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ORIGINAL ARTICLE

Phenotyping of VIGS-mediated gene silencing in rice using a vector derived from a DNA virus

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Abstract

Key message **Target genes in rice can be optimally silenced if inserted in antisense or hairpin orientation in the RTBV-derived VIGS vector and plants grown at 28 °C and 80% humidity after inoculation.**

Abstract Virus induced gene silencing (VIGS) is a method used to transiently silence genes in dicot as well as monocot plants. For the important monocot species rice, the Rice tungro bacilliform virus (RTBV)-derived VIGS system (RTBV-VIGS), which uses agroinoculation to initiate silencing, has not been standardized for optimal use. Here, using RTBV-VIGS, three sets of conditions were tested to achieve optimal silencing of the rice marker gene *phytoene desaturase* (*pds*). The effect of orientation of the insert in the RTBV-VIGS plasmid (sense, antisense and hairpin) on the silencing of the target gene was then evaluated using rice *magnesium chelatase* subunit H (*chl*H). Finally, the rice *Xa21* gene, conferring resistance against bacterial leaf blight disease (BLB) was silenced using RTBV-VIGS system. In each case, real-time PCR-based assessment indicated approximately 40–80% fall in the accumulation levels of the transcripts of *pds*, *chl*H and *Xa21*. In the case of *pds*, the appearance of white streaks in the emerging leaves, and for *chl*H, chlorophyll levels and F_v/F_m ratio were assessed as phenotypes for silencing. For *Xa21*, the resistance levels to BLB were assessed by measuring the lesion length and the percent diseased areas of leaves, following challenge inoculation with *Xanthomonas oryzae*. In each case, the RTBV-MVIGS system gave rise to a discernible phenotype indicating the silencing of the respective target gene using condition III (temperature 28 °C, humidity 80% and 1 mM MES and 20 μM acetosyringone in secondary agrobacterium culture), which revealed the robustness of this gene silencing system for rice.

Keywords Virus induced gene silencing · *Rice tungro bacilliform virus* · *Phytoene desaturase* · *chlH* · *Xa21* · Bacterial leaf blight · Agroinoculation

Abbreviations

VIGS	Virus induced gene silencing
pds	*Phytoene desaturase*
Xoo	*Xanthomonas oryzae* pv. *oryzae*
RTBV	*Rice tungro bacilliform virus*
RNAi	RNA interference
MLL	Mean lesion length
%DLA	Percentage diseased leaf area

Introduction

Virus induced gene silencing (VIGS) is a powerful loss of function technology for functional characterization of plant genes. It exploits host RNA interference (RNAi) pathway to cleave the introduced recombinant viral transcript containing plant gene fragments, into siRNAs which subsequently degrades the endogenous target gene transcript in a sequence specific manner (Lu 2003; Burch-Smith et al. 2004; Unver and Budak 2009). VIGS transiently down-regulates the expression of target genes, which leads to the emergence of rapid loss of function phenotype in plants

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within a matter of 2–3 weeks. This system is now considered as powerful tool for plant functional genomics, especially for those species which are recalcitrant to transformation (Bernacki et al. 2010; Zhang et al. 2010). Because the method is rapid, robust and lends itself to high-throughput techniques, VIGS acts as an outstanding alternative platform for large-scale functional screening of target genes (Ye et al. 2009; Yuan et al. 2011). The significance of VIGS for the functional analysis of genes in dicots (Arabidopsis, bean, tobacco, tomato, etc.) as well monocots (barley, rice, wheat, maize etc.) is well characterized (Holzberg et al. 2002; Scofield et al. 2005; Van Der Linde et al. 2011; Lee et al. 2012; Mei et al. 2016; Wang et al. 2016). For example, VIGS vectors have been derived from *Tobacco rattle virus* (Ratcliff et al. 2001; Liu et al. 2002; Bachan and Dinesh-Kumar 2012), *Apple latent spherical virus* (Sasaki et al. 2011), *Bean pod mottle virus* (Zhang et al. 2009, 2010) for dicots (tomato, apple, soybean) and *Rice tungro bacilliform virus* (Purkayastha et al. 2010), *Barley stripe mosaic virus* (Holzberg et al. 2002; Lee et al. 2012), *Brome mosaic virus* (Ding et al. 2006; Van Der Linde et al. 2011), *Cucumber mosaic virus* (Wang et al. 2016) and *Foxtail mosaic virus* (Liu et al. 2016; Mei et al. 2016) for monocots (rice, maize and wheat) and employed for functional characterization of genes. Interestingly, among the recently developed VIGS vectors, the *Bamboo mosaic virus* (BaMV)-based vector has been used for the functional analysis of genes in monocots as well as dicots (Liou et al. 2014).

Environmental factors play a crucial role for plant growth and development and also influence VIGS efficiency in plants. TRV-based gene silencing efficiency in tomato is enhanced at low temperature and low humidity (Fu et al. 2006). Similar observations were reported in cotton plants (Tuttle et al. 2008). Conversely, gene silencing has also been reported to be inhibited at low temperature in *Nicotiana benthamiana* protoplasts transfected with *Cymbidium ringspot virus* (Szittya et al. 2003). VIGS-mediated gene silencing efficiency is dependent upon the concentration of agroinocula and temperature (Wang et al. 2013) and insert orientation (Lacomme et al. 2003; Hein et al. 2005; Zhang et al. 2010). Therefore, conditions need to be standardized to achieve optimal and consistent silencing with any VIGS system.

We had earlier developed a gene silencing system for rice using RTBV based VIGS system (pRTBV-MVIGS, Purkayastha et al. 2010). To standardize the optimal conditions for the use of this system, we took inoculation method, temperature and humidity into account using the marker gene, *phytoene desaturase* (*pds*). For further extension of pRTBV-MVIGS mediated silencing, an additional marker gene, *magnesium chelatase* (*chl*) was also used, which produces a visible phenotype upon silencing. Magnesium chelatase participates in the biosynthetic pathway for chlorophyll synthesis and chloroplast development by catalyzing the insertion of Mg^{2+} into protoporphyrin IX, the last common intermediate precursor in chlorophyll biosynthesis (Zhang et al. 2006). Its deficiency causes yellowing of leaf (chlorosis) in higher plants. We used the gene encoding one of the three subunits of magnesium chelatase, *chl*H of rice in sense, antisense and hairpin orientation in pRTBV-MVIGS system, to investigate the effect of insert orientation on the efficiency of VIGS in rice. Finally, we silenced the gene *Xa21*, well known to confer resistance to the widespread bacterial pathogen *Xanthomonas oryzae* pv. *oryzae*, (*Xoo*) responsible for Bacterial leaf blight (BLB, Song et al. 1997), in rice cv. PB6 (Ellur et al. 2016) to study the potential of this VIGS system for rice functional genomics.

Materials and methods

Construction of recombinant viral vector

The recombinant pRTBV-MVIGS containing as-*pds* partial fragment (530 bp from rice cloned in antisense orientation, has already been described earlier (Purkayastha et al. 2010). The partial *O. sativa chl*H gene (Genbank accession no. EU569725.1) was amplified from rice cDNA using gene-specific primers having appropriate restriction enzyme sites and cloned in sense (318 bp), antisense (350 bp) and hairpin orientations (sense, 318 bp + antisense 318 bp) in the vector pHannibal (Table 1; Wesley et al. 2001). The inserts were then subcloned into pRTBV-MVIGS. Rice (*O. sativa*) *Xa21*gene fragment (560 bp, Genbank accession no. AB212798.1) was PCR amplified with gene specific primers (Table 1), using cDNA made from rice cv. PB6 carrying intact *Xa21*. PCR amplified *Xa21* fragment was cloned first in T/A vector, followed by subcloning in pRTBV-MVIGS vector respectively in antisense orientation. All constructs (empty pRTBV-MVIGS as well as recombinants) were transformed in the *Agrobacterium* (strain EHA-105) for agroinoculation in rice plants.

Agroinoculation for silencing *pds* and *chl*H

Rice plants cv. PB1 (20 day old) were agroinoculated (Purkayastha et al. 2010) with different compositions of resuspension buffers and kept at variable temperature and humidity (Table 2) to standardize the silencing efficiency of the *pds* gene. The effect of growing the secondary culture of agrobacterium (sub-culturing primary agrobacterium culture into larger volume of LB broth) in the presence of acetosyringone was also assessed. To study the

Table 1 Sequence and origin of primers used to amplify *chl*H subunit fragment of Mg chelatase gene and *Xa21* fragment

Primer name	Primer sequence (5′ → 3′)	Position on *Oryza sativa chlH, Xa21, UBQ5* nucleotide sequences
*chl*H Sense-FP	**ACGCGT**CTTGGTGAACATAGCTTCCC	81–100
*chl*H Sense-RP	**TTAATTAA**TCTCTCTGTCTGCTCTGATGAACT	374–398
*chl*H Antisense-FP	**TTAATTAA**GCCACCAAGTGCCA	51–64
*chl*H Antisense-RP	**ACGCGT**TCTCTCTGTCTGCTC	485–400
*chl*H Hairpin S-FP	**GAATTCACGCGT**CTTGGTGAACATAGCTTCCC	81–100
*chl*H Hairpin S-RP	**GGTACCATCGAT**TCTCTCTGTCTGCTCTGATGAACT	374–398
*chl*H Hairpin AS-FP	**TCTAGATTAATTAA**CTTGGTGAACATAGCTTCCC	81–100
*chl*H Hairpin AS-RP	**GGATCC**TCTCTCTGTCTGCTCTGATGAACT	374–398
RT *chl*H-FP	TGCTTGGTTTTGGTTTATC	326–344
RT *chl*H-RP	CAAGCTTCCCAGCTCATTA	456–474
Xa21 AS-FP	**TTAATTAA**GATCAAGCAGACCAGAGG	1540–1558
Xa21 AS-RP	**ACGCGT**CAGTGATTCTTCTA	2084–2100
RT *Xa21*-FP	GATAACAGAGGGAACGA	2323–2339
RT *Xa21*-RP	AGGCAACATCAAGTAGTA	2456–2473
RT *UBQ5*-FP	ACCACTTCGACCGCCACTACT	508–528
RT *UBQ5*-RP	ACGCCTAAGCCTGCTGGTT	558–576

Primers for sense, antisense and hairpin *chl*H gene fragment are indicated as *chl*H Sense FP/RP, *chl*H Antisense FP/RP and *chl*H hairpin S FP/RP, *chl*H AS FP/RP respectively. Primers for antisense *Xa21* gene fragment are indicated as *Xa21* AS-FP/RP. Primers for real-time PCR indicated as RT*chl*H-FP/RP, RT*Xa21*-FP/RP and RT*UBQ5*-FP/RP. Nucleotides in bold represent the added restriction enzyme sites

Table 2 Comparative silencing efficiency of *pds* gene showing leaf-streaking phenotype at variable temperature, humidity and composition of resuspension buffer in rice plants inoculated with pRTBV-MVIGS and pRTBV-MVIGS-as*pds*, at 20 days post inoculation

Composition of resuspension buffer[a]	Temperature (°C)	Humidity (%)	No. of plants showing white streaking/no. of plants inoculated
Resuspension buffer I	26	60	2/32
Resuspension buffer I	27	70	8/20
Resuspension buffer III (secondary culture supplemented with 1 mM MES and 20 μM acetosyringone)	28	80	30/50

Control plants inoculated with pRTBV-MVIGS ($n = 50$) did not show any streaking phenotype

[a] Resuspension buffer I: 5 mM $MgCl_2$, 5 mM MES, 100 μM acetosyringone; Resuspension buffer II: 10 mM $MgCl_2$, 10 mM MES, 200 μM acetosyringone; Resuspension buffer III: 10 mM $MgCl_2$, 10 mM MES, 500 μM acetosyringone

effect of insert orientation on *chl*H silencing efficiency, The secondary agrobacterium culture for each construct was supplemented with 1 mM MES and 20 μM acetosyringone and the resulting pellet was reconstituted in resuspension buffer (10 mM $MgCl_2$, 10 mM MES and 500 μM acetosyringone) having OD 0.6–1.0 and incubated for 3 h at room temperature. Rice plants were agroinoculated with 1 ml syringe at the meristematic region and kept at 28 °C and 80% humidity in a growth chamber.

Silencing phenotype and real-time PCR

The plants inoculated with recombinant pRTBV-MVIGS having *pds* and *chl*H partial cDNA fragments were observed for corresponding gene silencing phenotypes in the emerging leaves. The emerging leaves showing silenced phenotype were harvested and further validated for gene silencing through quantitative estimation of the corresponding transcript levels by real-time PCR. The harvested leaves were used for total RNA isolation using RNeasy plant mini kit (Qiagen) and cDNA was synthesized from 2 μg isolated RNA using high capacity cDNA synthesis kit (Applied Biosystems).

The Real-time PCR primers (Table 1) specific to *pds*, *chl*H and the endogenous control ubiquitin 5 (*UBQ5*) were designed using Primer Express® software (Applied Biosystem). cDNA from the plant inoculated with pRTBV-MVIGS and pRTBV-MVIGS-*pds*/*chl*H was used for qRT-PCR using Fast SYBR® Green Master Mix (Applied Biosystem) according to manufacturer's instructions. The

relative transcript accumulation of *pds* and *chl*H in the emerging leaves from pRTBV-MVIGS-*pds*/*chl*H inoculated plants were calculated and compared from pRTBV-MVIGS inoculated plants using comparative C_T method (Schmittgen and Livak 2008).

Chlorophyll content and chlorophyll fluorescence

Chlorophyll content was estimated from the emerging leaves at 20 dpi in the pRTBV-MVIGS and pRTBV-MVIGS-*chl*H inoculated plants using methods of Arnon (1949) and Hiscox and Israelstam (1980) with slight modifications. Chlorophyll was extracted without grinding the leaf tissues by heating the 100 mg of leaf samples in 3 ml DMSO at 65 °C in a water bath for 1 h, the extracted solution volume was made up to 5 ml with DMSO. The extracted chlorophyll was measured at wavelengths of 645 and 663 nm using spectrophotometer for each sample respectively as described (Arnon 1949). Chlorophyll fluorescence was measured from the emerging intact leaves in 30 min dark adapted pRTBV-MVIGS and pRTBV-MVIGS-*chl*H inoculated plants using portable Pulse modulated chlorophyll fluorescence meter (Walz GmbH, Effeltrich Germany). Chlorophyll fluorescence was recorded in triplicate for each emerged leaf from both pRTBV-MVIGS and pRTBV-MVIGS-*chl*H inoculated plants. The chlorophyll fluorescence values (F_v/F_m) obtained, represent the maximum quantum yield of Photosystem II (PS II), which is the ratio of variable (F_v) to maximum fluorescence (F_m, Puteh et al. 2013).

Agroinoculation, detection of *Xa21* silencing and BLB disease susceptibility

Rice cv PB6 (BLB susceptible) and PB6 carrying *Xa21* gene plants (BLB resistant) were first inoculated with *Xoo* (BXO43) using clip inoculation method (Babu et al. 2003) to analyse the BLB disease susceptibility at 7 days post inoculation in susceptible and resistant plants. pRTBV-MVIGS and pRTBV-MVIGS-*Xa21* were agroinoculated in 20 days old PB6-*Xa21* plants using standard method of rice agroinoculation (Purkayastha et al. 2010). The agroinoculated rice plants were screened for *Xa21* silencing at 15 day post agroinoculation through real time PCR as described above using gene-specific primers for *Xa21* and ubiquitin (*UBQ5*, as an internal control, Accession no. AK061988). The mock-silenced (using pRTBV-MVIGS) and *Xa21*-silenced (using pRTBV-MVIGS-*Xa21*) plants were challenged with *Xoo* using clip inoculation method and kept 28 °C temperature and 80% humidity. BLB disease incidences were measured at 7 day post-*Xoo* inoculation in both non-silenced and *Xa21*-silenced plants by measuring the mean lesion lengths (MLL) and average percentage diseased leaf area (%DLA) in the *Xoo* inoculated plants (two leaves/plant). The %DLA was calculated using the formula: %DLA = lesion area/leaf area × 100.

Results

Variable growth conditions affect *pds* silencing in rice

The efficiency of pRTBV-MVIGS mediated rice *pds* silencing at variable temperature, humidity and composition of resuspension buffer ($MgCl_2$, MES and acetosyringone) was determined under three different sets (I, II and III) of plant growth conditions (Table 2). At 20 day post inoculation (dpi), varying percentages of plants (6–60%) inoculated with pRTBV-MVIGS-*pds* displayed white streaks (photobleaching) in the emerging leaves, the plants inoculated with empty pRTBV-MVIGS and un-inoculated plants did not show any streaks in emerging leaves (Fig. 1A, B). Out of three sets (I, II, III) used, set III produced the most efficient gene silencing.

The accumulation of *pds* transcripts in plants inoculated with pRTBV-MVIGS and pRTBV-MVIGS-*pds* (showing white streaking phenotype) was determined by real time PCR from set III. Relative *pds* transcript levels for individual samples were plotted as a bar diagram (Fig. 1C), which showed 40–80% decrease in transcript accumulation of *pds* in pRTBV-MVIGS-*pds* inoculated plants as compared to pRTBV-MVIGS plants. The percentage silencing values shown represents the average of three samples for each plant. The relative *pds* transcript accumulation obtained thus corroborate *pds* silencing phenotype in inoculated rice plants.

VIGS mediated *chl*H silencing develops orientation specific phenotype

Rice plants inoculated with pRTBV MVIGS-Sense/Antisense/Hairpin *chl*H constructs showed leaf-yellowing phenotypes in the emerging leaves at 20 dpi with variable intensities. The antisense and hairpin *chl*H constructs were found to exhibit almost equal leaf-yellowing, which was more intense as compared to plants inoculated using sense *chl*H construct (Fig. 2a–d; Table 3). The plants inoculated with pRTBV-MVIGS (empty vector) did not display any leaf yellowing in the emerging leaves, similar to un-inoculated plants.

Silencing of *chl*H affects chlorophyll content and maximum quantum yield (F_v/F_m)

The amount of chlorophyll *a*, *b* and total chlorophyll were measured in emerging leaves of plants inoculated with

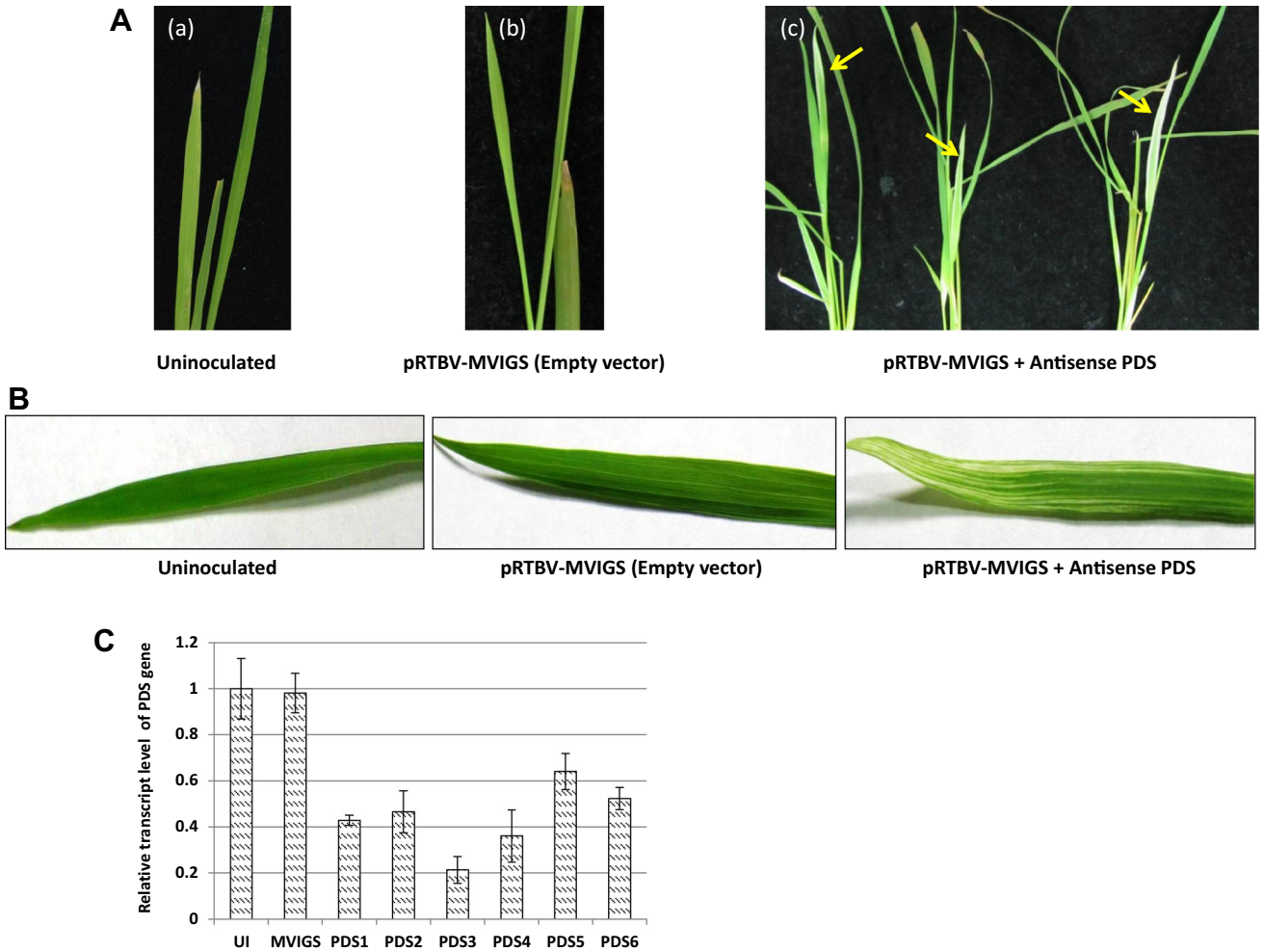

Fig. 1 **A** Phenotype in the emerging leaves of rice plants at 20 dpi. *a* Leaf of a un-inoculated plant did not show any silencing phenotypic symptoms, *b* leaf of pRTBV-MVIGS (empty vector) inoculated plant did not show any silencing phenotype and *c* leaves of pRTBV-MVIGS + Antisense-*pds* inoculated plant showed white streaking phenotype pointed in yellow arrowhead. **B** Enlarged picture of individual leaf at 20 dpi: Un-inoculated, pRTBV-MVIGS (empty vector) and pRTBV-MVIGS + Antisense-*pds* inoculated. **C** *pds* transcript accumulation in plants inoculated with pRTBV-MVIGS and pRTBV-MVIGS-Antisense-*pds*, labels at *x*-axis shows the individual plants. *UI* un-inoculated plant, *MVIGS* pRTBV-MVIGS, empty vector and *pds* (1–6) pRTBV-MVIGS-Antisense-*pds*. Relative transcript level of samples is shown at *y*-axis. The error bars indicate the standard deviations of the mean of three independent biological replicates (color figure online)

either pRTBV-MVIGS or pRTBV-MVIGS-*chl*H (sense/antisense/hairpin). In plants inoculated with pRTBV-MVIGS, three different forms of chlorophyll (*a*, *b* and total) measurements were found to be in the range of 0.32–0.35, 0.18–0.2 and 0.44–0.48 mg per g of fresh leaf weight respectively in plants. On the other hand, plants inoculated with pRTBV-MVIGS-*chl*H showed chlorophyll *a*, *b* and total chlorophyll in a range of 0.22–0.3, 0.12–0.16 and 0.32–0.4 mg per g of fresh leaf weight respectively across sense, antisense and hairpin constructs, representing significantly low chlorophyll accumulation as compared to pRTBV-MVIGS inoculated plants. Average chlorophyll contents (chl-*a*/chl-*b*/total-chl) in the plants agroinoculated with antisense *chl*H ($0.23 \pm 0.02/0.13 \pm 0.01/0.34 \pm 0.02$) and hairpin *chl*H ($0.24 \pm 0.02/0.13 \pm 0.01/0.34 \pm 0.02$) were comparable, but lower than plants inoculated with sense *chl*H ($0.28 \pm 0.01/0.14 \pm 0.01/0.37 \pm 0.02$) milligram per gram of fresh leaf weight respectively. Each value represents the mean $\pm$ standard deviation of six replicates with statistical significance between means for each construct ($p < 0.01$; Student's *t* test) (Fig. 3a–c).

In addition to chlorophyll contents, chlorophyll fluorescence from pRTBV-MVIGS and pRTBV-MVIGS-*chl*H was also determined using pulse modulated chlorophyll fluorescence meter to validate the reduction in chlorophyll biosynthesis. Chlorophyll fluorescence (F_v/F_m) represents the maximum quantum yield of PS II (first protein complex in the light-dependent reactions of oxygenic photosynthesis) located in the thylakoid membrane of plant leaves. The

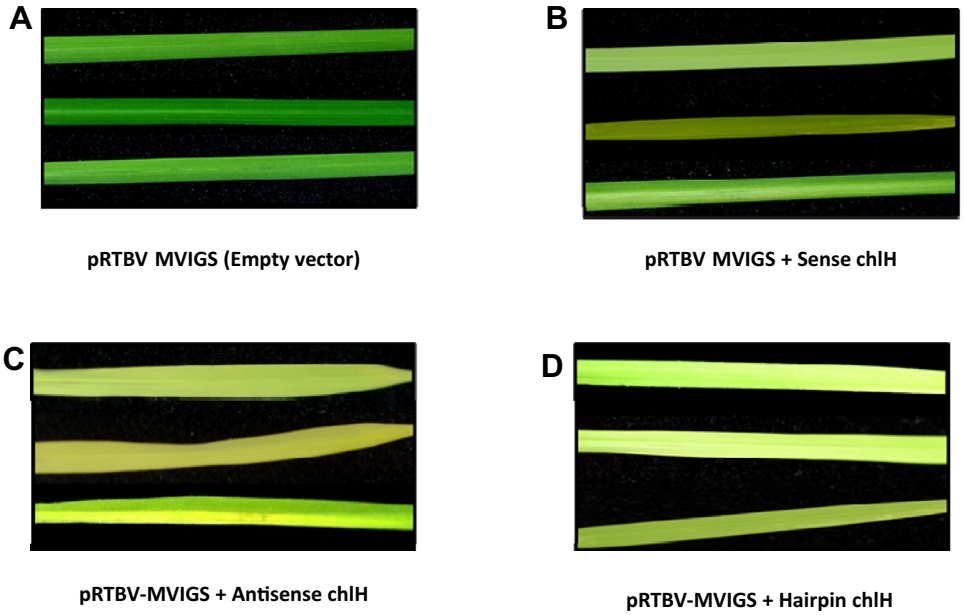

Fig. 2 Silencing phenotypes in the emerging leaves of rice plants inoculated with **a** pRTBV-MVIGS (empty vector), **b** pRTBV-MVIGS + Sense-*chl*H, **c** pRTBV-MVIGS + Antisense-*chl*H and **d** pRTBV-MVIGS + Hairpin-*chl*H

Table 3 Number of plants inoculated and number of plants showing leaf yellowing phenotype inoculated with pRTBV-MVIGS, pRTBV-MVIGS + Sense(S) *chl*H, pRTBV-MVIGS + Antisense(AS) *chl*H and pRTBV-MVIGS + Hairpin(HP) *chl*H constructs in three (s. no. 1–3) independent experiments

S. no.	Number of plants showing leaf yellowing in emerging leaves/number of plants inoculated		
	S-*chl*H	AS-*chl*H	HP-*chl*H
1	10/30	16/30	14/30
2	8/20	13/20	12/20
3	15/40	19/40	18/40

Ten rice plants were inoculated with pRTBV-MVIGS (empty vector) in each independent experiment and none of them showed leaf yellowing phenotype

pRTBV-MVIGS inoculated plants showed the F_v/F_m values above 0.8 while these values varied between 0.7–0.8 among sense, antisense and hairpin *chl*H inoculated plants. Like chlorophyll contents, average F_v/F_m values were comparable in plants inoculated with antisense (0.75 ± 0.01) and hairpin (0.74 ± 0.01) *chl*H, but lesser than those inoculated with sense *chl*H (0.79 ± 0.01) showing mean $F_v/F_m \pm$ standard deviation of six replicates with statistical significance between means for each construct ($p < 0.05$; Student's *t* test, Fig. 3d).

Transcript accumulation in *chl*H silenced plants

In order to determine the dependence of the orientation of *chl*H on the efficiency of silencing, the chl*H* transcript accumulation in plants inoculated with pRTBV-MVIGS-sense *chl*H/antisense *chl*H/hairpin *chl*H showing leaf yellowing phenotype at 20 dpi, was validated through real-time PCR. The decrease in relative *chl*H transcript accumulation was 20–50% in plants inoculated with pRTBV-MVIGS-sense *chl*H, 40–80% in plants inoculated with pRTBV-MVIGS-antisense *chl*H and 30–75% in plants inoculated with pRTBV-MVIGS-hairpin *chl*H respectively as compared to the transcript level in pRTBV-MVIGS inoculated plants (Fig. 4a–c). The *chl*H transcript accumulation obtained in plants inoculated with sense, antisense and hairpin *chl*H thus corresponded with their respective leaf yellowing phenotypes.

Silencing the BLB resistant gene, *Xa21*

Bacterial leaf blight disease susceptible and resistant rice cvs. PB6 and PB6 carrying *Xa21* (PB6-*Xa21*), were clip inoculated with *Xoo*, which resulted in severe necrotic lesions in PB6 plants as compared to mild lesions in the resistant variety PB6-*Xa21* at 7 day post clip inoculation (Fig. 5a). Further, *Xa21* was silenced in PB6-*Xa21* plants by agro-inoculating with pRTBV-MVIGS-*Xa21* (*Xa21* in antisense orientation), along with PB6-*Xa21* plants inoculated with pRTBV-MVIGS alone as control. At 15 dpi, 50–90% decrease in the relative transcript accumulation of *Xa21* was observed in pRTBV-MVIGS-*Xa21* as compared to pRTBV-MVIGS alone in agro-inoculated plants (Fig. 5b).

BLB disease susceptibility was scored in plants inoculated with pRTBV-MVIGS (non-silenced) and those with pRTBV-MVIGS-*Xa21* (*Xa21* silenced) to validate the effect of *Xa21* silencing. Both non-silenced and *Xa21*-silenced plants were then challenged with *Xoo*. At 7 dpi,

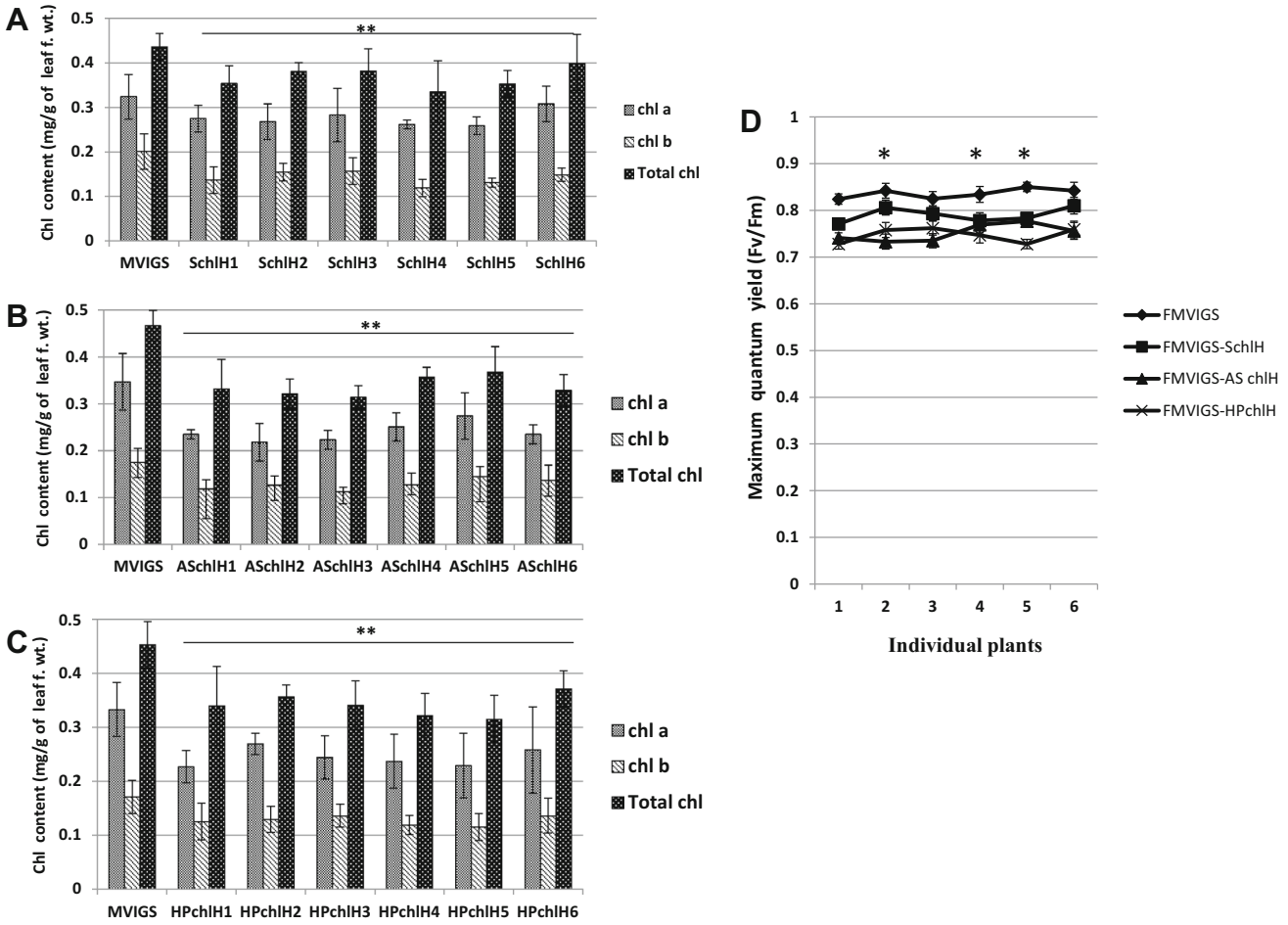

Fig. 3 Chlorophyll contents and chlorophyll fluorescence in inoculated plants at 20 dpi. Chlorophyll contents (chl *a*, chl *b* and total chl) in pRTBV-MVIGS + Sense-*chlH* (**a**), pRTBV-MVIGS + Antisense-*chl*H (**b**), pRTBV-MVIGS + Hairpin-*chl*H (**c**) inoculated plants as compared to pRTBV-MVIGS (empty vector) inoculated plants. In **a**–**c**, MVIGS and S/AS/HP-*chl*H (1–6) at the *x*-axis represent individual plants inoculated with pRTBV-MVIGS (empty vector) and pRTBV-MVIGS-S/AS/HP-*chl*H constructs. The experiment had three biological replicates and *bar* values with *error bars* indicate the mean value ± standard deviation with statistically significant differences between means for each construct and empty vector ($*p < 0.01$; Student's *t* test). **d** Comparison of chlorophyll fluorescence represented as F_v/F_m ratio in pRTBV-MVIGS and pRTBV-MVIGS + Sense/Antisense/Hairpin-*chlH* inoculated plants. The F_v/F_m ratio in pRTBV-MVIGS inoculated plants was taken as control. Numbers 1–6 at *x*-axis indicates individual plants. The experiment was conducted in three biological replicates and *line graph* with *error bars* indicate the mean value ± standard deviation with statistically significant differences between means for each construct ($*p < 0.05$; Student's *t* test)

more severe symptoms (Fig. 5c) were observed in plants silenced for *Xa21* (lesion lengths of 3.0–11.5 cm) as compared to non-silenced plants (1.0–3.2 cm), the difference being statistically significant ($p < 0.001$; Student's *t* test). The leaf lesion lengths, total leaf length and leaf width were also measured in all inoculated leaves of the plants to determine the mean lesion lengths and percentage diseased leaf area (%DLA) in both non-silenced and *Xa21*-silenced plants respectively. A total of fifteen plants were tested for analyzing lesion length and %DLA. The average mean lesion length (average cm ± SD) of the total number of non-silenced and *Xa-21* silenced plants was measured to be 1.75 ± 0.71 and 5.02 ± 2.42 respectively. Similarly, average %DLA of non-silenced and *Xa-21* silenced plants was calculated to be 8.75 ± 4.3 and 21.9 ± 9.7 showing statistical significance in the values ($p < 0.05$; Student's *t* test) respectively. Figure 6a, b shows the increased mean lesion lengths and %DLA upon *Xa21* silencing in a graphical manner (Fig. 6a, b).

Discussion

Virus induced gene silencing is potentially a very powerful tool to determine gene functions in the important food crop rice, especially to meet the emerging challenges of global warming and shrinking land and water resources, factors most likely to impact rice productivity in the not-so-distant

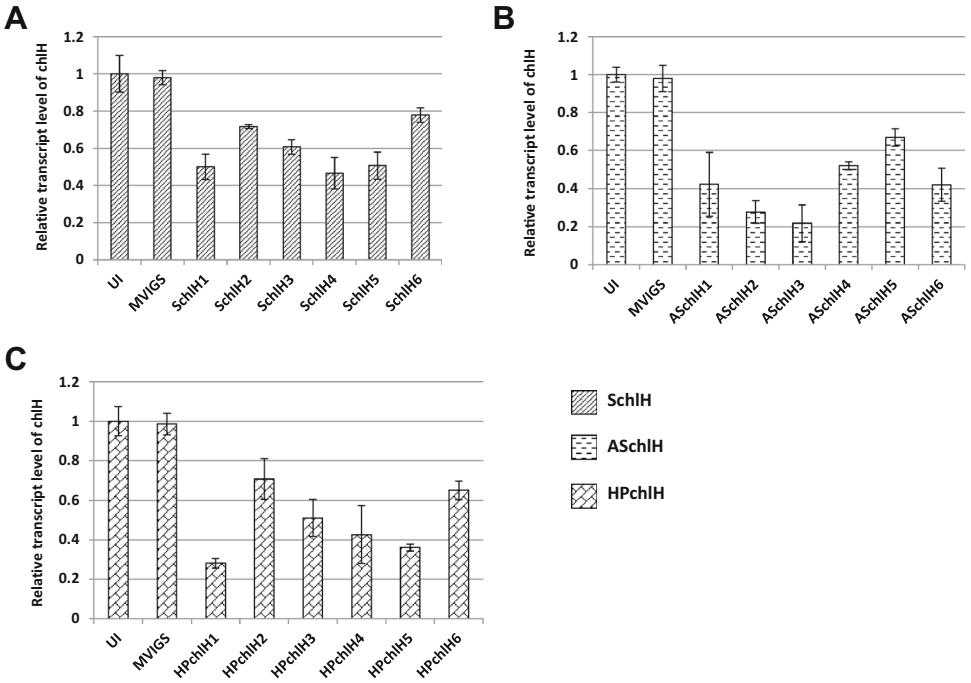

Fig. 4 Bar graph representation of *chl*H silencing in agroinoculated plants: **a** relative *chl*H transcript accumulation of pRTBV-MVIGS + Sense-*chl*H inoculated plants as compared pRTBV-MVIGS inoculated plants. **b** Relative *chlH* transcript accumulation of pRTBV-MVIGS + Antisense-*chl*H inoculated plants as compared pRTBV-MVIGS inoculated plants. **c** Relative *chl*H transcript accumulation of pRTBV-MVIGS + Hairpin *chlH* inoculated plants as compared pRTBV-MVIGS inoculated plants. In **a**–**c**, UI (un-inoculated), MVIGS and S/AS/HP-*chl*H (1–6) at the *x*-axis represent individual plants inoculated with pRTBV-MVIGS (empty vector) and pRTBV-MVIGS-S/AS/HP-*chl*H constructs. The *error bars* indicate the standard deviation of the mean value and experiment was repeated three times

future. Here, we standardize the optimal gene silencing conditions for use of the pRTBV-MVIGS system developed earlier by us for rice (Purkayastha and Dasgupta 2009; Purkayastha et al. 2010; Kant et al. 2015). Here, first using the marker gene *pds*, we tested various conditions of agroinoculation and growth conditions of rice plants, on the efficiency of gene silencing. The inclusion of 20 μM acetosyringone in the growth medium of the agrobacterium during its growth prior to inoculation to the plant (secondary culture) dramatically increased the *pds* silencing efficiency, as assessed by the number of plants showing the streaking phenotype in the emerging leaves. Acetosyringone is a known inducer of T-DNA transfer from Agrobacterium to plant cells (Godwin et al. 1991; He et al. 2010; Manfroi et al. 2015), but its enhancing effect, when added at the secondary culture may indicate a priming event of the T-DNA transfer process, in addition to the actual transfer process during the agro-inoculation. The enhancement of the proportion of plants showing streaking phenotype (indicative of *pds* silencing) upon slight increase of the temperature at which plants were grown following agro-inoculation, is contrary to some of the earlier reports of enhancement of VIGS silencing at lower temperatures (Fu et al. 2006; Nethra et al. 2006; Tuttle et al. 2008). However, as shown by (Szittya et al. 2003), the RNA silencing machinery is activated at higher temperatures, which should result in better VIGS-mediated silencing. Hence, our results of enhancement of the proportion of plants showing *pds* silencing at higher temperatures is supported by the above report, although variations of this general theme cannot be ruled out in plants adapted to varying temperatures. Therefore, we conclude that condition III is the most efficient, among the three conditions used, for gene silencing in rice using pRTBV-MVIGS.

Next, we used the marker gene *chl*H to study the effect of orientation of the insert in the VIGS vector on silencing efficiency. Our observation demonstrates that antisense and hairpin orientations gave rise to higher silencing as compared to sense orientation is somewhat similar to the reports of Holzberg et al. (2002) and Lacomme et al. (2003), both groups having used the BSMV system, that genes inserted in hp or sense-antisense orientation, show higher silencing than when inserted in antisense or sense orientations. Pacak et al. (2010), using the BMV system, Zhang et al. (2009, 2010), using the BPMV system reported that antisense orientations are superior than sense or hairpin in gene silencing. Unlike BSMV, BMV and BPMV, which are single-stranded positive-sense RNA viruses, RTBV is a double-stranded DNA pararetrovirus, which replicates by reverse transcribing its terminally

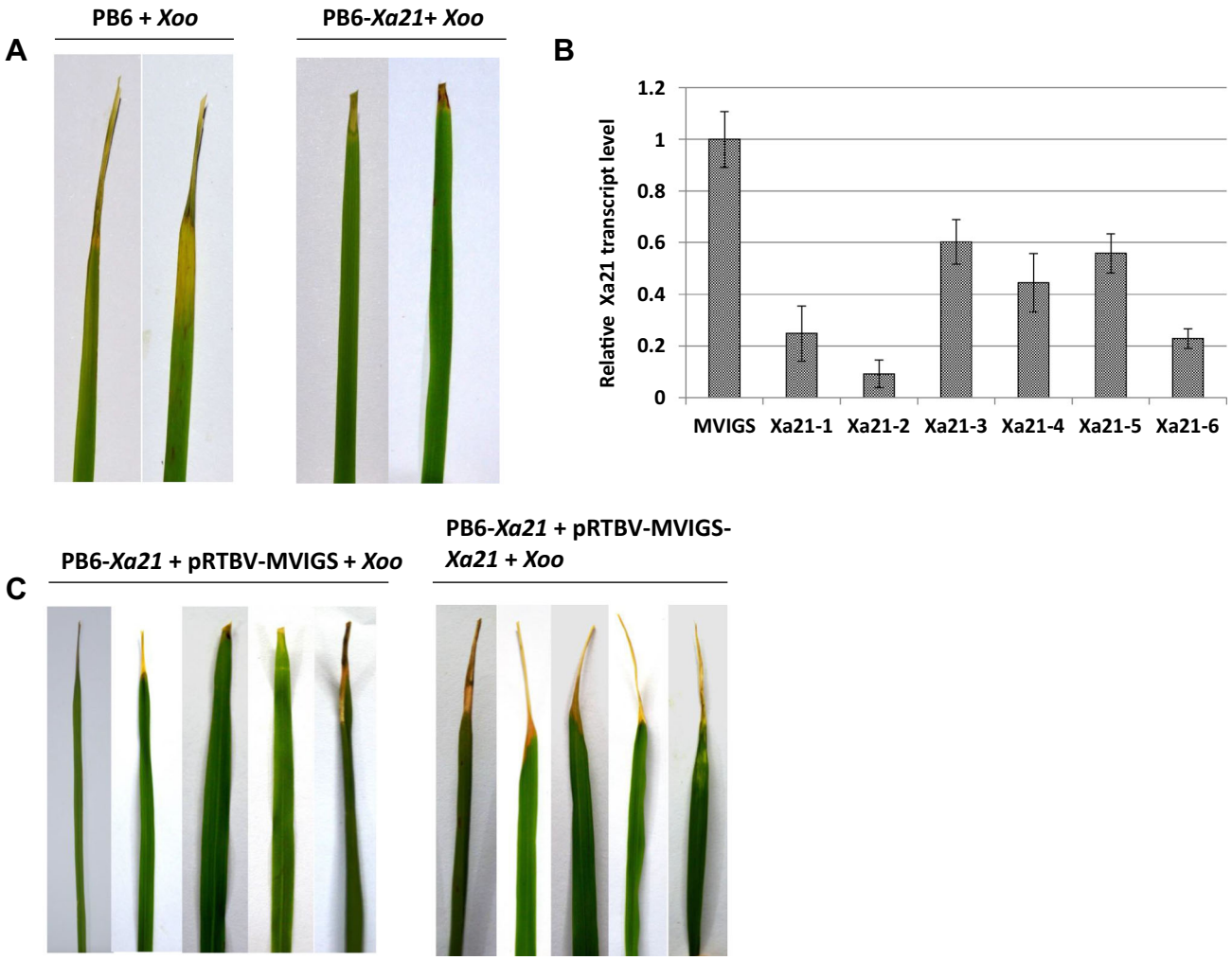

Fig. 5 BLB disease susceptibility in non-silenced and silenced plants: **a** PB6 (susceptible) and PB6-*Xa21* (resistant) plant leaves inoculated with *Xoo*. **b** Non-silenced (pRTBV-MVIGS inoculated) PB6-*Xa21* plants showed nominal disease symptoms with very less necrotic leaf lesion in *Xoo* inoculated leaves. **c** *Xa21*-silenced (pRTBV-MVIGS-*Xa21* inoculated) PB6-*Xa21* plants showed much greater disease symptoms with higher lesion lengths in the *Xoo* inoculated leaves as compared to non-silenced plants. MVIGS and *Xa21* (1–6) at *x*-axis represent individual plants inoculated with pRTBV-MVIGS and pRTBV-MVIGS-*Xa21* respectively. The error bars indicate the standard deviation of the mean value and experiment was repeated three times

redundant transcript (Hay et al. 1991). The interaction of the replication intermediates of pararetroviral nucleic acids with the plant DCLs has not been reported, but the accumulation of abundant small RNAs from pararetroviral transcripts (Blevins et al. 2011; Rajeswaran et al. 2014) from regions of the transcripts showing strong secondary structures, point towards the involvement of DCLs recognizing such structures. Using the same logic, intermolecular double-stranded molecules formed between the *chl*H mRNA and antisense *chl*H RNA formed from the pRTBV-MVIGS construct might also be recognized and processed into small RNAs by DCLs, which may be responsible for the observed silencing of *chl*H. The sense *chl*H-mediated silencing is inefficient, possibly because of inefficient recognition by DCLs or similar proteins. The RNAi machinery may also recognize the hairpin construct efficiently, thereby giving almost the same level of silencing as the antisense construct.

Despite the 40–80% reduction in the *chl*H transcript levels, the reduction of chlorophyll levels was modest (less than 25%) and so was the reduction in the quantum yield (F_v/F_m ratio). Fu et al. (2006) reported a much higher (90%) reduction in chlorophyll content following TRV-based silencing of *pds* gene in tomato. Similarly, Liu and Page (2008) reported an average of 90% fall in the nicotine levels in tobacco leaves following TRV-based silencing of *putrescine N-methyl transferase*, required in the nicotine biosynthetic pathway. The moderate fall in the chlorophyll content observed here could be due to mild silencing in pRTBV-MVIGS, compared to TRV. It could also be due to differences in the metabolic fluxes in the pathways being compared. Interestingly, the *chl*H-silenced phenotypes

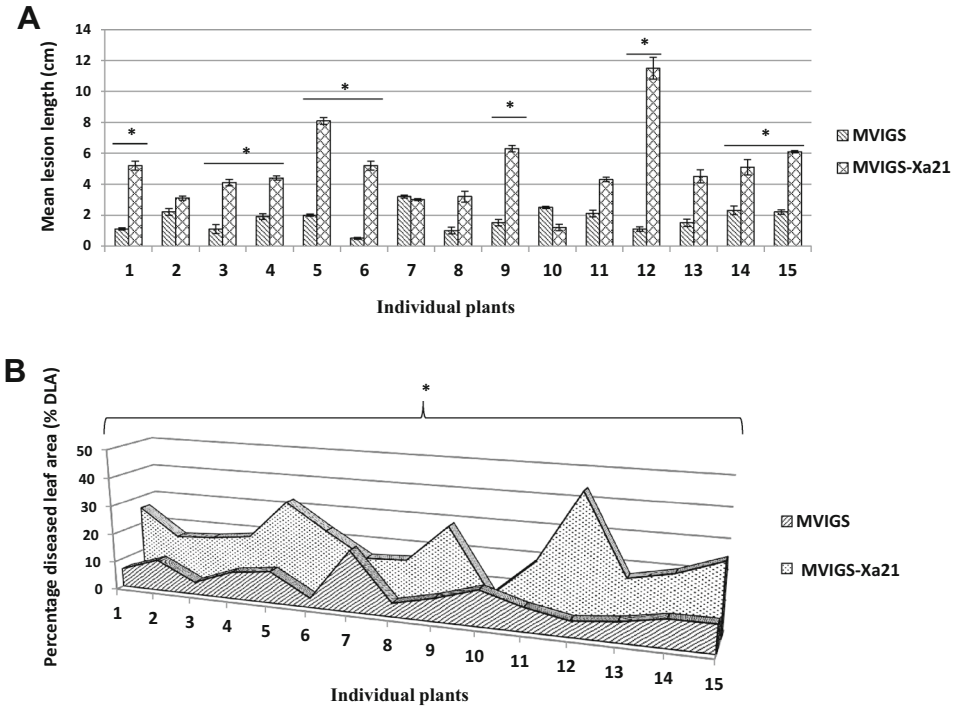

Fig. 6 Evaluation of BLB disease pathogenicity: **a** Graphical representation of mean lesion lengths (two leaves/plant) with *error bars* (mean ± standard deviation) for *Xoo* inoculated leaves in non-silenced (pRTBV-MVIGS inoculated) and *Xa21* silenced (pRTBV-MVIGS-*Xa21* inoculated) plants. An *asterisk* represents a statistically significant difference in values between two treatments (*$p < 0.001$; Student's *t* test). **b** Average percentage diseased leaf area (%DLA, two leaves/plant) of *Xoo* inoculated leaves in non-silenced (pRTBV-MVIGS inoculated) and *Xa21* silenced (pRTBV-MVIGS-*Xa21* inoculated) plants. Numbers (1–15) at *x*-axis in **a** and **b** represent individual plants inoculated with pRTBV-MVIGS and pRTBV-MVIGS-*Xa21*. An *asterisk* represents statistically significant differences in the values between two treatments (*$p < 0.05$; Student's *t* test). The experiment was repeated thrice

observed here (leaf yellowing following inoculation of the antisense and hairpin *chl*H constructs, Fig. 2c, d) were similar to the reported rice *chl1* and *chl9* mutant phenotypes (Zhang et al. 2006), in which the *chl*D and *chl*I subunits carried missense mutations.

VIGS mediated functional analysis of genes responsible for bacterial pathogens in monocots are limited; however few recent reports exist in dicots. For example; silencing of *NbMADS1* in *N. benthamiana* using *potato virus X* (PVX) VIGS vector showed compromised regulation of defense responses to $Harpin_{Xoo}$ (bacterial elicitor of the pathogenicity (*hrp*) gene of *Xoo*; Zhang et al. 2016). The SUMOE2 conjugating enzyme (*SCE*I) in *Solanum peruvianum* appeared to be crucial in the defense against *Clavibacter michiganensis* subsp. *michiganensis* (*Cmm*) colonization using *Tomato Mottle Virus* (ToMoV) based VIGS (Esparza-Araiza et al. 2015). Silencing of genes encoding ribosomal proteins, RPL12 and RPL19 in *N. benthamiana* showed disease resistance to non-host bacteria *P. syringae* pv. tomato using TRV-VIGS (Nagaraj et al. 2015). There are also few reports on VIGS mediated silencing of genes against fungal pathogens in monocots; for example, silencing of three genes in barley associated with resistance against powdery mildew, a fungal disease (Hein et al. 2005) and one against the fungal maize smut disease, caused by *Ustilago maydis* in maize (Van Der Linde et al. 2011). Hence, we decided to use the pRTBV-MVIGS system to silence the well-characterized *Xa21* gene, conferring resistance against the bacterial pathogen *Xoo*, responsible for causing BLB in rice. This would allow us to assess the use of this gene silencing system in characterizing other disease resistance genes. Silencing of *Xa21* resulted in similar levels of reduction in *Xa21* transcript accumulation in the variety PB6-*Xa21* as observed for *pds* and *chl*H, illustrating the similarity of the silencing process in all three cases. In most *Xa21*-silenced plants, there was an increase in the symptoms of BLB in the leaves upon challenge with *Xoo* (Figs. 5, 6), clearly indicating the robustness of the pRTBV-MVIGS gene silencing system for the study of disease resistance genes. It compares well with the levels of susceptibility reported after silencing of disease resistance genes using established silencing systems, such as TRV in Arabidopsis (Burch-Smith et al. 2006) and with newly-described VIGS systems, such as ALSV in tobacco (Igarashi et al. 2009), BSMV in barley (Hein et al. 2005) and BMV in maize (Van Der Linde et al. 2011). In this report, we have shown that silencing three rice genes, *pds*, *chl*H and *Xa21* resulted in the same extent

of reduction in the target transcript accumulation, namely 40–80% and hence reinforces the VIGS system as a robust gene silencing system for rice. Although, on one hand, silencing of *chl*H results in decrease in chlorophyll content and F_v/F_m ratio, the decrease is not the same. On the other hand, silencing of *Xa21* transcript accumulation to the same extent, results in increased susceptibility to BLB to a different level.

In this report, the conditions for efficient VIGS-mediated gene silencing in rice, using the pRTBV-MVIGS system, have been standardized. To achieve an efficient gene silencing, the condition III, described in Table 2 should be followed. The robust pRTBV-MVIGS-based gene silencing system for rice can now be used widely for silencing any gene of choice and can be expected to contribute immensely for rice functional genomics.

Author contribution statement ID conceived the work, RK constructed the clones, conducted the experiments and compiled the results, RK and ID analyzed the results and RK and ID wrote the manuscript.

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Compliance with ethical standards

Ethical standards The work was performed keeping in mind all applicable ethical standards.

Conflict of interest The authors declare that no conflicts of interests exist related to any of the authors pertaining to this work.

References

Arnon DI (1949) Copper enzymes in isolated chloroplasts. Polyphenoloxidase in *Beta vulgaris*. Plant Physiol 24:1–15

Babu RM, Sajeena A, Vidhyasekaran P et al (2003) Differential induction of chitinase and beta-1,3-glucanase in rice in response to inoculation with bacterial leaf blight pathogen (*Xanthomonas oryzae* pv. *oryzae*). J Plant Dis Prot 110:105–112

Bachan S, Dinesh-Kumar SP (2012) Tobacco rattle virus (TRV)-based virus-induced gene silencing. Methods Mol Biol 894:83–92

Bernacki S, Karimi M, Hilson P, Robertson N (2010) Virus-induced gene silencing as a reverse genetics tool to study gene function. Methods Mol Biol 655:27–45

Blevins T, Rajeswaran R, Aregger M et al (2011) Massive production of small RNAs from a non-coding region of Cauliflower mosaic virus in plant defense and viral counter-defense. Nucleic Acids Res 39:5003–5014

Burch-Smith TM, Anderson JC, Martin GB, Dinesh-Kumar SP (2004) Applications and advantages of virus-induced gene silencing for gene function studies in plants. Plant J 39:734–746

Burch-Smith TM, Schiff M, Liu Y, Dinesh-Kumar SP (2006) Efficient virus-induced gene silencing in Arabidopsis. Plant Physiol 142:21–27

Ding XS, Schneider WL, Chaluvadi SR et al (2006) Characterization of a Brome mosaic virus strain and its use as a vector for gene silencing in monocotyledonous hosts. Mol Plant Microbe Interact 19:1229–1239

Ellur RK, Khanna A, Yadav A et al (2016) Improvement of Basmati rice varieties for resistance to blast and bacterial blight diseases using marker assisted backcross breeding. Plant Sci 242:330–341

Esparza-Araiza MJ, Bañuelos-Hernández B, Argüello-Astorga GR et al (2015) Evaluation of a SUMO E2 conjugating enzyme involved in resistance to *Clavibacter michiganensis* subsp. michiganensis in *Solanum peruvianum*, through a tomato mottle virus VIGS assay. Front Plant Sci 6:1–11

Fu DQ, Zhu BZ, Zhu HL et al (2006) Enhancement of virus-induced gene silencing in tomato by low temperature and low humidity. Mol Cells 21:153–160

Godwin I, Todd G, Ford-Lloyd B, Newbury HJ (1991) The effects of acetosyringone and pH on Agrobacterium-mediated transformation vary according to plant species. Plant Cell Rep 9:671–675

Hay JM, Jones MC, Blakebrough ML et al (1991) An analysis of the sequence of an infectious clone of rice tungro bacilliform virus, a plant pararetrovirus. Nucleic Acids Res 19:2615–2621

He Y, Jones HD, Chen S et al (2010) Agrobacterium-mediated transformation of durum wheat (*Triticum turgidum* L. var. *durum* cv Stewart) with improved efficiency. J Exp Bot 61:1567–1581

Hein I, Barciszewska-Pacak M, Hrubikova K et al (2005) Virus-induced gene silencing-based functional characterization of genes associated with powdery mildew resistance in barley. Plant Physiol 138:2155–2164

Hiscox JD, Israelstam GF (1980) Erratum: a method for the extraction of chlorophyll from leaf tissue without maceration. Can J Bot 58:403

Holzberg S, Brosio P, Gross C, Pogue GP (2002) Barley strip mosaic virus-induced gene silencing in a monocot plant. Plant J 30:315–327

Igarashi A, Yamagata K, Sugai T et al (2009) Apple latent spherical virus vectors for reliable and effective virus-induced gene silencing among a broad range of plants including tobacco, tomato, *Arabidopsis thaliana*, cucurbits, and legumes. Virology 386:407–416

Kant R, Sharma S, Dasgupta I (2015) Virus-induced gene silencing (VIGS) for functional genomics in rice using rice tungro bacilliform virus (RTBV) as a vector. Plant Gene Silencing Methods Mol Bio 1287:201–217

Lacomme C, Hrubikova K, Hein I (2003) Enhancement of virus-induced gene silencing through viral-based production of inverted-repeats. Plant J 34:543–553

Lee W-S, Hammond-Kosack KE, Kanyuka K (2012) Barley stripe mosaic virus-mediated tools for investigating gene function in cereal plants and their pathogens: virus-induced gene silencing, host-mediated gene silencing, and virus-mediated overexpression of heterologous protein. Plant Physiol 160:582–590

Liou MR, Huang YW, Hu CC et al (2014) A dual gene-silencing vector system for monocot and dicot plants. Plant Biotechnol J 12:330–343

Liu E, Page JE (2008) Optimized cDNA libraries for virus-induced gene silencing (VIGS) using tobacco rattle virus. Plant Methods 4:5

Liu Y, Schiff M, Dinesh-Kumar SP (2002) Virus-induced gene silencing in tomato. Plant J 31:777–786

Liu N, Xie K, Jia Q et al (2016) Foxtail mosaic virus-induced gene silencing in monocot plants. Plant Physiol 171:16.00010

Lu R (2003) Virus-induced gene silencing in plants. Methods 30:296–303

Manfroi E, Yamazaki-Lau E, Grando MF, Roesler EA (2015) Acetosyringone, pH and temperature effects on transient genetic transformation of immature embryos of Brazilian wheat genotypes by *Agrobacterium tumefaciens*. Genet Mol Biol 38:470–476

Mei Y, Zhang C, Kernodle BM et al (2016) A foxtail mosaic virus vector for virus-induced gene silencing in maize. Plant Physiol 171:760–772

Nagaraj S, Senthil-Kumar M, Ramu VS et al (2015) Plant ribosomal proteins, RPL12 and RPL19, play a role in nonhost disease resistance against bacterial pathogens. Front Plant Sci 6:1192

Nethra P, Nataraja KN, Rama N, Udayakumar M (2006) Standardization of environmental conditions for induction and retention of post-transcriptional gene silencing using tobacco rattle virus vector. Curr Sci 90:431–435

Pacak A, Strozycki PM, Barciszewska-Pacak M et al (2010) The brome mosaic virus-based recombination vector triggers a limited gene silencing response depending on the orientation of the inserted sequence. Arch Virol 155:169–179

Purkayastha A, Dasgupta I (2009) Virus-induced gene silencing: a versatile tool for discovery of gene functions in plants. Plant Physiol Biochem 47:967–976

Purkayastha A, Mathur S, Verma V et al (2010) Virus-induced gene silencing in rice using a vector derived from a DNA virus. Planta 232:1531–1540

Puteh AB, Saragih AA, Ismail MR et al (2013) Chlorophyll fluorescence parameters of cultivated (*Oryza sativa* L. ssp. *indica*) and weedy rice (*Oryza sativa* L. var. *nivara*) genotypes under water stress. Aust J Crop Sci 7:1277–1283

Rajeswaran R, Golyaev V, Seguin J et al (2014) Interactions of rice tungro bacilliform pararetrovirus and its protein P4 with plant RNA silencing machinery. Mol Plant Microbe Interact 27:1370–1378

Ratcliff F, Martin-Hernandez AM, Baulcombe DC (2001) Tobacco rattle virus as a vector for analysis of gene function by silencing. Plant J 25:237–245

Sasaki S, Yamagishi N, Yoshikawa N (2011) Efficient virus-induced gene silencing in apple, pear and Japanese pear using Apple latent spherical virus vectors. Plant Methods 7:15

Schmittgen TD, Livak KJ (2008) Analyzing real-time PCR data by the comparative CT method. Nat Protoc 3:1101–1108

Scofield SR, Huang L, Brandt AS, Gill BS (2005) Development of a virus-induced gene-silencing system for hexaploid wheat and its use in functional analysis of the Lr21-mediated leaf rust resistance pathway 1. Plant Physiol 138:2165–2173

Song WY, Pi LY, Wang GL et al (1997) Evolution of the rice Xa21 disease resistance gene family. Plant Cell 9:1279–1287

Szittya G, Silhavy D, Molnár A et al (2003) Low temperature inhibits RNA silencing-mediated defence by the control of siRNA generation. EMBO J 22:633–640

Tuttle JR, Idris AM, Brown JK et al (2008) Geminivirus-mediated gene silencing from Cotton leaf crumple virus is enhanced by low temperature in cotton. Plant Physiol 148:41–50

Unver T, Budak H (2009) Virus-induced gene silencing, A post transcriptional gene silencing method. Int J Plant Genom 2009:198680

Van Der Linde K, Kastner C, Kumlehn J et al (2011) Systemic virus-induced gene silencing allows functional characterization of maize genes during biotrophic interaction with *Ustilago maydis*. New Phytol 189:471–483

Wang J-E, Li D-W, Gong Z-H, Zhang Y-L (2013) Optimization of virus-induced gene silencing in pepper (*Capsicum annuum* L.). Genet Mol Res 12:2492–2506

Wang R, Yang X, Wang N et al (2016) An efficient virus-induced gene silencing vector for maize functional genomics research. Plant J 86:102–115

Wesley SV, Helliwell C, Smith N et al (2001) Construct design for efficient, effective and high-throughput gene silencing in plants. Plant J 27:581–590

Ye J, Qu J, Bui HTN, Chua N-H (2009) Rapid analysis of *Jatropha curcas* gene functions by virus-induced gene silencing. Plant Biotechnol J 7:964–976

Yuan C, Li C, Yan L et al (2011) A high throughput barley stripe mosaic virus vector for virus induced gene silencing in monocots and dicots. PLoS One 6:e26468

Zhang H, Li J, Yoo JH et al (2006) Rice Chlorina-1 and Chlorina-9 encode ChlD and ChlI subunits of Mg-chelatase, a key enzyme for chlorophyll synthesis and chloroplast development. Plant Mol Biol 62:325–337

Zhang C, Yang C, Whitham S, Hill JH (2009) Development and use of an efficient DNA-based viral gene silencing vector for soybean. Mol Plant Microbe Interact 22:123–131

Zhang C, Bradshaw JD, Whitham S, Hill JH (2010) The development of an efficient multipurpose bean pod mottle virus viral vector set for foreign gene expression and RNA silencing. Plant Physiol 153:52–65

Zhang H, Teng W, Liang J et al (2016) MADS1, a novel MADS-box protein, is involved in the response of *Nicotiana benthamiana* to bacterial harpinXoo. J Exp Bot 67:131–141