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Functional analysis of a rice tapetum specific (RTS) gene promoter variant identified in rice cultivar IR64

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ABSTRACT

A novel variant of Rice Tapetum Specific (RTS) promoter, RTS64 isolated from indica rice cv. IR64, differed from reported RTS promoter from IR54 (RTS54) in the nucleotide sequence as well as in number and distribution of cis-acting regulatory elements. The new promoter contains motifs implicated in strong anther-specific expression. RTS64 promoter showed enhanced expression in anther compared to reported RTS54 promoter as indicated by Gus reporter assay. This novel anther-specific promoter, thus, would be a better candidate in genetic engineering strategies that require a high level of anther-specific expression.

Key words: *RTS54, RTS64, GUS, anther-specific expression, IR64, IR54*

INTRODUCTION

Crop species of the poaceae grass family (*viz.,* rice, wheat, maize, sorghum and millet) are the staple food for a large population of the world. Hence, improvement of agronomic performance of these crops remains a major challenge for plant breeders to meet food supply for growing population of the world (Siddiq, 2000; Khush, 2004; Agarwal et al., 2016). Transgenic technology can complement conventional breeding techniques to fulfill this target. Till date, most of the promoters used in plant genetic engineering approaches show constitutive expression; for examples, CaMV35S from cauliflower mosaic virus (Ghosh et al., 2016), Act1 from rice (Ganguly et al., 2014), and Ubi1 from rice (Bhattacharyya et al., 2012). Constitutive expression of a foreign gene may have deleterious effect on the host plant, leading to abnormal morphology (Sinha et al., 1993), altered grain weight and composition (Kurek et al., 2002), affects root growth and auxin response (Zhang et al., 2008), conferring hypersensitivity to abiotic stresses (Quilis et al., 2008). Tissue-specific expression would more likely to save the host plant from huge yield penalty as a consequence of mass diversion of the cellular machinery towards expressing the target protein throughout the plant (Anami et al., 2013). Therefore restricting the gene expression in specific target tissues is considered one of the strategies to avoid those problems related to constitutive expression (Molla et al., 2016). Green tissue specific promoter- rice PD54O-544 (Molla et al., 2013), maize PEPC (Hagh et al., 2009), phloem specific promoter- brassica GS3A (Koramutla et al., 2016), root specific promoter Rcc3 (Xu et al., 1995). Several pollen or anther-specific promoters were isolated and characterized from various plant species, *viz.,* TA29 tapetum specific promoter from tobacco (Koltunow et al., 1990), TP10 from tobacco (Rogers et al., 1992), PS1 from rice (Zou et al., 1994), Zm13 from maize (Hamilton et al., 2000), and TaPSG719 from wheat (Chen et al., 2010). Characterization of pollen or anther specific promoters from rice bears immense significance for the study of

pollen development in general or for deployment in genetic manipulation strategies to achieve higher grain yield through heterosis. The early stage pollen specific promoter RTS was used to drive the barnase gene expression to successfully generate male sterile transgenic rice (Luo et al., 2006). This strategy can be further extended to expressing other cytotoxic gene(s) in a anther- specific manner to produce male sterile lines for hybrid seed production.

The RTS gene was identified in *indica* rice cv. IR54 from a differentially screened cDNA library (rice panicles vs leaf) (Luo et al., 2006). The 1274 bp 5' upstream regulatory region of RTS gene possessed a number of putative cis-acting elements that drive specific expression of target gene in anther tissue of rice (Luo et al., 2006). In an attempt to re-isolate RTS promoter from an agronomically important *indica* cv. IR64, we discovered significant changes at nucleotide level from the reported sequence along with a 44 bp extra stretch in RTS promoter from IR64. Bioinformatic analyses revealed the difference between cis-acting regulatory elements in promoters from IR64 and IR54 genotypes. As a case study, the newly isolated promoter was subjected to comparative spatio-temporal expression profiling with reported RTS (Luo et al., 2006) promoter. The newly isolated variant was found to possess relatively high expression level in anther of rice than the previously reported RTS promoter as assessed through histochemical analysis and reporter assay. The present study stands significant regarding the comprehensive characterization of a new variant of anther-specific RTS promoter element from rice and this new promoter can be used in future genetic engineering approaches with a particular focus on production of hybrid crops.

MATERIALS AND METHODS

Plant materials

Oryza sativa cv. IR64 and cv. IR54 were used as plant materials for promoter isolation and plant transformation, as the case may be.

Bacterial strains and plasmids

The super virulent *Agrobacterium tumefaciens* strain EHA105 (Cheng et al., 1998) and binary vector pCAMBIA1391Z (Cambia, Canberra; GenBank: AF234312.1) were used for rice transformation experiments. *Escherichia coli* strain DH10B (BRL), and TA cloning vector (Invitrogen) were used for cloning of the isolated 5' regulatory sequences according to the manufacturer's instructions and a standard protocol (Sambrook et al., 1989).

Isolation of 5' upstream DNA sequence of RTS gene from rice genotypes IR64/ IR54 and bioinformatic prediction based deletion of promoters

Genomic DNA of IR64 and IR54 rice genotypes were used as templates for PCR amplification of 5' upstream sequence of RTS gene using sequence specific oligos (Luo et al. 2006; GenbankAcc # U12171; Table 1). The amplified PCR products were cloned separately into TA cloning vector (Invitrogen) and sequenced. The 5' upstream sequences of RTS gene from IR64 (henceforth, referred to as RTS64) and IR54 (henceforth, referred to as RTS54) rice genotypes were subjected to analysis using promoter module searching programs, *viz.,* Athena (O'Connor et al., 2005; http://www.bioinformatics2.wsu.edu/Athena); promoter scan (Prestridge 1995; http://bimas.dcrt.nih.gov/molbio/proscan/). For prediction of cis-acting regulatory elements, the following programs were used: PLACE (Higo et al., 1999; http://www.dna.affre.go.jp/PLACE);

Table 1. List of oligo nucleotides used in the study

Primers name		5 to 3 Nucleotide sequence	Tm	Purpose
1	RTS FP	GTCAAAGCTTGAGCTCACCGGCGAGGCGGTGCGTCT	65°C	Forward primer for PCR amplification of RTS promoter
2	RTS RP	AGCTGTCGACGAGCACCGCCGCGGCGGCAGCAAC	67°C	Reverse primer for PCR amplification of RTS promoter
3	GS FP	AGCTGATAGCGCGTGACAAA	57°C	Forward primer for PCR amplification of gusA gene for probe
4	GS RP	CTAGCTTGTTTGCCTCCCTG	59°C	Reverse primer for PCR amplification of gusA gene for probe

PlantCARE (Lescot et al., 2002; http://www.bioinformatics.psls.ugent.be/webtools/PlantCARE/html/). The sequence of RTS64 was submitted to Genbank under the accession number KT361609.

Construction of plant transformation vectors in pCAMBIA1391Z

The full-length sequences from IR64 and full-length promoter from IR54 genotypes (HindIII/SalI fragment in each case) were fused in translational frame with gusA gene to generate binary vectors, pCAMBIA1391Z::RTS64/gus, and pCAMBIA1391Z::RTS54/gus, respectively and subjected to plant transformation.

Agrobacterium-mediated rice transformation and generation of T_1 progeny plants

Standard *Agrobacterium tumefaciens* mediated rice transformation protocol was followed as described previously in Bhattacharyya et al., (2012). The *Agrobacterium* strain EHA105 (harboring target plasmid) was grown over night in AB medium, supplemented with 10mg/L of rifampicin and 50mg/L of kanamycin along with acetosyringone (AS) (3', 5'-dimethoxy 4'-hydroxy-acetorphenone; Aldrich Chemical Co.). The pellet of young culture (0.8 OD) was subsequently washed with 10µM $MgS0_4$ and re-suspended in AAM medium at an optical density of 3-5XI09 cells/ml at 600nm. Twenty days old embryogenic calli were infected with *Agrobacterium* suspension culture and kept there for 45 min with mild shaking. Thereafter the calli masses were transferred to co-cultivation medium containing of AS (150µM) and thereafter incubated in dark for 48 h at 25°C. Regenerated plants were established in the greenhouse after root development and proper acclimatization and further grown to maturity. To obtain the T_1 progeny plants, representative T_0 transgenic rice lines were self-pollinated under containment condition (to avoid cross-pollination), and the harvested seeds from T_0 plants were germinated in half strength MS media with hygromycin B (50 mg/L). The goodness of fit of the observed segregation ratio of the transgene was tested against the Mendelian segregation ratio (3:1) using the formula: $\chi^2 = \sum$[(observed frequency- expected frequency)^2 /expected frequency].

Southern blot analysis

Genomic DNA was isolated from the fresh leaves of T_0 and T_1 plants following the protocol of Doyle and Doyle (1990). Southern hybridization was performed according to Sambrook et al. (1989). 10 µg genomic DNA was digested with restriction enzyme (BamHI) and α-[32P]-dCTP-labeled gusA gene as a probe. 700bp fragment of gusA gene was used as probe. The fragment was amplified through PCR from the gusA gene using specific oligos (Table 1).

Western blot analysis

Protein was extracted from anther of representative T_1 transgenic lines of different category using extraction buffer and the quantity was estimated by the standard method (Bradford, 1976). The rabbit polyclonal antibody Anti-β-Glucuronidase (N-Terminal) (Sigma-Aldrich, G5420) was used as primary antibody (1:1000 dilution), mouse monoclonal plant Actin antibody (Sigma, mabGPa) was used as loading control (1:500 dilution) and anti-isotype IgG-POD (Horseradish peroxidase) (1:2000 dilution) was used as the secondary antibody for the Western blot. Western blot analysis was carried out using the Western Chromogenic Kit (Roche Molecular Biochemical) according to the manufacturer's instructions.

Estimation of Gus activity

Histochemical and fluorometric analyses of GUS activity were conducted following the method of Jefferson et al. (1987). Fluorometric estimation was carried out with the help of spectrofluorometer (PerkinElmer LS45) according to manufacturer's protocol.

Oligonucleotides used in the study

Oligonucleotides used in the study were listed in Table 1 .

Statistical analysis

Unpaired Student's t-test was used for analysis of statistical significance. Significance was evaluated at level $P<0.05$ (*), as the case may be.

RESULTS AND DISCUSSION

In silico analyses of 5' upstream sequences of RTS gene from IR64 and IR54 rice genotypes

The 5' upstream region of RTS gene was isolated from

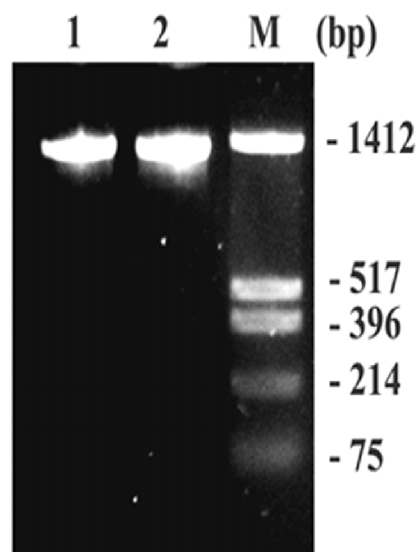

Fig. 1. Ethidium bromide stained agarose gel (1%) showing PCR amplification of 5' upstream regions of RTS gene from rice genotypes IR54 and IR64, respectively. lane 1: RTS54 promoter, lane 2: RTS64 promoter. Lane M: EcoRI-HindIII digested λ DNA marker; UC: Untransformed control.

two different rice genotypes (Fig. 1), *viz.,* IR64 and IR54, using sequence specific oligos (Table 1). Upon alignment, the sequence from IR64 (RTS64) was found to be ~1319 bp long as compared to earlier reported sequence from IR54 (1274 bp; RTS54). The RTS64 was found to contain an additional 44 bp stretch (from -1066 to -1023, assigning transcription start site to be +1) and 6 single nucleotide changes at positions -1185, -1177, -1170, -768, -658, and -184 (Fig. 2). Bioinformatic prediction based comparative analysis of the isolated fragments indicated the presence of TATA and CAAT-box elements and multiple cis-acting regulatory elements in both the sequences (Fig. 2; Table 2). In addition to common anther tissue specific cis-regulatory elements (POLLEN1LELAT52 element, GCN4 motif) present in both RTS64 and RTS54 (Fig. 2; Table 2), a few additional cis-elements were identified in RTS64 *viz.,* CACTFTPPCA1, S Box, CGACG element, A Box, TCCACCT motif and unnamed 6 motif (Fig. 2). Also, some of the essential minimal and cis-elements

Table 2. Distribution of cis-acting regulatory elements of RTS64 and RTS54 promoters

Promoter element		RTS64	RTS54	Function
Sp1 element	Element identified in extra 44bp sequence of RTS64	5	-	light responsive element
TCCACCT motif		+	-	unknown
Unnamed motif		+	-	unknown
S Box ATRBCS		+	-	ABI4 binding site, important for sugar and ABA responsiveness.
CGACG		+	-	function as a coupling element for the G box element
CGCG box		2	-	Ca++/calmodulin binding site
CACTFTPPCA1		+	-	A key compound of Mem1
HEXAMERATH4		+	-	unknown
A Box	Extra element identified in full sequence of RTS64 for single nucleotide changed	+	-	cis-acting regulatory element
CCGTCC box		+	-	cis-acting regulatory element related to meristem specific activation
Unnamed6		+	-	Unknown
MNF1	Deleted element from RTS64	-	+	Light responsive element
TATA box	Copy number of element increased due to single nucleotide change in RTS64	20	18	core promoter element around -30 of transcription start
Unnamed 4		21	17	Unknown
as box	Common cis-acting regulatory element	2	2	shoot-specific expression and light responsiveness
MSA like		+	+	cis-acting element involved in cell cycle regulation
LTR element		2	2	involved in low-temperature responsiveness
I Box		+	+	light responsive element
CAAT Box		16	16	common cis-acting element in promoter and enhancer element regions
POLLEN1LELAT52		8	8	responsible for pollen specific activation
AuxRR Core		+	+	involved in auxin responsiveness
Box II		+	+	light responsive element
MBS		2	2	MYB binding site involved in drought-inducibility
GCN4 motif		+	+	cis-regulatory element involved in endosperm expression

(+) indicate Present and (-) indicate absent

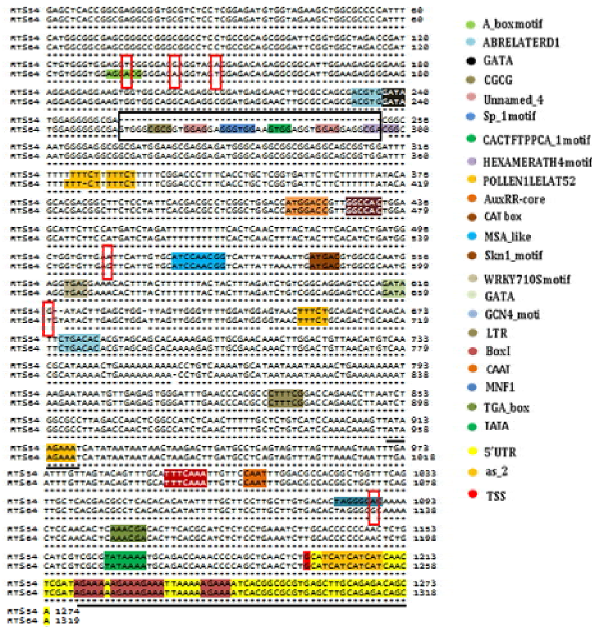

Fig. 2. Nucleotide sequence alignment of RTS64 and RTS54 promoters. The black box indicates the additional 44 bp stretch in RTS64. Red boxes indicate the single nucleotide changes between RTS64 and RTS54. Various cis-acting regulatory elements are indicated in different color schemes as listed in the figure. The alignment is performed by ClustalW version 2.1 alignment tool.

were found to be present in higher copy number in RTS64 than RTS54 *viz.,* TATA box (20 vs 18), unnamed 4 motifs (21 vs 17). RTS64 also contained five copy of sp1 element that remained absent in RTS54. However, one motif (MNF1) was absent from RTS64 sequence due to single nucleotide change (Table 2).

Generation and Seed transmission of gusA gene through T_1 generation and progeny analysis

To test the promoter activity of RTS64 and reported anther specific promoter RTS54, molecular constructs were generated by the transcriptional fusion of reporter gusA gene under regulatory control of individual fragments (Fig. 3a, b). The number of independent primary plant transformants generated following the optimized Agrobacterium-mediated rice transformation protocol. Four from both cases of RTS64, RTS54 were selected after stringent screening and were grown to maturity for subsequent molecular analysis.

Southern blot analysis confirmed the presence of the transferred gusA gene in the putative transformants in T_0 generation (Fig. 4a, b). Results revealed transgene integration at single as well as at multiple sites. However, plant lines with single insertion sites of the transgene were chosen from each category to have common premises for comparison. Progeny analysis from 40 T_1 seeds from each category indicated

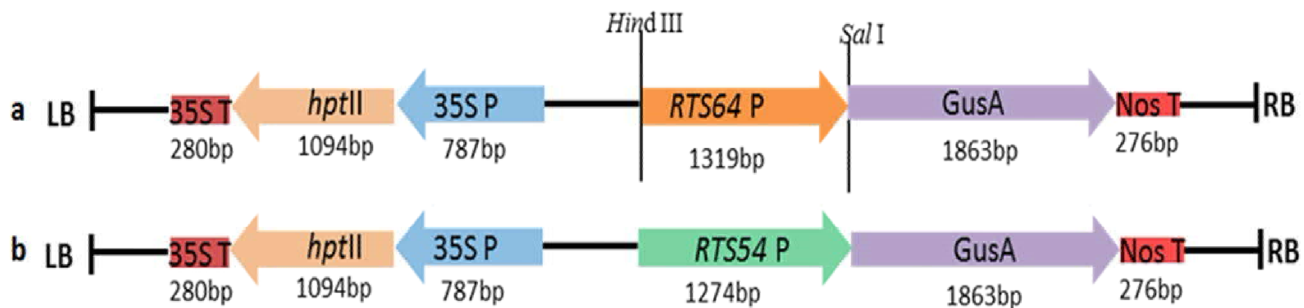

Fig. 3. Schematic representation of the T-DNA regions of the promoter::gusA expression cassettes used for transgenic rice plant generation. a pCAM1391Z::RTS64/gusA, b pCAM1391Z::RTS54/gusA

Table 3. Segregation analysis of representative transgenic plants of each category

Construct	Transgenic line	No. of seeds incubated	$hptII^r$	$hptII^s$	Segregation ratio (r:s)	Chi square (χ^2) value	p-value
RTS64	RTS64#1	40	28	12	3:1	0.13	0.7150
	RTS64#2	40	28	12	3:1	0.53	0.4652
	RTS64#3	40	31	9	3:1	0.13	0.7150
RTS54	RTS54#2	40	28	12	3:1	0.53	0.4652
	RTS54#3	40	32	8	3:1	0.53	0.4652

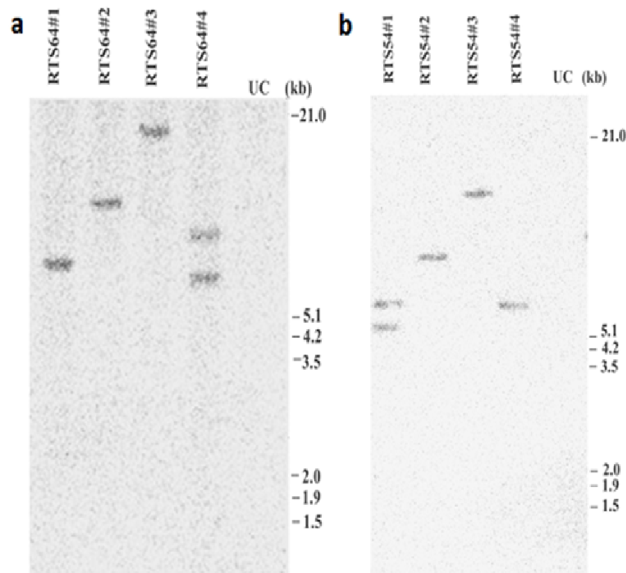

Fig. 4. Southern blot analysis of BamHI digested genomic DNA of independent T_0 plants from each category probed with radiolabeled gusA gene. (a) RTS64 and (b) RTS54. UC: Untransformed control. Approx. sizes of the EcoRI-HindIII digested λ DNA marker bands are marked

a 3:1 segregation ratio for gusA transgene (followed by scoring the linked hygromycin resistance gene) implying transgene integration at single chromosome (Table 3). During the progeny analysis, the observed values of gusA transgene integration did not show significant difference from expected values (P>0.05). This provided evidence for stable integration of the transgene as well as its transfer to next generation through seeds.

Expression and fluorometric estimation of gusA gene expression in T_1 transgenic lines

Representative T_1 transgenic rice lines from various category were subjected to spatio-temporal expression profiling of the gusA transgene (in anther, leaf and root tissue) (Fig. 5a,b). The Western blot analysis from anther of full-length RTS64 and RTS54 harboring plant lines detected a 66 kDa protein band as expected and no band was detected in untransformed control line (Fig. 5a). Western blot confirmed the relatively higher level of anther-specific expression for RTS64 than RTS54 promoters. Fluorometric Gus (β-glucuronidase) assay was performed to quantify the level of soluble GusA protein in anther, leaf and root tissues of T_1 transgenic rice lines harboring different promoter:: reporter cassettes. The transgenic lines with RTS64 promoter (RTS64#1a) showed the formation of 233.28 pmol 4 MU/min/μg in anther, whereas for RTS54 promoter (RTS54#3a) the formation level was 205.21 pmol 4 MU/min/μg. The results indicated a relatively higher level of anther-specific expression for RTS64 promoter (Fig. 5b). However, the expressions in other tissue like leaf and root were found to be minimal to negligible for RTS promoters, indicating strong anther specific regulation of RTS64 promoter element. GUS activity in untransformed lines was below the detection level.

Histochemical gus activity assay from T_1 transgenic lines

The gusA gene expression was finally monitored in anther, leaf and root tissues of T_1 transgenic and control

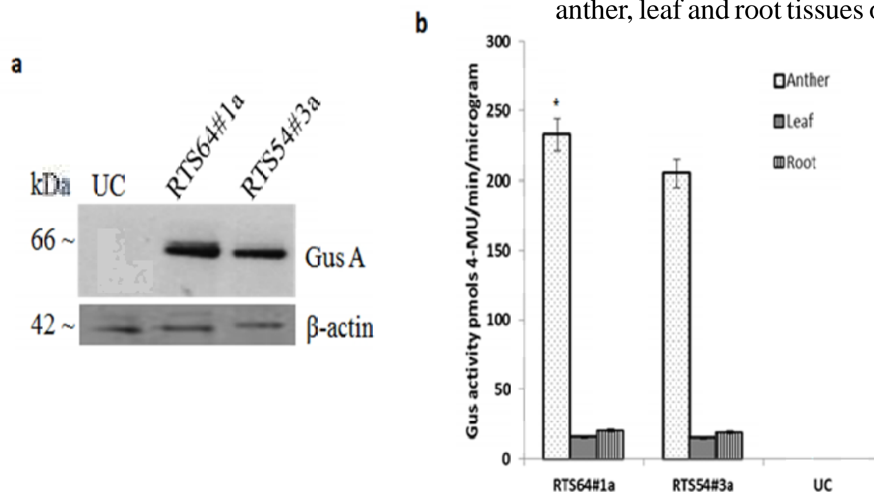

Fig. 5. (a) Western immunoblot analysis of GUS protein in anthers of transgenic rice lines containing RTS64 and RTS54. (b) Fluorometric GUS activity assay for two promoters. *P-value <0.05; UC: Untransformed control line

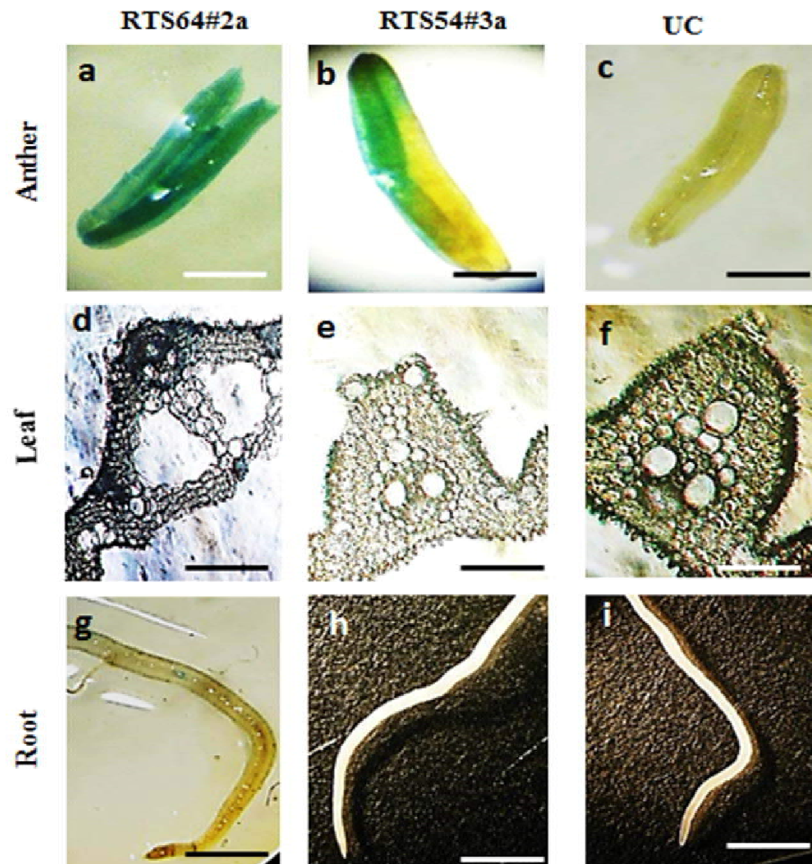

Fig. 6. Histochemical localization of GUS activity in representative T_1 transgenic rice plants in different tissue parts. RTS64 promoter: (a) anther, (d) leaf cross section, (g) root. RTS54 promoter: (b) anther, (e) leaf cross section, (h) root. Untransformed control: (c) anther, (f) leaf cross section, (i) root. Bars = 3.6 mm in the pictures of roots and 50 μm in the pictures of leaf cross section and 2 mm in the picture of anther

rice plants by the histochemical assay. Gus expression was clearly evident in anther of transgenic rice plants of different category with relatively higher expression in transgenic line carrying the full length RTS64 promoter then RTS54 promoter (Fig. 6a, b). However, negligible gus expression was detected in leaf and root tissues of plant lines with RTS64 and RTS54 promoter elements (RTS64#1a, and RTS54#3a) (Fig. 6d, e, g, h). This was internally consistent with the earlier molecular level analyses (Fig. 5a, b) and provided evidence for the higher level of anther-specific expression for RTS64 as compared to RTS54 promoter.

In an earlier study, a 1274 bp fragment at the 5' upstream of the RTS gene was shown to drive anther tissue specific expression in rice (Luo et al., 2006). Though the study by Luo et al., (2006) identified a few putative cis-acting regulatory elements, however, till date there is no report of a comprehensive anther-specific characterization of the identified promoter element in terms of any reporter gene based assay. Also, there is a lack of quantitative comparative profiling of the RTS promoter to validate further its specific utility in driving anther-specific gene expression. Also, detailed bioinformatics based characterization of putative cis-acting regulatory elements was not carried out in earlier studies.

Usually, the type, number, and position of cis-acting elements within a promoter region can determine the expression profile of the gene regulated by that promoter (Cai et al., 2007). The new promoter contained TATA-box and CAAT-box that are essential for promoter function. Also, multiple cis-acting elements were identified in RTS64. The RTS64 promoter consisted of five copies of POLLENILELAT52 at plus

strand and three copies at minus strand. These elements are reported to drive pollen specific gene expression (Bate and Twell, 1998). One GCN4 motif, responsible for endosperm-specific expression, was also found in RTS64 and RTS54. The additional 44 bp sequence in RTS64 contained a number of cis-acting regulatory elements, *viz.,* sp1 (light response) and TCCACCT motifs, CACT element (a key compound of Mem1), CGACG element (coupling element of G-box), CGCG element (Ca++/calmodulin binding site), S-box (ABI4 binding site, important for sugar and ABA responsiveness) and an unnamed motif without any reported function. All these elements were absent in RTS54. This in-silico analyses thus, provided the groundwork for further anther-specific characterization of the promoter elements. Among this two full-length promoters under study, RTS64 promoter was found to direct the high level of anther-specific gus expression, while RTS54 promoter showed relatively less anther-specific expression as detected by Gus histochemical assay as well as fluorometric assay. Thus, the high-level anther-specific expression of the RTS64 promoters might be attributed to the presence of 44bp extra stretch and SNPs. The even higher level of anther-specific expression for RTS64 promoter may originate from additional essential promoter elements and cis-elements in RTS64 as compared to RTS54, *viz.,* TATA box (20 vs 18), unnamed 4 motifs (21 vs 17), Sp1 element (5 vs 0). However, the detailed insight into the individual effect of these elements in promoter expression warranted a closer investigation and remained beyond the scope of the present study.

CONCLUSION

However, the present study expands the inventory of anther-specific promoters. Furthermore, the study remains the first report of comprehensive anther-specific expression profiling of RTS promoter from rice in comparison to reported RTS54 promoter. This novel anther-specific RTS64 promoter would be an ideal choice for expressing male sterility inducing gene(s) to produce hybrid seed in a variety of crop species, including rice.

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