Effect of orientation of transcription of a gene in an inverted transferred DNA repeat on transcriptional gene silencing in rice transgenics—a case study

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Abstract We studied transgene silencing in two transgenic rice plants, OSM25 and COT-OSM4, which harboured two different types of right border (RB)-centered inverted transferred DNA (T-DNA) repeats (IRs). The T-DNA in OSM25 has three genes *gus*, *OSM* and *hph*, all under the transcriptional control of the *Cauliflower mosaic virus* 35S promoter (P35S). The *gus* gene, which is proximal to the RB, is in a convergent orientation of transcription in the IR. OSM25 displayed silencing of all three transgenes. Nuclear run-on transcription analysis revealed that silencing of *gus*, *OSM* and *hph* genes in OSM25 operates at the transcriptional level. P35S showed hypermethylation in OSM25 plants. COT-OSM4 has P35S-driven *gus* and *hph* genes in its T-DNA. The *hph* gene, which is proximal to the RB, is in a divergent orientation of transcription in the IR. Unlike in OSM25, the transgenes in COT-OSM4 showed no silencing. These findings show that convergent orientation of transcription of a gene at the origin of an IR is important for transgene silencing.

Keywords Convergent genes · Divergent genes · DNA methylation · Inverted T-DNA repeats · Transcriptional gene silencing

Abbreviations

<i>gus</i>	β -glucuronidase gene
<i>hph</i>	Hygromycin phosphotransferase gene
IR	Inverted T-DNA repeat
JF	Junction fragment
LB	Left T-DNA border
<i>OSM</i>	Tobacco osmotin gene
P35S	<i>Cauliflower mosaic virus</i> 35S promoter
RB	Right T-DNA border

Introduction

Agrobacterium tumefaciens-mediated plant transformation offers the advantage of defining the transferred DNA (T-DNA) between the right (RB) and left (LB) T-DNA borders (Gelvin 2003). The formation of inverted T-DNA repeats (IRs) upon *A. tumefaciens*-mediated plant transformation was first reported in tobacco calli (Kwok et al. 1985). Complex T-DNA structures, particularly those with IRs, are often coupled with transgene silencing (Stam et al. 1997). Gene silencing occurs at the posttranscriptional level (PTGS) or at the transcriptional level (TGS). In PTGS the promoter is active in transcription but the mRNA is degraded, whereas in TGS, the promoter is densely methylated and hence is inactivated (Fojtova et al. 2003). In an IR, the gene which lies proximal to the origin of an IR is most often subjected to PTGS (Hobbs et al. 1990).

Here, we report that two transgenic rice plants with structurally different RB-centered IRs display differences in transgene silencing. The IRs differ in the orientation of transcription of the transgene which is proximal to the origin of the IR. A transgenic rice plant OSM25 harbours an RB-centered IR with three P35S-driven transgenes, *gus*, *OSM* and *hph*. The *gus* gene at the origin of the IR is in a convergent orientation of transcription. OSM25 exhibited transgene silencing by

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The COT-OSM4 plant was obtained by co-transformation of the scutellum-derived callus of *Oryza sativa* L. ssp. *indica* cv Pusa Basmati1 with the *A. tumefaciens* strain C58C1 Rif^r (pGV2260::pSSJ1, pRP7) (Sripriya et al. 2011). pGV2260::pSSJ1 T-DNA harbours the P35S-driven *hph* and *gus* genes (Fig. 2c). Rice plants were grown in pots with clay soil in a greenhouse with natural light, under 12-h day and 12-h night cycles.

Rice DNA (2.5 µg) was digested with restriction enzymes and electrophoresed in 0.8 % [w/v] agarose gels. DNA was transferred to the Zeta-probe nylon membrane (Bio-Rad, Hercules, USA). The probe DNA was labeled with [α - 32 P]dCTP (Board of Radiation and Isotope Technology, Mumbai, India) using Megaprime™ labeling kit (GE Healthcare UK Limited, Little Chalfont, UK). DNA blotting, hybridization and washes were done as described by Sridevi et al. (2006).

Fig. 2 Inverted transferred DNA (T-DNA) repeats in transgenic rice plants. **a** pGSD2 T-DNA. The *gus* probe is indicated as a **bold line**. Distance between the RB and the restriction sites *Hind*III (3.0 kb-H), *Bam*HI (3.0 kb-B), *Eco*RI (3.4 kb-E) and *Xho*I (4.5 kb-X) are marked in *broken lines*. **b** Southern blot analysis of OSM25 using the [α - 32 P]dCTP-labeled *gus*. **c** pGV2260::pSSJ1 T-DNA. The *hph* probe is indicated as a **bold line**. Distance between RB and restriction sites of *Sac*I (1.7 kb-Sc), *Eco*RI (2.1 kb-E), *Sal*I (4.4 kb-SI) and *Bgl*II (5.1 kb-Bg) are marked in *broken lines*. **d** Southern blot analysis of COT-OSM4 using the [α - 32 P]dCTP-labeled *hph* probe

Total RNA extracted (Pawlowski et al. 1994) from leaves was estimated in a spectrophotometer. Total RNA (10 μ g) was electrophoresed in 1.2 % [w/v] agarose gels with 1 % [v/v] formaldehyde. RNA was transferred to a nylon membrane (Roche Diagnostics GmbH, Mannheim, Germany). Hybridization and washes were done as described by Pawlowski et al. (1994).

Nuclear run-on transcription analysis

Nuclei isolation, labeling of ongoing transcription with [α - 32 P]UTP (Board of Radiation and Isotope Technology, Mumbai, India), extraction of labeled nuclear transcripts, hybridization, and washes were done as described by Meng and Lemaux (2003).

Results and discussion

Ten transgenic rice plants (OSM2, OSM3, OSM5, OSM6, OSM8, OSM20, OSM25, OSM36, OSM53, and OSM54) were obtained by transformation with *A. tumefaciens* LBA4404 (pSB1, pGSD2). DNA samples were digested with *Hind*III and the blots were probed with the *gus* and *OSM* genes to analyze RB and LB junction fragments (JFs), respectively. Six plants (OSM2, OSM8, OSM20, OSM36, OSM53, and OSM54) were identified as single-copy transgenic plants, OSM5 as a two-copy transgenic plant, and OSM3 and OSM6 as multi-copy transgenic plants (Fig. 1). The number of JFs which hybridized to the *gus* probe (Fig. 1b, c) and the *OSM* probe (Fig. 1d, e) were the same in all transgenic plants, except OSM25. OSM25 displayed one RB JF for the *gus* probe and two LB JFs for the *OSM* probe (Fig. 1b and d), suggesting integration of an RB-centered IR.

DNA of OSM25 was digested with multiple restriction enzymes and Southern blotting was performed with the *gus* probe to confirm the nature of the IR. DNA of OSM25 was digested with *Bam*HI, *Eco*RI, *Hind*III and *Xho*I. If an RB-centered IR is formed, the *gus* probe-hybridizing fragments are expected to be twice the distance between restriction site and the RB (Fig. 2a). In OSM25, 6.0-kb *Bam*HI, 6.8-kb *Eco*RI, 6.0-kb *Hind*III, and 9.0-kb *Xho*I fragments hybridized (Fig. 2b) confirming the formation of an RB-centered IR.

Co-transformation of rice with *A. tumefaciens* (pGV2260::pSSJ1, pRP7) yielded 15 transgenic plants (Sripriya et al. 2011). One of these, COT-OSM4, displayed one *Eco*RI-released 4.2-kb RB JF with the *hph* probe, which was twice the distance (2.1 kb) between the restriction site and the RB (Fig. 2c) (Sripriya et al. 2011). In other 14 transgenic plants, the fragments which displayed hybridization were not of 4.2 kb size, indicating the absence of RB-centered IRs. Therefore, the T-DNA insertion in COT-OSM4 was proposed to carry an RB-centered IR. To confirm the IR in COT-OSM4, DNA was digested with *Eco*RI, *Sac*I, *Bgl*II and *Sal*I. The blot was probed with the *hph* gene. In COT-OSM4, as expected for an IR, 4.2-kb *Eco*RI, 3.4-kb *Sac*I, 10.2-kb *Bgl*II and 8.8-kb *Sal*I fragments hybridized (Fig. 2d). Thus, the RB-centered IR in COT-OSM4 is confirmed.

Transgenes residing in IRs are highly prone to gene silencing (Stam et al. 1997; Hobbs et al. 1990; Fojtova et al. 2006; Mroczka et al. 2010). In view of the possibility that silencing of transgenes in an IR may initiate at the T₁ or T₂ generations and intensify in homozygous plants (Skarn et al. 2006), homozygous T₂ plants were established for OSM25 and COT-OSM4 as described by Sridevi et al. (2006). Of the 21 OSM25 T₁ plants analyzed, six were null, nine were hemizygous, and six were homozygous, fitting to the 1:2:1 Mendelian segregation ratio for a single T-DNA locus.

A homozygous T₂ OSM25 plant was selected to study transgene silencing caused by the IR. A single copy transgenic

rice plant, OSM20 was used as a positive control. Northern blotting was done with the *gus*, *OSM*, and *hph* probes. OSM20 showed high levels of *gus*, *OSM*, and *hph* transcripts (Fig. 3a, b, c). In OSM25, *gus*, *OSM*, and *hph* transcripts did not accumulate. Thus, all three transgenes in OSM25 were silenced. The T-DNA of the binary vector pGSD2 (Fig. 1a), from which OSM25 was derived, has three transgenes (*gus*, *OSM*, and *hph*) under the transcriptional control of P35S. Expression of multiple transgenes under the transcriptional control of one strong promoter such as P35S is conceived as one of the important causes for gene silencing. In order to test this possibility, five single-copy transgenic rice plants (OSM8, OSM20, OSM36, OSM53, OSM54), and two two-copy transgenic rice plants (OSM5, OSM25), all derived by pGSD2 transformation, were subjected to Northern blotting with the *OSM* probe. All plants, except OSM25, showed accumulation of the *OSM* transcript (Fig. 4a, b). Thus, silencing of transgenes in OSM25 is due to T-DNA IR and not due to multiplicity of the 35S promoter.

Two homozygous COT-OSM4 T₂ rice plants with IR were selected to study the transgene expression. A transgenic tobacco expressing P35S-driven *gus* and *hph* transgenes (lab collection) was used as a positive control. Northern blot analyses showed high levels of *gus*, and *hph* transcripts in the transgenic tobacco plant (Fig. 3d, e). Interestingly, unlike in OSM25, both COT-OSM4 plants accumulated *gus* and *hph* transcripts. Thus, the transgenes in COT-OSM4 were not silenced till the T₂ generation.

Nuclear run-on transcription assay (Meng and Lemaux 2003) was done to study whether the *gus*, *OSM*, and *hph* genes in OSM25 were subjected to PTGS or TGS. To ensure that the actively transcribed T-DNA genes are detected, nuclei from the OSM20 plant, which expressed the *hph*, *OSM*, and *gus* genes (Fig. 3a, b, c), was included as a positive control. In this assay, the labeled total transcripts from the nuclei of the control plant, OSM20 (non-silenced), and OSM25 (silenced) plants were hybridized to independent DNA blots that contained *Hind*III-digested plant genomic DNA (5 µg), λ-*Hind*III DNA (2.5 µg), and digested plasmids (2.5 µg each) containing the *hph*, *OSM*, and *gus* genes present in the T-DNA. Plant DNA with high homology with the total transcripts served as the positive hybridization control and λ-*Hind*III with no homology, served as the negative hybridization control. Suspension of the nuclei in a transcription buffer containing unlabeled NTPs and [α-³²P]UTP allows the ongoing transcription to continue and provides labeled transcripts from genes which are actively transcribed while the nuclei are isolated. No or negligible reinitiation of transcription occurs after nuclei isolation. The labeled total transcripts were extracted and used as probe to hybridize to the DNA blot.

As expected, intense hybridization signal was observed in the *Hind*III-digested plant DNA lane (positive control) in all the three blots (Fig. 5a, b, c) indicating that the total transcripts

Fig. 3 Transgene silencing in OSM25 and COT-OSM4. **a**, **b** and **c** Northern blot analyses of OSM25 and OSM20 with [α - 32 P]dCTP-labeled, 1.8-kb *gus* (**a**), 0.96-kb *OSM* (**b**) and 1.05-kb *hph* (**c**) probes. Lanes with RNA of control plant (C), OSM25 (25), and OSM20 (20). **d** and **e** Northern blot analysis in COT-OSM4 with [α - 32 P]dCTP-labeled, 1.8-kb *gus* (**d**) and 1.05-kb *hph* (**e**) probes. Lanes with total RNA of control plant (C), COT-OSM4/1 (1), COT-OSM4/2 (2), and tobacco plant (P) expressing *gus* and *hph*

from the nuclei were effectively labeled. No signal was observed in the λ -HindIII (negative control) in all the blots indicating the absence of cross hybridization. In OSM20, the nuclear transcripts hybridized to the *gus*, *OSM*, and *hph* gene fragments (Fig. 5c). Thus, the nuclear run-on transcription experiment worked very well. No hybridization to the *gus*, *OSM*, and *hph* genes to the control plant nuclear transcript was observed (Fig. 5a). In the OSM25 nuclear transcript

hybridized blot, no hybridization was seen for the *gus*, *OSM*, and *hph* genes (Fig. 5b). Lack of signal for the *gus*, *OSM*, and *hph* genes indicates that the transcription of the *gus*, *OSM*, and *hph* genes did not occur in the OSM25 nuclei. Thus, the *gus*, *OSM*, and *hph* genes are inferred to be under TGS in OSM25.

Methylation of the 35S promoters in OSM25 was studied by Southern blotting using the methylation-sensitive restriction enzymes *AluI* and *MspI*. The 35S promoter was used as the probe. Absence of methylation in the promoter will lead to complete digestion and hence multiple, short DNA fragments of expected sizes will hybridize, whereas methylation in the promoter will lead to incomplete digestion and hence longer DNA fragments will hybridize to the probe. DNA samples (5 μ g) from OSM20 (non-silenced) and OSM25 (silenced) were digested with *AluI* and *MspI* and subjected to Southern blotting using the P35S probe. In OSM20 (non-silenced), short DNA fragments (<1.0 kb) hybridized (Fig. 6) indicating the absence of methylation in the P35S region. However, in OSM25 (silenced), fragments of higher molecular weight hybridized for both enzyme digestions. Incomplete digestion with *AluI* and *MspI* indicates that the 35S promoter in OSM25 is methylated. As reported by Fojtova et al. (2003), P35S of TGS silenced genes in OSM25 are subjected to hypermethylation.

Fig. 4 Northern blot analysis of OSM T₀ plants with the [α - 32 P]dCTP-labeled, *OSM* probe. Lanes C, control plant RNA. Numbers on top represent T₀ plants. **a** and **b** represent two independent Northern blot analyses

Fig. 5 Nuclear run-on transcription analysis in OSM25 and OSM20. *HindIII*-digested genomic DNA from the control plant (*P*), λ -*HindIII* (λ), plasmid borne gene sequences of *gus* (*g*), *OSM* (*o*), and *hph* (*h*)

were electrophoresed. The blots were probed with [α - 32 P]UTP-labeled total transcripts isolated from (a) control plant nuclei, (b) OSM25 nuclei, and (c) OSM20 nuclei

Although both OSM25 and COT-OSM4 harboured IRs, transgenes in OSM25 were silenced but those in COT-OSM4 were not silenced. This suggests that the direction of transcription of the gene which is proximal to the origin of the IR is an

important silencing determinant. The *gus* gene, which is proximal to the origin of the IR in OSM25, is in a ‘convergent’ orientation which can generate a double-stranded RNA (dsRNA) by a read through process in which transcription is continued beyond the transcription termination site. The dsRNA thus generated may have served as the trigger of silencing in OSM25. In contrast, COT-OSM4 lacks such structural ability to make dsRNA because the *hph* gene proximal to the origin of the IR is in a ‘divergent’ orientation. The importance of convergent orientation of a gene for it to be silenced in an IR has been shown earlier (Mroccka et al. 2010; Hobbs et al. 1990). Mroccka et al. (2010) showed that a read-through dsRNA served as the trigger of PTGS. Although IRs are very frequently associated with gene silencing, we cannot totally rule out the rare possibility of flanking genomic sequences at the site of T-DNA integration triggering silencing in OSM25.

The convergent orientation of transcription of the *gus* gene in OSM25 is expected to cause PTGS. However, nuclear run-on transcription analysis revealed that the *gus* as well as *OSM* and *hph* genes were subjected to TGS. An IR locus, *locus1* in tobacco, epigenetically switched from PTGS to TGS upon prolonged vegetative propagation in tissue culture (Fojtova et al. 2003; Krizova et al. 2009). The P35S promoter of the TGS silenced *nptII* gene was hypermethylated. Similarly, the *gus* gene proximal to the origin of the IR in OSM25 may have initially been a PTGS locus and subsequently became a TGS locus by hypermethylation. Our analysis with methylation-sensitive enzymes clearly showed that 35S promoters in OSM25 are methylated (Fig. 6).

Fig. 6 Methylation analysis in OSM25 and OSM20. DNA (5 μ g) was digested with *AluI* (a) and *MspI* (b). The blot was probed with [α - 32 P]dCTP-labeled P35S sequence. Lanes *U*, undigested DNA from OSM25; *Pr*, P35S (50 pg); OSM25 digested DNA (25); OSM20 digested DNA (20)

This study with OSM25 and COT-OSM4, both harbouring RB-centered IRs, shows that convergent orientation of transcription of the gene proximal to the origin of the IR is crucial to trigger silencing.

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References

- Fojtova M, Van Houdt H, Depicker A, Kovarik A (2003) Epigenetic switch from posttranscriptional to transcriptional silencing is correlated with promoter hypermethylation. *Plant Physiol* 133:1240–1250
- Fojtova M, Bleys A, Bedrichova J, Van Houdt H, Krizova K, Depicker A, Kovarik A (2006) The *trans*-silencing capacity of invertedly repeated transgenes depends on their epigenetic state in tobacco. *Nucl Acid Res* 34:2280–2293
- Gelvin SB (2003) *Agrobacterium*-mediated plant transformation: the biology behind the “gene-jockeying” tool. *Microbiol Mol Biol Rev* 67:16–37
- Hobbs SLA, Kpodar P, DeLong CMO (1990) The effect of T-DNA copy number, position and methylation on reporter gene expression in tobacco transformants. *Plant Mol Biol* 15:851–864
- Krizova K, Fojtova M, Depicker A, Kovarik A (2009) Cell culture-induced gradual and frequent epigenetic reprogramming of invertedly repeated tobacco transgene epialleles. *Plant Physiol* 149:1493–1504
- Kwok WW, Nester EW, Gordon MP (1985) Unusual plasmid DNA organization in an octopine crown gall tumor. *Nucl Acid Res* 13:459–471
- Meng L, Lemaux PG (2003) A simple and rapid method for nuclear run-on transcription assays in plants. *Plant Mol Biol Rep* 21:65–71
- Mroccka A, Roberts PD, Fillatti JJ, Wiggins BE, Ulmasov T, Voelker T (2010) An intron sense suppression construct targeting soybean *FAD2-1* requires a double-stranded RNA-producing inverted repeat T-DNA insert. *Plant Physiol* 153:882–891
- Pawlowski K, Kunze R, DeVries S, Bisseling T (1994) Isolation of total, poly(A) and polysomal RNA from plant tissues. In: Gelvin SB, Schilperoort RA (eds) *Plant molecular biology manual*, 2nd edn. Kluwer Academic Publishers, Dordrecht, pp D5: 1–13
- Skarn M, Eike MC, Meza TJ, Mercy IS, Jakobsen KS, Aalen RB (2006) An inverted repeat transgene with a structure that cannot generate double-stranded RNA, suffers silencing independent of DNA methylation. *Transgenic Res* 15:489–500
- Sridevi G, Parameswari C, Rajamuni P, Veluthambi K (2006) Identification of hemizygous and homozygous transgenic rice plants in T₁ generation by DNA blot analysis. *Plant Biotechnol* 23:531–534
- Sripriya R, Sangeetha M, Parameswari C, Veluthambi B, Veluthambi K (2011) Improved *Agrobacterium*-mediated co-transformation and selectable marker elimination in transgenic rice by using a high copy number pBin19-derived binary vector. *Plant Sci* 180:766–774
- Stam M, de Bruin R, Kenter S, van der Hoorn RAL, van Blokland R, Mol JNM, Kooter JM (1997) Post-transcriptional silencing of chalcone synthase in *Petunia* by inverted transgene repeats. *Plant J* 12:63–82