PCR (RBC Bioscience, Taiwan) using VP28_ESP_F and
4 VP28_ESP_R primers. The formation of dsRNA was confirmed by observing degradation of dsRNA by RNase III
5 treatment.

6 Quantitative RT-PCR (qRT-PCR) was performed using the same set of primers as for RT-PCR (KAPA
7 Biosystem, USA). Isolated dsRNA was incubated at 95 °C for 5 min and immediately chilled on ice for 2 min prior
8 to addition into the qRT-PCR reaction to ensure denaturation of dsRNA. A series of ten-fold dilution of purified
9 dsRNA from the bacterial expression was used to generate a standard curve. The amount of standard dsRNA was
10 calculated according to a formula obtained from the standard curve (R^2=0.958).

11

12 ***2.6 In vivo protection assays of VP28-dsRNA expressing C. reinhardtii against WSSV in shrimp***

13 ***2.6.1 Preparation of animal and viral inoculum***

14 Specific Pathogen Free (SPF) Post larval (PL) (23 day old) *Penaeus vannamei* shrimp were purchased from a
15 major hatchery farm (supplier wishes to remain anonymous) and acclimatized in 10 parts per trillion (ppt) salinity
16 artificial seawater for 7 days prior to use as an animal model. Average animal mass and length were approximately
17 0.03 g and 1.5 cm respectively on the first day of experiment. To prepare the WSSV inoculum for the challenge assay,
18 a mixed gender group of 20 shrimps (10-15 g each) were fed with WSSV infected homogenized shrimp tissue to a
19 rate of 10% of total body mass. Visibly infected shrimps were euthanized and muscle and pleopod tissue collected.
20 WSSV infection was confirmed by the IQ2000 PCR-based detection kit (GeneReach Biotechnology Corp., Taiwan).
21 Infected shrimp muscle tissue was homogenized and kept at -20 °C for later use in the oral challenge assay.

22

23 ***2.6.2 Efficiency of transformants for viral protection in shrimp***

24 360 animals were used per experiment. These were divided into four feeding groups of 90, representing three
25 replicates of 30 animals. Each replicate was cultured in its own glass tank containing 2 L of 10 ppt artificial seawater
26 with continuous aeration. Groups one and two represented negative and positive controls respectively and were both
27 fed with commercial feed twice daily at 5% body weight throughout the experiment. Groups three and four were

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1 supplemented with microalgae by daily submerging 10^9 cells of TN72-SS and TN72-dsVP28, respectively, in addition
2 to commercial feed.
3 The WSSV challenge was conducted on day four of the experiment. Groups two, three, and four were
4 exposed to oral administration of WSSV infected tissue at a rate of 50% total body mass. Group one was not exposed.
5 Animal survival was observed daily, and at 5 days post infection (dpi) all living specimens were collected for viral
6 load assay.
7
8 ***2.6.3 Sample collection and analysis***
9 Animals still alive at 5 dpi were euthanized and stored at -20 °C for later total DNA extraction. Individual
10 specimens were treated in lysis buffer (50 mM Tris–HCl, pH 9.0, 100 mM EDTA, 50 mM NaCl, 2% SDS and 1 µg/ml
11 proteinase K) followed by phenol-chloroform (1:1) extraction (Sritunyalucksana et al. 2006). 200 ng of each sample
12 were subjected to quantitative PCR (qPCR) (KAPA Biosystem, USA) using a 7500™ Real-Time PCR System
13 (Applied Biosystems) for evaluating WSSV copy number. The WSSV specific primers used were WSSV_447_F and
14 WSSV_447_R, and are listed in Table 1. In order to generate standard curve for quantification, qPCR was conducted
15 on series of ten-fold dilutions of the WSSV447 fragment subcloned into the pGEM-T Easy Vector (manufacturer),
16 and were performed in duplicate. qPCR of experimental samples was then conducted and WSSV copy number
17 calculated according to formula obtained from the standard curve (R^2=0.99). Survival percentage and WSSV copy
18 number were statistically compared by ONE-WAY ANOVA and mean differences were tested by Least Significant
19 Different (LSD) (SPSS ver.22, IBM).
20
21 **3. Results**
22 ***3.1 Nature of the p2xTRBL dsRNA expression vector***
23 The p2xTRBL vector is based on the principle of convergent promoters elements, each facing an in-sense
24 terminator located on the opposite side of a Golden Gate cloning region, and downstream of the opposite facing
25 promoter (Figure 1A). For this vector, the promoter of the *Chlamydomonas reinhardtii* chloroplast gene *rrnS*, which
26 encodes the 16S ribosomal RNA, was chosen as it has been shown to be the most transcriptionally active promoter in
27 the *C. reinhardtii* chloroplast (Blowers et al. 1990). Insertion of the target sequence is mediated by Golden Gate
28 cloning using the type IIS restriction enzyme Esp3I as discussed below. Once assembled, the expression cassette

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comprising the target sequence flanked by the promoters and terminators, can then be excised using a second type IIS enzyme, BsaI and transferred to a chloroplast integration vector such as pSS116 through a second Golden Gate cloning step, as illustrated in Figure 1A. The incorporation of different antibiotic selection markers on the two vectors avoids the need to purify the excised cassette prior to cloning into pSS116.

3.2 The Golden Gate assembly method allows for quick and highly efficient construction of plasmids

The insertion of the *VP28* PCR product into the p2xTRBL vector, and of the resulting expression cassette into the pSS116 vector, were both conducted by one-pot-one-step Golden Gate reactions. This method allows for the directional cloning of one or more fragments into a vector without having to separate digestion and ligation steps: the use of type IIS restriction endonuclease (RE) sites ensure that once the desired plasmid is assembled the enzyme recognition sites are lost, precluding it from any further digestion. Both vectors also featured a colorimetric negative screening device, such that successfully assembled plasmids lose the bacterial mRFP expression cassette located between the pair of RE sites and thus yield white colonies, as opposed to the pink colonies generated by the parental plasmids (Figure 1B). Using a 1:8 molar ratio of p2xTRBL to *VP28* DNA we achieved 90% successful assembly based on colony colour. All selected white colonies (26) were shown by PCR analysis to be correctly assembled p2xTRBL-VP28.

Downstream cloning of the expression cassette into pSS116 was conducted using a similar method, but with BsaI used as the restriction endonuclease instead of Esp3I, and colonies selected on ampicillin as opposed to tetracycline. Assembly as judged by pink/ white selection was at a similarly high level (98% white colonies), and again all picked colonies gave the expected PCR products for the recombinant plasmid, pSS116_VP28. A diagnostic digestion of the purified plasmids using XbaI and BstXI further confirmed this.

*3.3 The dual-***rrnS** *promoter system is capable of dsRNA production in E. coli*

After assembly of the pSS116_VP28 plasmid in the standard *E. coli* strain DH5α, the plasmid was transformed into the RNase III deficient strain HT115(DE3) to allow accumulation of dsRNA (Papić et al. 2018). Cultures containing either pSS116_VP28 or the recipient pSS116 were grown to mid-log phase (8 hours), and the purified dsRNA analysed by agarose gel electrophoresis. As shown in Figure 2a, a dsRNA band of the expected size of ~0.4 kb is seen for the *VP28* dsRNA from the pSS116_VP28 culture, whereas no such band was observed from the pSS116

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culture. Total dsVP28 accumulation was estimated from band intensity to be approximate 500 µg. Furthermore, RT-PCR using specific primers to the *VP28* sequence was conducted to confirm the presence of VP28 dsRNA. An amplicon of ~0.3 kb representing just the VP28 sequence without the upstream and downstream transcribed regions was observed from pSS116-VP28, while this amplicon was not seen for the pSS116 line (Fig. 2b).

3.4 Generation of C. reinhardtii transplastomic lines

The pSS116 chloroplast integration vector contains *C. reinhardtii* chloroplast sequences flanking the cloning site such that the cloned DNA is targeted into the genome at a neutral site between *psbH* and *trnE2*. Furthermore, a wild-type copy of *psbH* on the right flank serves as a selectable marker, rescuing the Δ*psbH* mutant TN72 to phototrophy (Wannathong et al. 2016). Chloroplast transformants of TN72 were generated using pSS116-VP28, and pSS116 as a control, with the first colonies becoming visible on minimal medium after six weeks. A total of seven colonies were observed for pSS116-VP28 and only two for pSS116 transformation.

After the second re-streak on minimal medium, DNA was extracted and PCR analysis conducted, with amplification of the endogenous gene *rbcL* used as a positive control for extraction (Fig. 3a). The *VP28* DNA was successfully amplified from the pSS116_vp28 lines, and not seen from the pSS116 lines (Fig. 3b). As the copy number of the chloroplast genome in *C. reinhardtii* cells is typically ~80 (Gallaher et al. 2018), and transformation initially results in a heteroplasmic state with both transformed and original copies of the genome within the cell (Purton 2007), then homoplasmy in the transformant lines was assessed using primers specific for the original TN72 genome. The absence of a band from the pSS116_VP28 and pSS116 lines suggesting homoplasmy (Fig. 3c).

One representative transformant line containing the 2xTRBL_VP28 cassette was selected for dsRNA investigation and named TN72-dsVP28, with a corresponding pSS116 line chosen as a negative control and named TN72-SS.

3.5 VP28 dsRNA is produced by the transgenic line, and significantly more as compared to a previous line using convergent psaA promoters

The yield of dsRNA in the TN72-dsVP28 strain was analysed following total RNA extraction, and DNase I and RNase A treatment to digest all DNA and ssRNA leaving only dsRNA, as previously demonstrated in Charoonnart et al. (2019). The specific presence of VP28 dsRNA in the TN72-dsVP28 RNA sample, but not in an equivalent sample

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1 from the TN72-SS control strain, was investigated by RT-PCR. TN72-dsVP28 gave the expected amplicon at 307 bp,
2 whereas no band was seen for TN72-SS (Fig. 4a). Furthermore, this amplicon was lost when the dsRNA template was
3 treated with the dsRNA specific enzyme, RNase III (Fig. 4b), confirming that VP28 dsRNA was indeed present in the
4 TN72_VP28 line.
5 To investigate whether growth phase had an effect on dsRNA expression, semi quantitative RT-PCR was
6 performed on samples harvested after four and five days of culture. These data show significantly higher expression
7 of dsRNA from the 5 day culture suggesting accumulation of dsRNA in early stationary phase (Fig. 4c). Fully
8 quantitative qRT-PCR was then used on the sample harvested on the 5th day to give a conclusive dsRNA yield. Melt
9 curve analysis using purified dsRNA from the HT115(DE3)_VP28 bacterial expression system gave a melting
10 temperature (Tm) of 82.40±0.32 °C while dsRNA obtained from TN72-dsVP28 strain gave a Tm of 82.48±0.16 °C.
11 Quantitative analysis gave 41.13 pg VG28 dsRNA from a 500 ng dsRNA input, which corresponds to 119 µg of *VP28*
12 dsRNA from 1 L of TN72-dsVP28 culture (OD_{750} at 3.9) while previous expression cassette using psaA promoter in
13 Charoonnart et al. (2019) reported 16 ng specific dsYHV obtained from 1 L of culture (OD_{750} at 1.8).
14
15 ***3.6 Supplementation of either strain of C. reinhardtii provides protection against WSSV***
16 To evaluate the efficiency of TN72-dsVP28 in protecting against WSSV, a feeding experiment following by a
17 WSSV challenge assay was performed. At three days post infection (3 dpi), shrimp in the positive group (commercial
18 feed, full WSSV challenge) displayed 50% survival, compared to 95% for the negative group (commercial feed, no
19 WSSV challenge). Shrimp with feed supplemented with TN72-dsVP28 showed a 95.23±0.32% survival rate, but
20 surprisingly, the control group feed the TN72-SS line also showed very high survival rate of 93.10±0.00 (Fig. 5a).
21 At the end of the experiment (5 dpi) a viral load assay was conducted on surviving animals. Of all shrimp sampled
22 from negative group (n = 3) were found no infection whereas 7 of 9 shrimp sampled from survival in positive group
23 showed high viral load (>10^8 WSSV copies). The TN72-dsVP28 supplementing group showed slightly lower levels
24 of viral load on the last day of experiment suggested higher ratio of light infection in TN72-dsVP28 supplementing
25 group (10 from 15 shrimps) than TN72-SS supplementing and positive group which had more than 50% of shrimp
26 had high WSSV copies number (Fig. 5b).
27
28 **4. Discussion**

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1 The novel vector p2xTRBL and corresponding transformation vector pSS116 represent a significant improvement
2 in the construction and efficacy of dsRNA expressing lines of *C. reinhardtii*. The use of convergent *rrnS* promoters
3 marks a significant improvement over a previous design where *psaA* promoters were employed (Changko et al. 2020;
4 Charoonnart et al. 2019; Gangl et al. 2015; Zedler et al. 2015). This is to be expected, as the *rrnS* promoter has been
5 shown previously to be the most transcriptionally active promoter in the *C. reinhardtii* chloroplast (Rasala et al. 2011);
6 however, it should also be noted that the p2xTRBL cassette also represents the first fully purpose-built *C. reinhardtii*
7 dsRNA expression system. Charoonnart et al. (2019) previously constructed a dsRNA expressing cassette by
8 modifying pSRSapI to contain convergent *psaA* promoters, but as the original vector was designed for protein
9 synthesis it not only contained unnecessary 5'UTR regions, but also only the sense promoter had a corresponding
10 terminator. The antisense promoter on the other hand, will drive transcription until a suitable native terminator is
11 reached, lending the dsRNA an extended ssRNA "tail" as well as wasting cellular resources.

12 Since chloroplast promoters are prokaryotic in origin, they can also be expressed in *E. coli*, with the *rrnS* promoter
13 being no exception. This presented a useful opportunity to validate the dsRNA-expressing cassette, the pSS116_VP28,
14 prior to algal transformation, and also to produce a stock of VP28 dsRNA to be used as a positive control. Building
15 on several reports where convergent bacteriophage T7 promoters have been used to produce dsRNA in *E. coli* (García
16 et al. 2015; Kim et al. 2015), we transformed the pSS116_VP28 vector into the RNase III deficient *E. coli* strain
17 HT115(DE3). The resulting system was shown to be able to produce reasonable amount of dsRNA, but the amount
18 was lower than that produced from hairpin expressing cassettes (Chen et al. 2018). Nonetheless, hairpin cassette is not
19 stable in plastid genome due to the presence of protein RecA that might loop out the inverted repeat in hairpin cassette
20 (Nakazato et al. 2003). A convergent cassette was therefore used in expressing dsRNA in chloroplast of *C. reinhardtii*.

21 The use of traditional antibiotic resistance selection markers are highly regulated against in aquaculture, in order
22 to reduce the spread of such cassettes by horizontal gene transfer. The ability for Chloroplast transformants to be
23 selected for without such markers is hence a large advantage, as has be demonstrated numerous times (Changko et al.
24 2020; Charoonnart et al. 2019; Gangl et al. 2015; Zedler et al. 2015). Despite these benefits, the transformation with
25 pSS116_VP28 suffered from long incubation times and low transformation efficiency. The incubation period was 2
26 weeks longer than previous *psaA* convergent promoter cassette (4 weeks), and those reported protein production
27 (Gangl et al. 2015). The delay of transformation and low efficiency may have been due to the presence of the
28 convergent *rrnS* promoters, with the inverted repeat potentially interfering with homologous recombination; however,

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1 the matter is ultimately of little import, as colonies were recovered and shown to all be correct. Moreover, the
2 transformant strains rapidly reached homoplasmy, showing no evidence of the recipient genotype after only two re-
3 streakings. The result was similar to the two transformant strains containing *rrnS* convergent promoter covering RdRp
4 fragment of YHV and ORF366 fragment of WSSV.

5 The resultant transplastomic line, TN72-VP28, was shown to express VP28 specific dsRNA by RT-PCR
6 following selective degradation of DNA and ssRNA. It was then not possible to amplify the VP28 sequence after the
7 use of RNAse III to specifically degrade dsRNA. Combined, these two results give strong evidence for the production
8 of VP28 dsRNA in the *C. reinhardtii* chloroplast. Subsequent RT-qPCR was used to quantify the VP28 dsRNA, giving
9 a yield of 119 µg of *VP28* dsRNA from 1 L culture at OD_{750} at 3.9. Though dsRNA yield obtained was very much
10 lower than bacterial system, but it was worth since the transgenic cell could be use as direct feed and, on the fact that,
11 the system was marker-less. This is also representing a ~10,000-fold increase on the above discussed work using the
12 *psaA* promoter, although as this work expressed a different target sequence it is not possible to make a direct
13 comparison. It is of note that levels of dsRNA were observed to increase from day four to day five of the cultivation
14 period. Further work will be needed to confirm this trend, and give a more complete picture of dsRNA expression
15 over a full growth period. It would also be of interest to investigate whether other abiotic factors can influence dsRNA
16 accumulation, for example the effect of light levels or media composition.

17 The result revealed that both transformant strains had high survival percentage at the last day of experiment,
18 however TN72-VP28 feeding group conferred slightly better protection. TN72-SS was background strain of TN72-
19 VP28, therefore it could be implied that better protection are the results of dsRNA-mediated defence. Though the
20 survival percentage did not presence much difference, WSSV copy number has supported the effect of dsRNA on
21 viral inhibition that is higher ratio of low infection was found in TN72-VP28 supplementing group. However, it is
22 notably that TN72-SS also conferred significant protection comparing to positive group. Natural products from *C.*
23 *reinhardtii* may contain some beneficial bioactive molecules (Fields et al. 2020). Similar to another work, wild type
24 *C. reinhardtii* containing feed provided partial protection in a period after WSSV challenging comparing to positive
25 group (WSSV challenge, no algae) prior reaching 100% mortality at the end of the experiment (Kiataramgul et al.
26 2020; Somchai et al. 2016). An optimization of algal delivery and level of WSSV infection should be performed to
27 obtain an applicable method for viral protection in the pilot and farm scale.

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The *Penaeus vannamei* shrimp is known to be highly susceptible to WSSV throughout its life cycle, but the post larval (PL) stage of development is considered to be when it is must venerable to viral attack according to data collection from farms in Southern of Thailand by Department of Fisheries. It is therefore essential to ensure PL stocks are healthy and disease-free before transferring them to the grow-out pond. Since WSSV can be horizontal transmitted, WSSV transmission in TN72-SS group was potentially quicker than TN72-VP28 and eventually 100% mortality more rapidly. Feeding PL shrimp with dsRNA-producing microalgae delayed massive mortality of shrimp from viral infection. Together with good farm management, shrimp farmers may be able to rescue shrimp from mortality and gain some profits from the crop instead of completely discarding the whole crop. Further study would be feasibility of scaling-up culture to commercial level and downstream processing to produce inactivated-dry biomass for industrial application.

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6. Statement of informed animal rights

Experiments related to shrimps used in this study were approved by BT-IACUC, protocol no. BT Animal 8/2562. All procedures performed in studies involving animal were in accordance with the guidelines of the Australian, New South Wales state government for the humane harvesting of fish and crustaceans.

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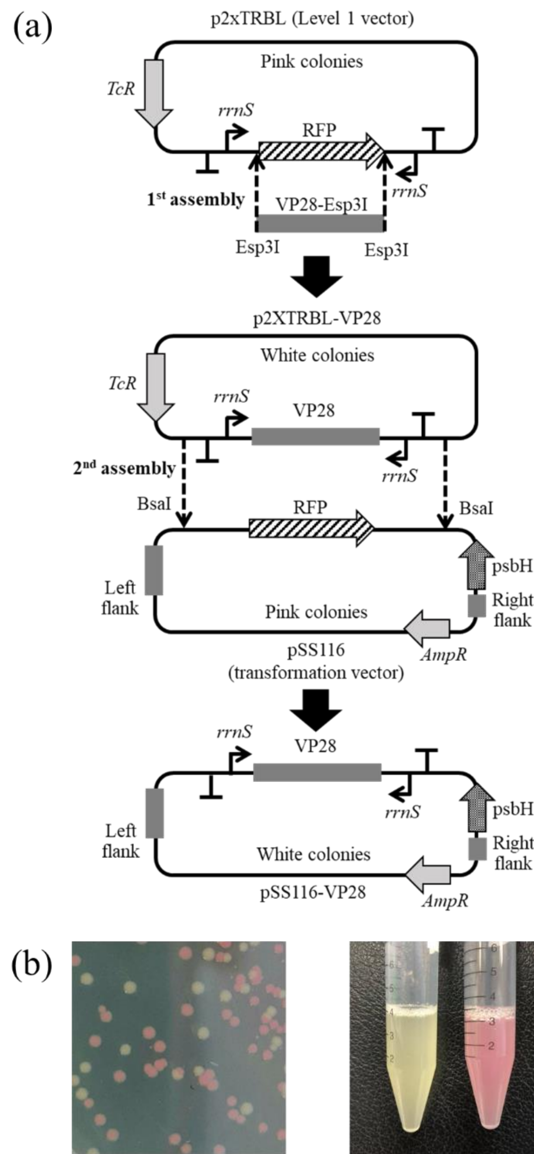
1 **Figure 1**



2

3 **Figure 1** Schematic diagram of the novel dsRNA expressing line 2xTRBL, and associated construction pipeline.
4 Illustration of the 'pink/white' screening system for identification of correctly assembled vectors (a). Cloning pipeline
5 for insertion of VP28 into p2xTRBL, then on into pSS116 (b).

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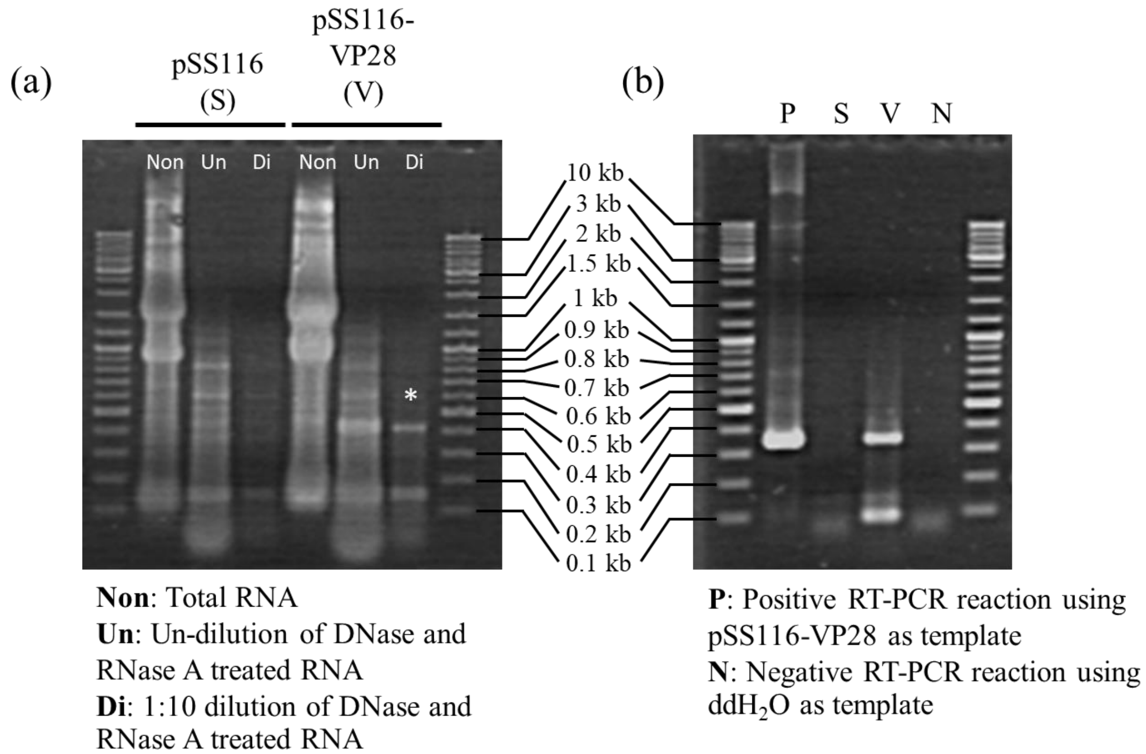
1 **Figure 2**



2

3 **Figure 2** Purified RNA from *E. coli* HT115(DE3) transformed with pSS116 (S) and pSS116-VP28 (V), showing total
4 RNA (Non), then presumably RNA digested with DNAase and RNAase A (Un) and 1:10 diluted and treated RNA
5 (Di) (a). RT-PCR of DNAse and RNAse A treated RNA with dsVP28 specific primers (b). Lane N and P represent
6 negative and positive reaction for PCR analysis using ddH2O and dsVP28, respectively, from bacterial system as a
7 template.

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Figure 3

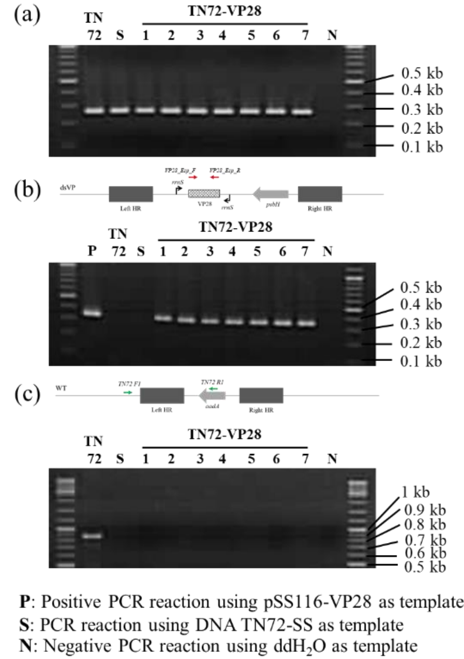

Figure 3 PCR analysis for selection of homoplasmic transgenic microalgae (TN72-VP281-7) containing dsRNA expressing cassette using rbcL_F and rbcL_R primers for chloroplast DNA control comparing to TN72 and original pSS116 transformed strain (S) (a), schematic of insertion region displaying VP28_Esp_F and VP28_Esp_R binding for VP28 fragment detection (b), and TN72_F and TN72_R for remaining plastid detection. Lane N and P represent negative and positive reaction for PCR analysis using ddH_2O and dsVP28, respectively, from bacterial system as a template.

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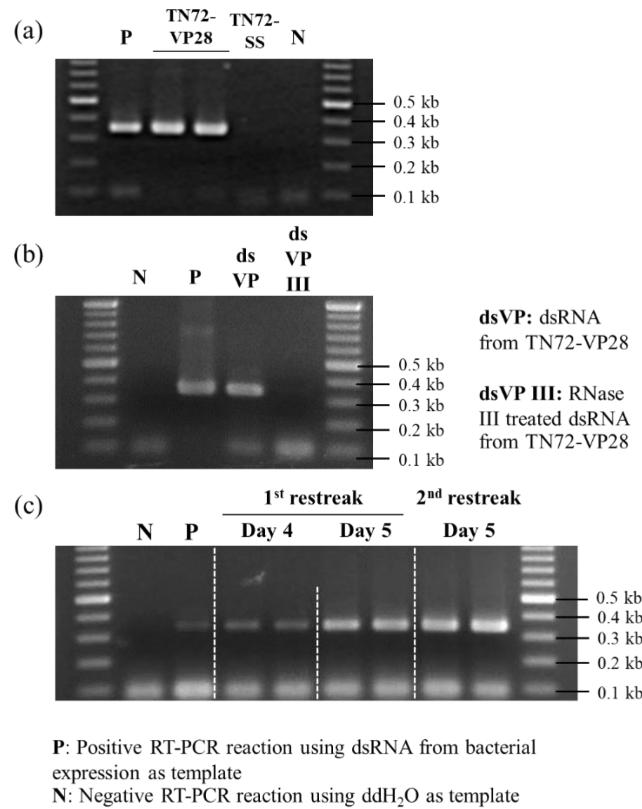
1 **Figure 4**



2

3 **Figure 4** RT-PCR analysis for detection of dsRNA from dsRNA-expressing cassette containing strain; (a) dsRNA
4 purified from TN72-VP28 shows the VP28 amplicon, whereas TN72-SS strain does not, (b) TN72_VP28 dsRNA
5 does not show the VP28 amplicon after RNase III treatment, and (c) the determination of dsRNA yield at different
6 harvesting time. Lane N and P represent negative and positive reaction for PCR analysis using ddH_2O and dsVP28,
7 respectively, from bacterial system as a template.

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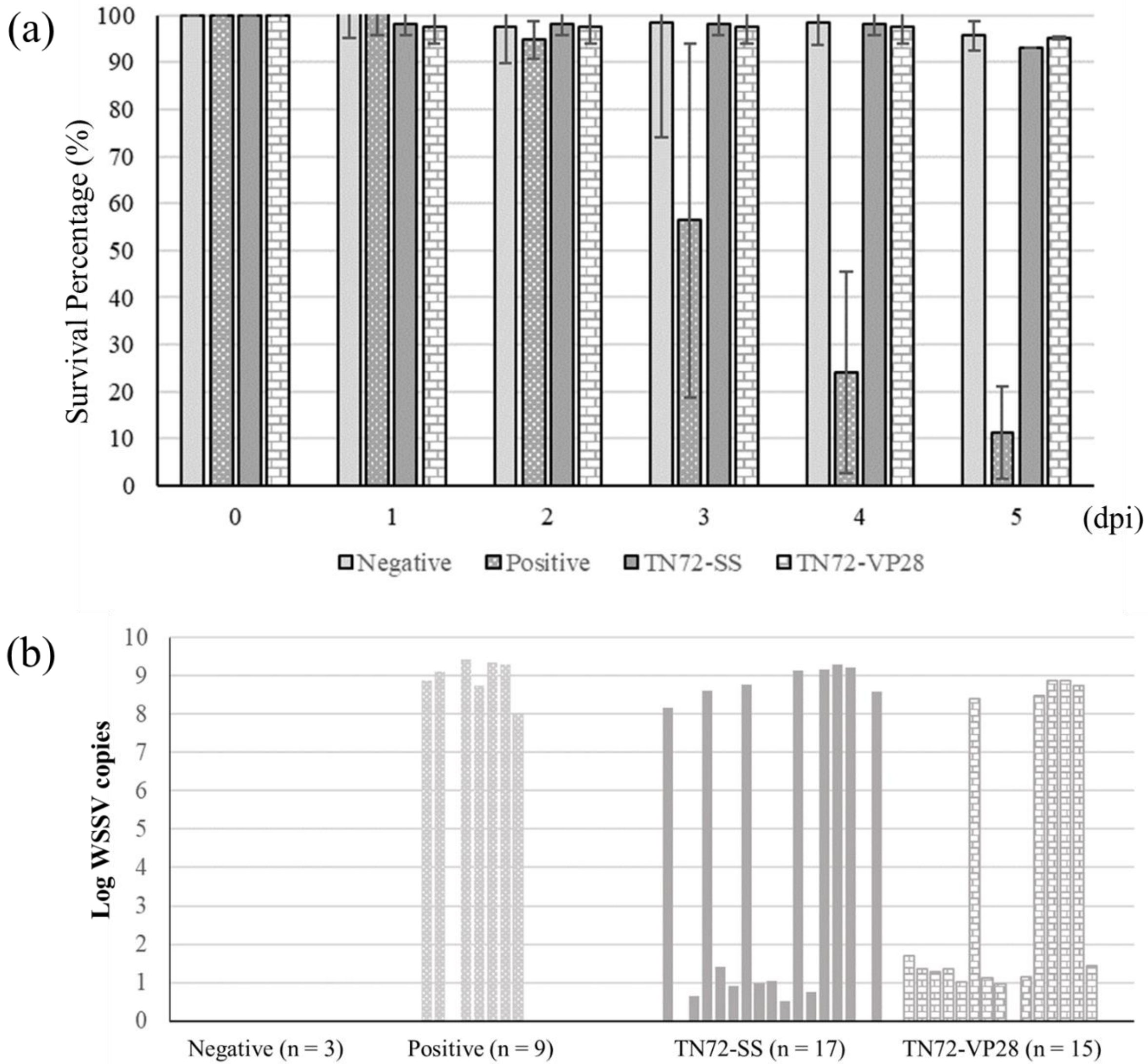
1 **Figure 5**



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3 **Figure 5** (a) Survival percentage of PL shrimp from feeding trial comparing between negative (no WSSV challenge)
4 and positive (WSSV challenge), TN72-SS and TN72-VP28 supplement prior WSSV oral challenge. Bar represents
5 standard deviation. (b) Quantitative RT-PCR indicates the number of WSSV copies from 100 ng of RNA extracted
6 from individual shrimp randomly sampled from survival animals in the wet lab experiment.

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1 **Table 1** List of primers used in the experiment

Primer names	Sequences	Function	References
VP28_Esp_F	5'-ATACGCGTCTCCGTCGATCTTTCTTTCACTCTTTCG-3'	Generation of target fragment for fusing with the shuttle vector	Own designed
VP28_Esp_R	5'-ATACGCGTCTCTCAAGCCACAGGAGTGATGACAA-3'		
RBCL_F	5'-GTCACCACCAGACATACGAAG-3'	Internal control for chloroplast DNA extraction	Charoonnart et al. (2019)
RBCL_R	5' GGTCACTACTTAAACGCTAC 3'		
TN72_F	5' GTCATTGCGAAAATACTGGTGC 3'	Homoplasmic status checking (wildtype-derived primers)	Wannathong et al. (2016)
TN72_R	5' CGGATGTAACTCAATCGGTAG 3'		
WSSV447_F	5'-ATGAGAATGAACTCCAACTTTAA-3'	WSSV load assay	Srivihok et al. (2019)
WSSV447_R	5'-CAGAGCCTAGTCTATCAATCAT-3'		

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