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Introgression of sheath blight disease tolerance from the transgenic rice event Pusa Basmati1-CG27 to the variety White Ponni through backcross breeding

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Abstract

Sheath blight disease caused by *Rhizoctonia solani* is a major constraint for rice production. In order to obtain transgenic resistance against sheath blight disease, an *indica* rice variety Pusa Basmati1 (PB1) was transformed with constitutively express chitinase (*chi11*) and β-1,3-glucanase (*GLU*) genes. A single-copy transgenic PB1-CG27 showed a high level of tolerance to *R. solani.* Here, we report the introgression of sheath blight tolerance from PB1-CG27 into a non-basmati rice variety, White Ponni (WP) through backcross breeding. In each of the four backcross generations, the presence of the transgene was checked by *hygromycin phosphotrans-ferase* (*hph*) and *β-glucuronidase* (*gus*) gene-specific foreground selection markers present in the T-DNA of the PB1-CG27 event. RAPD marker-based background selection was done to choose a BC_4F_1 plant that was similar to the WP recurrent parent. Morphological characters of the introgressed BC_4F_4 plants matched those of the recurrent WP parent. RT-PCR and western blot analyses confirmed the constitutive expression of the *chi11* and *GLU* genes in the introgressed homozygous BC_4F_3 plants. Bioassay with *R. solani* a high level disease tolerance (43 % disease reduction) revealed over the control WP plants.

Key words: Chitinase, β-1,3-glucanase, introgression, rice, sheath blight resistance

Introduction

Sheath blight disease (SBD) caused by *Rhizoctonia solani* Kühn is the second most important fungal disease of rice (Molla et al. 2013). Up to 50 % of yield loss has been reported under environment favourable for SBD development (Lee and Rush 1983). Chemical method applied to control *R*. *solani* infection is neither economical nor environment friendly. Breeding for SBD resistance in rice is a challenge because of non availability of germplasm with complete resistance (Kumar et al. 2003; Dubey et al. 2014), existence of high genetic variability of *R. solani* and its long term survival in the soil (Taheri et al. 2007). Genetic engineering is a promising strategy to develop SBD resistance in rice (Karmakar et al. 2016).

During pathogen infection, the host plant produces a group of pathogenesis-related (PR) proteins (van Loon et al. 2006). Constitutive expression of PR–proteins in crops by transgenic approach is an effective method to control fungal diseases (Honée 1999). Several previous studies reported the SBD tolerant transgenic plants by over expressing defense related genes such as chitinase (Lin et al. 1995; Kumar et al. 2003; Sridevi et al. 2003), *OsWRKY30* (Peng et al. 2012), *OsOXO4* (Molla et al. 2013), *AtNPR1* (non expresser of PR-1) (Molla et al. 2016), and chitinase gene *LOC_Os11g47510* from *indica* rice (Richa et al. 2017). Transgenic plants expressing a combination of PR protein genes confer higher SBD tolerance over those expressing a single PR-protein gene. Combined expression of rice chitinase (*chi11*) and tobacco β-1,3-glucanase (*GLU*) (Sridevi et al. 2008), thaumatin-like protein (*tlp*-D34) and *chi11* (Shah et al. 2013), rice chitinase (*OsCHI11*) and *oxalate oxidase* 4 (*OsOXO4*) (Karmakar et al. 2016) and tobacco osmotin (*ap24*) and rice *chi11* (Sripriya et al. 2017) genes conferred a higher level of SBD tolerance. Maruthasalam et al. (2007) developed a stacked transgenic rice line by expressing the *chi11, tlp* and *Xa21* genes. It displayed

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higher levels of resistance against sheath blight and bacterial blight diseases.

Marker-assisted backcross breeding is an effective method to transfer a transgenic trait from one parent to another. Roy et al. (2012) reported the successful transfer of a transgenic event comprising a hairpin RNA gene of RTBV (*Rice tungro bacilliform virus*) from PB1 to rice varieties IET4094 and IET4786. Upon RTBV challenge inoculation, BC_2F_1 plants displayed very mild tungro symptom as compared to the recurrent parents. The transgenic event conferring RTBV resistance has also been successfully introgressed in to a South Indian rice variety, ASD16 through marker-assisted backcross breeding (Jyothsna et al. 2013).

SBD tolerant PB1 transgenic rice line CG27 constitutively expresses higher level of chitinase (*chi11*) and β-1,3–glucanase (*GLU*) genes (Sridevi et al. 2008). The present study aimed at introgressing the SBD resistance from PB1-CG27 to a popular South Indian rice variety, White Ponni (WP) through backcross breeding. WP rice variety is preferred for its medium, slender white grain quality.

Materials and methods

Plant materials

Oryza sativa L. supsp. *indica* var. Pusa Basmati1 PB1-CG27 transgenic homozygous line (Sridevi et al. 2008) was used as a male donor parent. The T-DNA of the CG27 transgenic rice plant harbours P35S-*GLU*-35S3', P*Ubi1-chi11-chi11*3', P35S-*hph*-35S3' and P35S-int-*gus-nos*3' genes. WP was used as the recurrent female parent. All rice plants were maintained in a greenhouse at 23-30°C under natural light (12-h light/12-h darkness).

Backcrossing

Crossing was done by the clipping method (Coffman and Herrera 1980). The cross between the male donor parent (PB1-CG27) and female recurrent parent (WP) yielded the F_1 hybrid seed. The transgene-positive F_2 plants were crossed with the female (WP) parent to get the BC_1 seeds. The transgene-positive BC_1 plants were crossed with the WP parent to get BC_2 seeds. Similarly BC_3 and BC_4 seeds were obtained. The detailed backcrossing procedure is presented in Fig. 1.

Southern blot analysis

Total plant DNA was extracted from rice leaves (Rogers and Bendich 1988) and DNA was estimated in a fluorometer using Hoechst dye 33258. DNA transfer, probe labelling with [α-^{32}P]dCTP, hybridization and post hybridization washes were performed as described by Ramanathan and Veluthambi (1995).

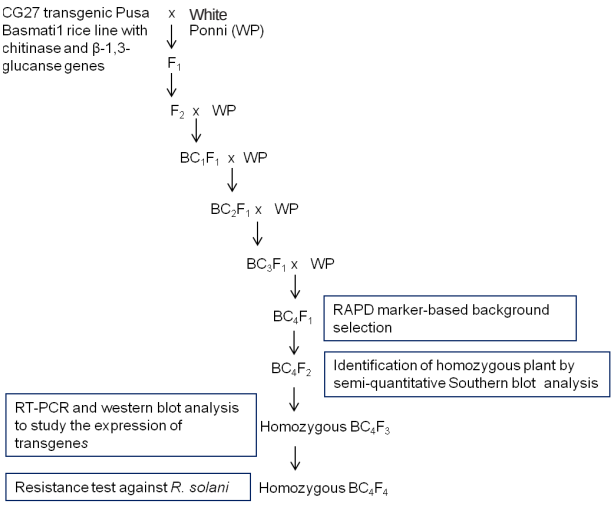

Fig. 1. Introgression scheme for the development of CG27 event-based SBD tolerant rice line in the WP genetic background

RAPD-based background selection

RAPD primers were used for background selection. The genetic similarity between the backcrossed progenies and the recurrent parent was analysed on the basis of polymorphism displayed by banding pattern. Eleven RAPD primers (OPC07, OPC15, OPD08, OPF06, OPF13, OPF14, OPF17, OPJ08, OPJ13, OPK11 and OPO11) were used in the analysis.

RNA extraction and RT-PCR analysis

Tri Reagent (Sigma-Aldrich, St. Louis, USA) was used to extract the total RNA which was treated with 2.5 U of DNase in 50 µl of 40 mM Tris-Cl (pH 7.5) at 37^{o}C for 30 min. A DNA-free RNA template was used for RT-PCR using SuperScript III One-Step RT-PCR system with Platinum *Taq* (Invitrogen, Carlsbad, USA). The primers KVP178 (5' GCGAGATCACC AACATCATCA 3') and KVP179 (5' TAGGGCCTCT GGTTGTAGCAA 3') for *chi11* and KVP 143 (5' CACACTCCTAGGATTACTAC 3') and KVP 144 (5' AATGGGATCAACAAACCACC 3') for *GLU* genes were used in RT-PCR.

Protein extraction and western blot analysis

Total protein was extracted from rice leaves and estimated using the Bradford method. Protein samples

(20 μg) were separated by 10 % SDS-PAGE. Western blot analysis was done as described earlier by Sridevi et al. (2008).

Bioassay with Rhizoctonia solani

Bioassay with *R. solani* was done as described by Sridevi et al. (2008). Forty five-days-old rice plants were infected with *R. solani*. SBD symptom was graded 7-day-post inoculation (dpi) in the 0 to 5 scale (Sriram et al. 1997). The disease index was calculated by using the formula,

$$\text{Disease Index} = \frac{\text{Mean grade point per plant x 100}}{\text{Maximum grade point (5)}}$$

Results

Backcrossing and foreground selection

An F_1 hybrid was obtained by crossing the homozygous CG27 PB1 transgenic rice line as the male donor parent and WP as the recurrent female parent (Fig. 1). The F_2 seeds were germinated on half-strength Murashige and Skoog (MS) medium supplemented with 50 mg/L hygromycin. The F_2 seedlings displayed 3:1 segregation ratio of hygromycin-resistance (Hyg^R) and sensitivity (Hyg^S). Five Hyg^R F_2 plants were analysed by Southern blotting to check the presence of T-DNA of the CG27 event. The plant DNA was digested with *Hin*dIII and the blot was probed with the *gus* sequence (Fig. 2a). As expected, the CG 27 x WP F_2 hybrid plants displayed hybridization to the 6.6-kb junction fragment (Fig. 2b) confirming the successful transfer of the CG27 event to the WP recurrent parent. The cross between the transgene-positive F_2 plants and the WP female recurrent parent plants (Fig. 1) yielded 52 BC_1 seeds, out of which, seven yielded GUS-positive seedlings. Five GUS-positive BC_1 plants were crossed with WP to get BC_2 seeds. Similar steps were followed to obtain BC_3 and BC_4 seeds (Fig. 1). To check the inheritance of the transgene in the backcrossed progenies, eight Hyg^R BC_4F_1 plants were analysed by Southern blotting. The BC_4F_1 plant DNA was digested with *Hin*dIII and the blot was probed with the *hph* gene (Fig. 2a). All eight BC_4F_1 plants showed hybridization to the expected 22-kb junction fragment (Sridevi et al. 2008) indicating stable inheritance of the T-DNA (Fig. 2c).

Background selection using RAPD markers

DNA from eight BC_4F_1 plants was subjected to RAPD marker-based DNA profiling to select the plant which had the maximum genetic similarity to the recurrent parent genome. Eleven primers were used for RAPD analysis. Out of 11 primers, ten primers showed polymorphism between the recurrent and donor parents (data not shown). RAPD profiling showed that all eight analysed backcrossed BC_4F_1 plants were genetically similar to the WP parent. Representative RAPD profiles obtained with primers OPF14 and OPJ13 are presented in Fig. 3. Plant-4, which showed maximum similarity with the recurrent parent genome, was selfed and the BC_4F_2 seeds were taken for further analyses.

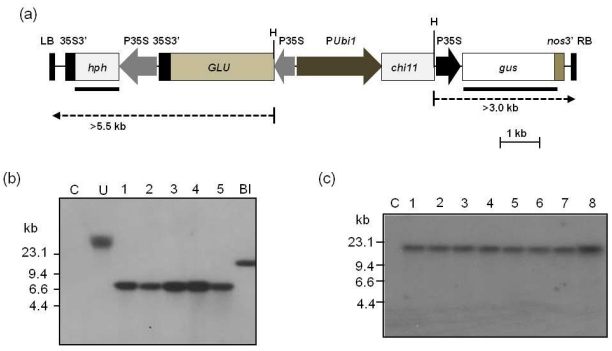

Fig. 2. Southern blot analyses of F_2 and BC_4F_1 plants derived from a cross between PB1 (CG27) and WP recurrent parent. (a) T-DNA map of the binary vector pNSP3. It harbours the *GLU, chi11, hph,* and *gus* genes. The *gus* and *hph* probes are marked in bold lines. The dotted arrows represent the expected junction fragments released upon *Hin*dIII (H) digestion. (b) Analysis of F_2 plants of the PB1 (CG27) x WP hybrid. Rice DNA (2.5 μg) was digested with *Hin*dIII and *gus* was used as probe. Lanes 1-5, PB1 CG27 x WP F_2 plant DNA; Lane U, undigested DNA of an F_2 plant; Lane C, DNA from control (non-transgenic) plant; Lane BI, binary plasmid pNSP3 (50 pg) digested with *Hin*dIII. (c) Analysis of BC_4F_1 plants. Rice DNA was digested with *Hin*dIII and *hph* was used as probe. Lanes 1-8, CG27 x WP BC_4F_1 plant DNA

Generation of homozygous transgene introgressed line

BC_4F_1 (plant-4) was selfed to obtain homozygous BC_4F_2 plants which can be used for analysing the expression of the *chi11* and *GLU* genes. Nine Hyg^R BC_4F_2 plants were subjected to DNA blot analysis to identify the homozygous plants by the method described by Sridevi et al. (2006). DNA of the control and the CG27 transgenic event backcrossed (BC_4F_2) plants was digested with *Hin*dIII and the blot was

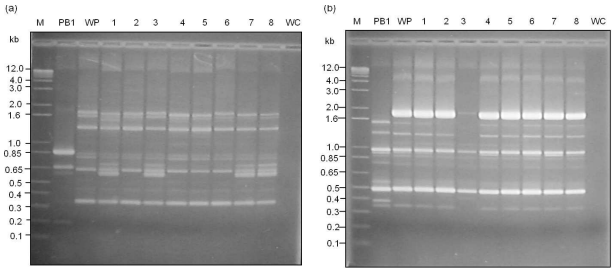

Fig. 3. Representative RAPD profiles of the CG27 x WP BC_4F_1 plants obtained with (a) OPF14 and (b) OPJ13 primers. M-DNA marker; PB1-Pusa Basmati1 control; WP-White ponni control; Numbers 1 to 8-BC_4F_1 plants; WC-water control

probed with the *gus* gene (Fig. 2a). Plant-3 and -7 showed more intense hybridization signal compared to other plants (Fig. 4). Therefore, plant-3 and -7 were

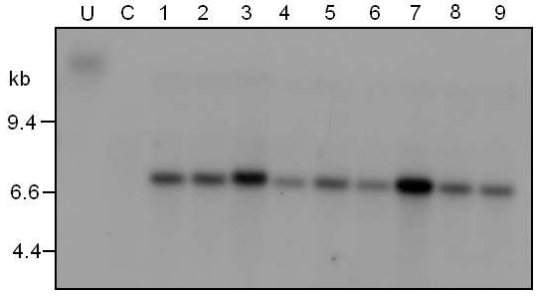

Fig. 4. Zygosity analysis of CG27 x WP BC_4F_2 plants by Southern blotting. Rice DNA (2.5 μg) was digested with *Hin*dIII and *gus* was used as probe. Lanes 1-9, BC_4F_2 plant DNA; Lane C, control plant DNA; Lane U, undigested BC_4F_2 plant DNA

identified as putative homozygous plants. Plant-7 was selfed and forwarded to the BC_4F_3 generation. The BC_4F_3 seeds (40 seeds) were germinated on half-strength MS medium containing 50 mg/L hygromycin. The seedlings showed 4:0 (Hyg^R/Hyg^S) segregation confirming the homozygous status of the CG27 event in the BC_4F_3 plants derived from plant-7 of the BC_4F_2 generation.

Expression analysis of the chi11 and GLU genes

RT-PCR was done on total RNA from control and BC_4F_3 homozygous plants by using *chi11* and *GLU* gene-specific primers. The BC_4F_3 plant expressed the *chi11* (170-bp amplicon) and *GLU* (540-bp amplicon) genes (Fig. 5a, b). *chi11* expression was very low in the control plant and its expression was elevated in the BC_4F_3 plants (Fig. 5a). *GLU* did not express in control plants, but its expression was high in the BC_4F_3 plant (Fig. 5b). Total protein of the BC_4F_3 homozygous plant was used for western blot analysis with the chitinase and β-1,3-glucanase antibodies. The BC_4F_3 plant accumulated high levels of the 35-kDa chitinase (Fig. 5c) and 32-kDa β-1,3-glucanase proteins (Fig. 5d), which were very low and absent, respectively, in the control plant.

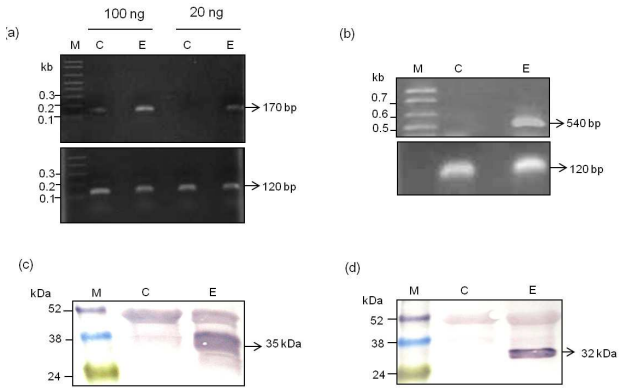

Fig. 5. Expression analyses of chitinase (*chi11*) and β-1,3-glucanase (*GLU*) genes in the CG27 x WP BC_4F_3 homozygous plant. RT-PCR of (a) *chi11* gene (100 ng and 20 ng RNA template) and (b) *GLU* gene (100 ng RNA template). (a) and (b) Bottom panels show amplification of the *ACTIN* transcript cDNA as an internal control. Western blot analysis to study the accumulation of (c) chitinase and (d) β-1,3-glucanase proteins. (a-d) Lane M, marker; Lane C, control plant sample and Lane E, BC_4F_3 plant sample

Bioassay with R. solani

Three tillers each from the 45-days-old control and BC_4F_4 homozygous plants were used for bioassay with *R. solani*. Sheath blight symptoms were scored 7-days-post *R. solani* inoculation. The mean disease indices for the control and BC_4F_4 plants were 52.65 ± 1.81 (n=11) and 29.96 ± 1.99 (n=11), respectively (Fig. 6). The difference of the disease index between the control and CG27 introgressed plants was statistically significant at 1% level. After normalizing the control plant disease index to 100, the percent disease index for the transgenic BC_4F_4 plant was determined as 57%. Thus, the BC_4F_4 plants showed 43% disease reduction.

Morphological characters

The donor (PB1), recurrent (WP) and BC_4F_4 plants were grown in the greenhouse. Plant height, tiller number, panicle number, days to flower post-transplantation, 100-seed weight and seed kernel

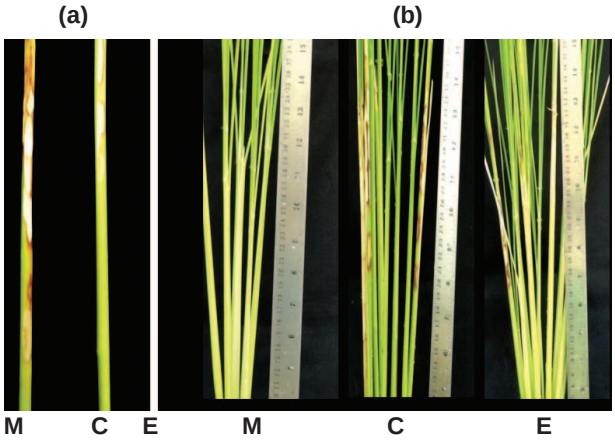

Fig. 6. Bioassay of control and CG27 x WP BC_4F_4 homozygous rice plants with *R. solani*. (a) Stems of rice plants with symptoms of *R. solani* infection. (b) Closer views of rice plants with sheath blight symptom. (a-b) M -Mock-inoculated control rice plant without *R. solani* inoculum. C-Control rice plant inoculated with *R. solani*. E-CG27 x WP BC_4F_4 homozygous rice plant inoculated with *R. solani*

length of the BC_4F_4 plants were equivalent to those of the recurrent WP parent (Table 1). No significant difference in the morphological characters was observed between the recurrent parent and the BC_4F_4 plants.

Table 1. Morphological characters of the donor parent, recurrent parent and homozygous BC_4F_4 plants with the introgressed CG27 event

Morphological characters	Donor parent PB1	Recurrent parent (WP)	Homozygous BC_4F_4 (Introgressed with CG27)
Plant height (cm)*	119±2.9	135±1.62	133±1.45
No. of tillers*	21±1.0	17±0.65	16±0.66
No. of panicles*	19 ±1.0	13±0.47	14±0.62
[a]Days to flower*	65±0.93	84±0.92	82±0.94
Seed kernel length** (mm)	8.9±0.1	5.9±0.3	6.0 ± 0.0
100-seed weight (g)	1.73±0.01	1.37±0.10	1.40±0.01

Values are given in mean ± SE (n*=6) (n**=10); [a]Seeds were germinated in sterile condition in conical flanks for 3 d in dark followed by 14 d in light in half-strength MS medium. Seedlings were transferred to clay soil in pots and grown in a greenhouse 'Days to flower' parameter was recorded from the day of transplantation to pots in the greenhouse

Discussion

Rice blast and sheath blight are two important fungal diseases of rice which cause significant yield loss (Dubey et al. 2014). Transgenic rice constitutively expressing two PR proteins, chitinase and β-1,3-glucanase, displayed a high level of SBD tolerance (Sridevi et al. 2008). In the present study, a transgenic event CG27, comprising the *chi11* and *GLU* genes was introgressed into the WP rice variety through backcross breeding. Chitinase hydrolyzes chitin in the fungal cell wall. β-1,3-Glucanase degrades the fungal cell wall and promotes the release of cell wall fragments that act as elicitors to trigger the plant defense response (Taheri and Tarighi 2011). Transgene-based introgression is an important strategy to transfer a valuable agronomical trait from one parent to another parent, which is recalcitrant to routine transformation procedures. For example, high-iron trait (*ferritin* gene overexpression) in the IR68144 rice variety was successfully introgressed into Swarna by backcrossing. The BC_2F_4 introgression line showed increased level of *ferritin* gene expression and it was agronomically similar to Swarna (Paul et al. 2014). Commercially released *Bt* cotton varieties in India were developed by a cross between the *cry1Ac* transgene-expressing Coker 312 and local commercial Indian cotton varieties (Kranthi et al. 2005). Pro-vitamin A transgenic trait was transferred from SGR2 R1 event to Swarna genetic background through marker assisted backcross breeding (Bollineni et al. 2014). Roy et al. (2012), while backcrossing the RTBV resistant transgenic trait from PB1 to IET4094 and IET4786 rice varieties, observed that in some of the backcrossed progenies the selectable marker gene was not detectable by PCR. It indicated that the marker gene (*hph*) might have been lost during backcrossing. To rule out the possibility of presence of truncated T-DNA in the backcrossed progenies, we did GUS- histochemical staining and Southern blotting in our study. The results clearly revealed that intact T-DNA is transmitted from the CG27 transgenic parent to the backcrossed progenies. Moreover, RT-PCR and western blotting analyses confirmed the expression of *chi11* and *GLU* genes in the introgressed progenies. RAPD profiling showed that BC_4F_1 plants were genetically similar to WP recurrent parent. In this study, RAPD markers have been used for background selection. It is, however, highly desirable to use multiple SSR markers for each chromosome to ensure efficient introgression of the CG27 event in WP. The introgressed BC_4F_4 plants were morphologically similar

to the recurrent WP parent and displayed a significant level of SBD tolerance in comparison to control plants.

Combined expression of two PR proteins invariably yielded higher level of SBD resistance in rice in comparison to expression of individual PR protein genes. Resistance levels of transgenic rice with the rice *chi11* + tobacco *GLU* genes was 52% (Sridevi et al. 2008), rice *chi11* + rice *tlp*-D34 genes was 61% (Shah et al. 2013), *OsOXO4* + *OsCHI11* genes was 63% (Molla et al. 2016) and rice *chi11*+ tobacco *ap24* (osmotin) genes was 57 % (Sripriya et al. 2017). In this work a useful SBD tolerant transgenic trait (event CG27) has been successfully introgressed into a popular rice variety WP, which is difficult to transform. The homozygous PB1 CG27 transgenic line provided 52% SBD tolerance, whereas the CG27 introgressed BC_4F_4 line yielded 43 % SBD tolerance. This observation suggests that the genetic background of rice plants may have an impact on the level of SBD resistance which is conferred by transgenes.

Authors' contributions

Conceptualization of research (KV, CP); Designing of the experiments (KV, CP, KP); Generation of CG27 event in PB1 rice (GS, KV); Backcross breeding and data collection on foreground and background selections (CP, KP); RT-PCR, western blotting and SBD bioassay (PK); Analysis of data and interpretation (PK, CP, KP, KV); Preparation of the manuscript (PK, KV).

Conflict of Interest

The authors declare no conflict of interest.

Acknowledgements

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