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GENETICS AND GENOMICS

Regulation of expression of rice thaumatin-like protein: inducibility by elicitor requires promoter W-box elements

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Abstract Rice thaumatin-like protein (Rtlp1) is a high-molecular-weight antimicrobial pathogenesis-related protein that plays a role in plant stress response. This study examines transcriptional regulation of *Rtlp1* using wild type and transgenic rice plants carrying a β-glucuronidase (*GUS*) reporter gene driven by the Rtlp1 promoter (pRtlp1GUS). The *Rtlp1* promoter is induced within 6 h after infection with rice blast fungus (*Magnaporthe grisea*). The *Rtlp1* promoter is also induced by salicylic acid (SA), methyl jasmonate (MeJA), wounding or an elicitor from rice blast fungus. The function of the pRtlp1GUS reporter gene was analyzed by deletion mapping and transient expression assays in cell culture. A 120 bp truncated fusion construct with six W-boxes (5′-TGAC-3′) demonstrated a strong dose-dependent elicitor-response. These results suggest that W-box elements are required for the response of the *Rtlp1* promoter to fungal elicitors.

Keywords Rice · Thaumatin-like protein · Promoter · Rice blast fungus · W-box · Elicitor

Introduction

Thaumatin-like proteins (TLP) are high-molecular-weight antimicrobial proteins closely related to osmotin that are classified as class 5 pathogenesis-related proteins (PR-5). TLP may exert its antifungal activity by permeabilizing the fungal cell membrane (Vigers et al. 1991, 1992; Malehorn et al. 1994; Abad et al. 1996). Yun et al. (1998) suggested that expression of tobacco osmotin in yeast kills microbes rapidly by subverting signal transduction during infection. Datta et al. (1999) reported that transgenic rice over-expressing *Tlp* exhibited enhanced resistance to *Rhizoctonia solani*, which causes rice sheath blight disease. Rice plants overexpressing *Tlp* also have enhanced resistance to rice blast (*Magnaporthe grisea*) (data not shown).

The signal transduction pathways involved in plant pathogen defense are complex. Pathogenesis-related (PR) proteins are key elements in pathogen defense, and it is important to understand mechanisms that regulate PR gene expression. PR gene expression has been studied in monocots including maize, rice and wheat; for example, Northern blot analysis has been carried out on maize proteinase inhibitor (Cordero et al. 1994), rice chitinase (Xu et al. 1996; Nishizawa et al. 1999), wheat lipoxygenase (Mauch et al. 1997), and rice PR1 (Agrawal et al. 2000). However, the function of promoters of rice PR-genes have not been characterized in detail. This study presents characterization of rice *Tlp1* expression in wild type and transgenic rice expressing an *Rtlp1* promoter—β-glucuronidase (*GUS*) fusion reporter gene. Deletion analysis was used to identify functionally important regions in the *Rtlp1* promoter and promoter activity was studied in transgenic plants challenged with rice blast fungus.

Materials and methods

Plant materials

Genomic DNA and total RNA were isolated from roots and leaves of 14 day-old rice seedlings (*Oryza sativa* L. cv.

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Sasanishiki) grown in a greenhouse. Cells from scutellum of mature rice seed were cultured in liquid AA medium (Toriyama and Hinata 1985) containing 3% sucrose and 1 mg/l 2,4-dichlorophenoxy acid (2,4-D) at 25°C. These cells were used to generate transgenic plants.

cDNA library construction and isolation of *Rtlp1* cDNA

Total RNA was prepared 4 days after subculture from cultured cells grown in suspension according to the method of Sambrook et al. (1989) with slight modifications. Poly(A) + RNA was purified using an oligo(dT)-binding latex particle, and cDNA was synthesized with either cDNA Synthesis System Plus (Amersham) or ZAP-cDNA Synthesis (Stratagene). cDNA was cloned by ligating double-stranded cDNA to linearized pBluescript SK- (Stratagene) or by in vivo excision from a cDNA library in ZAP II phage vector. Randomly selected cDNA clones were isolated using the alkali lysis method, and partial nucleotide sequences of cDNA inserts were determined using a Model 370A DNA Autosequencer (Applied Biosystems). The GenBank DNA database was searched for homologous DNA sequences using Blastn. A total of 2,000 clones were sequenced. One of them contained the full-length cDNA of Rtlp1 (GenBank no. X68197)

Isolation of total RNA and northern blot analysis

Total RNA was isolated from root, leaf and callus tissue using guanidine-thiocianate/phenol extraction (Sambrook et al. 1989). For northern blot analysis, 20 μg total RNA was separated by electrophoresis in a formaldehyde/1.5% agarose gel, and transferred to a nylon membrane (Hybond N+, Amersham). Filters were hybridized with *Rtlp1* cDNA 32P-labeled using the Random Primer DNA Labeling System (Amersham). Filters were visualized and hybridization signal quantified using a BAS-2000 phosphor image analyzer (Fuji).

Isolation of *Rtlp1* promoter using modified inverse PCR

Genomic DNA was isolated from rice leaves by urea-phenol extraction (Shure et al. 1983). Inverse PCR (Ochman et al. 1988) was used with modifications to isolate the 5′-flanking region of the *Rtlp1* gene. One μg genomic DNA was double-digested with restriction enzymes, protruding ends were converted to blunt-ends and the DNA fragments were circularized by self-ligation for 16 h at 16°C in 400 μL containing 66 mM Tris–HCl (pH7.6), 6.6 mM MgCl2, 10 mM dithiothreitol, 0.1 mM ATP and 10 U T4 DNA ligase. Samples were extracted with phenol/chloroform and precipitated with ethanol, resuspended in buffer and used as a PCR template. The 5′-flanking region of *Rtlp1* was amplified using primer pairs oriented in opposite directions in the cDNA sequence. PCR was carried out in a 100 μl reaction volume containing 20 μM primers (5′-AGTAAATTGTTAATGGCGTCTCCGGCCACC-3′, 5′-TGAACGAGCACCGGTTGGTGATGG TGAAGG-3′), 250 μM dNTPs, 10 mM Tris–HCl (pH 9.0), 50 mM KCl, 0.1% Triton X-100, 1.5 mM $MgCl_2$ and 2.5 U Ex Taq polymerase (Takara). Thermal cycling conditions were 94°C for 2 min, followed by 30 cycles of 94°C/60 s, 62°C/90 s, 72°C/120 s, and final extension at 72°C for 7 min. A 1.3 kb PCR fragment was amplified from genomic DNA, double-digested with *Pvu*II and *Dra*I and self-ligated. This fragment contained the *Rtlp1* 5′-flanking region, and was cloned into the pT7Blue vector (Invitrogen). Nucleotide sequence of the DNA insert was determined using a Model 377A DNA Autosequencer (Applied Biosystems).

Construction of a binary vector and transformation of rice plants

The 5′-flanking region of *Rtlp1* was cloned immediately upstream of the GUS reporter gene in vector pBI221 (Clontech) replacing the CaMV 35 S promoter. This fusion gene reporter plasmid is called pRtlp1GUS. pRtlp1GUS was digested with *Hin*dIII, and inserted into the *Hin*dIII-site of a binary vector EBisKH2 (Kanzaki et al. 2002) (pEKH/Rtlp1GUS). The binary plasmid pEKH/Rtlp1GUS contains the GUS gene driven by the rice *Rtlp1* promoter and the *hygromycin phosphotransferase* (Hpt) gene driven by the CaMV 35S promoter. pEKH/Rtlp1GUS was transferred into *Agrobacterium tumefaciens* strain EHA105 by electroporation. Rice plants were transformed and regenerated (*O. sativa* L. cv. Sasanishiki) according to Hiei et al. (1994) with slight modifications. Fifty microgram per milliliter *hygromycin* (Hyg) was used to select transformants . After acclimatization, transformants were grown in soil in a greenhouse.

Characterization of *Rtlp1* promoter using GUS assay

Seedlings of transgenic rice harboring pEKH/Rtlp1GUS (T0 generation) were transferred into an inoculation chamber at the four- to five-leaf stages (30 days after sowing), and each pot was inoculated by spraying 250 μl of a conidial suspension of the rice blast fungus *M. grisea* (race 007.0) adjusted to 5×10^5 spores/ml (containing 0.05% Tween-20). Inoculated plants were kept in a closed chamber (25°C, 100% relative humidity) for 20 h and transferred to a moist incubator at 25°C until the lesion developed. Leaves were frozen in liquid nitrogen and used for GUS assay. Three replicates were taken for each treatment.

Seedlings of transgenic rice (T0 generation) were transferred into an inoculation chamber at the four- to five-leaf stage (30 days after sowing), and plants were treated with either 5 mM salicylic acid (SA), sprayed with 100 μM methyl jasmonate (MeJA) (containing 0.05% Tween-20) or wounded mechanically with forceps. After each treatment, plants were kept in a closed chamber (25°C, 100% relative humidity). Leaves were collected 1, 2, 6 or 24 h after treatment, frozen immediately in liquid nitrogen and stored at −80°C. Rice cultured cells harboring pEKH/Rtlp1GUS were maintained as *hygromycin*-resistant calli and assayed for GUS assay. Each treatment was repeated 3 times. Elicitor was prepared from rice blast hyphae according to the method of Koga et al. (1998). Water soluble cell wall extract was isolated from 70 g fungal hyphae, and dissolved in 210 ml 20 mM potassium phosphate buffer (pH 6.5). Cell wall extract (330 μl) was applied to 20 ml cultured cells. Potassium phosphate buffer (20 mM, pH 6.5) was used as the negative control.

Construction of promoter fragment/TATAintGUS vectors and GUS transient assay

A DNA fragment containing the TATA-box and the GUS reporter gene was PCR-amplified using pIG121Hm (Tanaka et al. 1990) as template and ligated into pBI221 (Clontech) digested with *Hin*dIII and *Sac*I (pTATAintGUS). Different regions of the *Rtlp1* promoter were amplified from genomic DNA. PCR primers were used to create fragment termini to match *Hin*dIII or *Sph*I cleaved DNA ends, and promoter fragments were cloned into pT7Blue vector (Invitrogen). Fragments from the Rtlp1 promoter were digested with *Hin*dIII and *Sph*I, and inserted immediately upstream of the TATA-box of pTATAintGUS. The constructs were called pRtlp1M5intGUS, pRtlp1M4intGUS, pRtlp1M3intGUS, pRtlp1M2intGUS and pRtlp1M1intGUS. A cDNA fragment containing the parsley (*Petroselinum crispum*) gene encoding WRKY1 transcription factor was PCR amplified from pBT-35S-WRKY1 (a kind gift from Dr. I. Somssich, Rushton et al. 1996) and placed downstream of the maize *Ubi-1* promoter in pAHC17 (Christensen et al. 1992). The resulting plasmid pUbiPcWRKY1 was used to study transactivation of the *Rtlp1* promoter.

Transient GUS activity assay was carried out in embryogenic calli of japonica rice from scutella of mature seed embryo (*O. sativa* L. cv. Sasanishiki). The calli were transformed by particle bombardment using the Bio-Rad PDS 1000/He device as described in Zhang et al. (1996).

In young transgenic plants, GUS activity was detected in *hygromycin*-resistant calli using histochemical and fluorometric assays essentially as described by Jefferson et al. (1987). Protein concentration was determined by the method of Bradford (1976) with a Bio-Rad kit (Protein Assay kit; Bio-Rad).

Results

Pathogen-induced *Rtlp1* expression

A rice EST library was constructed from rice calli exposed to salt stress, and a cDNA clone, PSAL005, was isolated that has high sequence homology with Tlp genes in the PR-5 family. This gene was called *Rtlp1*. The coding region of *Rtlp1* is 723 bp in length and encodes a protein of 177 amino acids with a molecular mass of 18 kDa.

Expression of *Rtlp1* was examined in susceptible (Sasanishiki) and resistant (Toyonishiki) cultivars infected with rice blast fungus race 007.0 (Fig. 1). Northern blot analysis indicated that *Rtlp1* transcripts increased in susceptible cultivar Sasanishiki within 6 h after inoculation, and was expressed constitutively in the field-resistant cultivar Toyonishiki. A weak signal was detected in the buffer-treated sample (basal transcription from *Rtlp1*).

Isolation of the *Rtlp1* 5′-flanking region

The upstream flanking region of *Rtlp1* was isolated using modified inverse PCR (Ochman et al. 1988). Genomic DNA was digested with restriction enzymes that recognize AT-rich sequences and the termini were converted to blunt-ends for cloning. A 1.2 kb fragment of the 5′-flanking region of *Rtlp1* was isolated, sequenced and analyzed for motifs that are associated with transcriptional regulation (Fig. 2). A putative TATA-box motif is located 150 bp upstream of the start codon. Several other putative

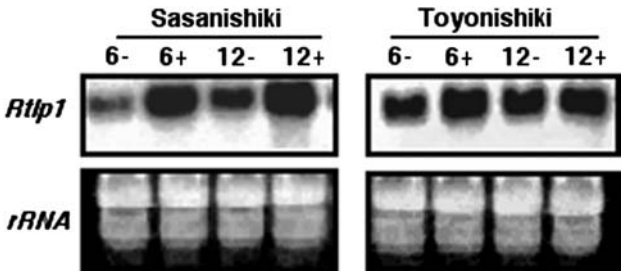

Fig. 1 Induction of *Rtlp1* by infection with rice blast fungus. At the four- to five-leaf stage, rice seedlings were transferred to an inoculation chamber and pots were inoculated by spraying with a suspension of conidia of the rice blast fungus (race 007.0). Control samples were sprayed with buffer. Total RNA was isolated from infected and uninfected leaves and used for northern blot analysis. Blots were probed with ^{32}P-labeled *Rtlp1* cDNA. *minus symbol* uninfected; *plus symbol* infected. Hours from treatment to harvest of RNA are shown. Sasanishiki is susceptible and Toyonishiki is field-resistant to the blast fungus race 007.0

regulatory motifs were identified including eight W-box motifs (elicitor responsive element, consensus sequence 5′-(T)TGAC(C)-3′; Rushton et al. 1996), a GCC-box motif (ethylene responsive element, consensus sequence 5′-(A)GCCGCC-3′; Ohme-Takagi and Shinshi 1995) and a MYB recognition element (SA-responsive element, MRE consensus sequence 5′-A(A/C)C(A/T)A(A/C)C-3′; Lois et al. 1989).

Rtlp1 promoter activity enhanced by rice blast infection

The *Rtlp1* promoter was fused with a GUS reporter gene (pRtlp1GUS) and a binary vector, pEKH/Rtlp1GUS, was constructed and transformed into rice embryogenic calli. Transformed rice plants were regenerated from *hygromycin*-resistant calli and GUS activity was detected by histochemical stain. Transgenic plants were challenged with rice blast infection and stained for GUS activity (Fig. 3). Kinetics of the response of the *Rtlp1* promoter and GUS expression are shown in Fig. 4. In uninfected plants, GUS activity was absent in leaves except for near sites of leaf wounding. In infected plants, GUS activity was strong near foci on rice blast-infected leaves 7 days after inoculation (Fig. 3). In addition, GUS expression was tenfold higher in cultured cells exposed to rice blast than in control uninfected cells. GUS activity was weak in the anther and the lemma in young panicle and in the lower part of the sheath (data not shown). A fluorometric GUS assay was used to quantify expression of pRtlp1GUS; the results indicate that expression from the *Rtlp1* promoter increases

Fig. 2 Nucleotide sequence of the *Rtlp1* 5′-flanking region. Nucleotide sequence is numbered with coordinate +1 at the translation start site. *Double underline* indicates putative TATA-box. *Boxes* indicate putative *cis*-acting consensus sequence elements: W-box (5′-TGAC-3′); GCC-box (5′-GCCGCC-3′); MRE (MYB recognition-element, 5′-A(A/C)C(A/T)A(A/C)C-3′). The coding region is *underlined*

Fig. 3 Histochemical analysis of GUS expression from a Rtlp1GUS reporter gene. **a** The leaves of transgenic rice harboring pEKH/Rtlp1GUS were inoculated with rice blast and incubated for 7 days. **b** Histochemical stain for GUS activity in infected leaves. **c** Histochemical stain for GUS activity in intact leaves of uninfected transgenic plants harboring pEKH/Rtlp1GUS

20-fold in leaves treated with rice blast conidia within 6 h after infection (Fig. 4).

Rtlp1 promoter activity in transgenic plants enhanced by jasmonic acid, wounding and elicitor

Transgenic plants harboring pEKH/Rtlp1GUS were sprayed with SA (5 mM) or MeJA (100 μM), which act as second messengers in plant defense signal transduction. GUS activity was three- to fourfold higher in SA- or MeJA-treated plants than in untreated control plants; GUS activity increased within 2 h after treatment (Fig. 5). As mentioned above, GUS activity also increased in plants wounded mechanically with forceps; wounding increased GUS activity fourfold within 1 h after treatment.

Transgenic rice cells grown in suspension culture harboring pEKH/Rtlp1GUS were also tested for responsiveness to elicitor. Even without elicitor-treatment the basal GUS

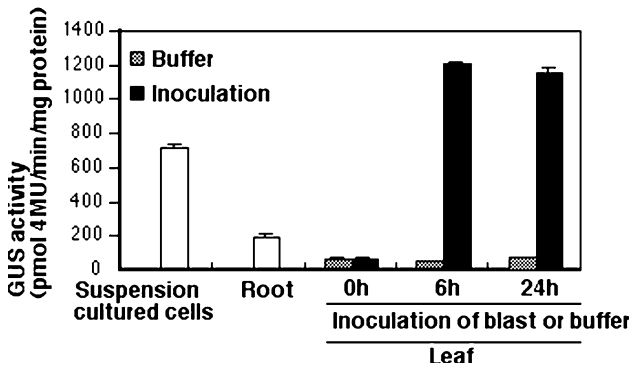

Fig. 4 Activation of *Rtlp1* promoter by blast fungus infection. Transgenic rice were treated as described in legend to Fig. 3. GUS activity was quantified fluorometrically in infected or uninfected leaves. The transgenic plants were inoculated with a suspension of conidia (containing 0.05% Tween20) of the rice blast fungus race 007.0. Plants were sprayed and incubated under conditions of high humidity at 25°C. Incubation times are indicated. Mean specific activity and standard error is shown

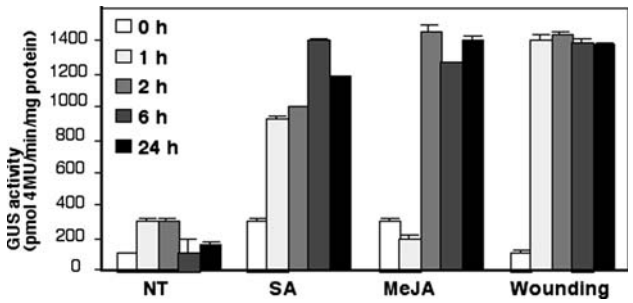

Fig. 5 Activation of *Rtlp1* promoter by elicitor or wounding. Transgenic rice plants harboring pEKH/Rtlp1GUS were treated with 5 mM SA or 100 μM MeJA by spraying, or mechanical wounding using forceps. Plants were incubated under conditions of high humidity at 25°C. GUS activity was quantified fluorometrically in treated or untreated leaves. Mean specific activity and standard error is shown

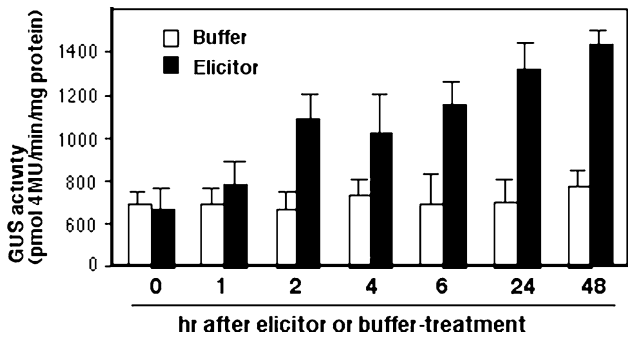

Fig. 6 Inducibility of *Rtlp1* promoter-GUS fusion gene in rice cultured cells. Rice cultured cells harboring pEKH/Rtlp1GUS were maintained as hyg-resistant calli, and then used for GUS assay after elicitor treatment. Elicitor from rice blast race 007.0 was prepared according to the method of Koga et al. (1998). Mean specific activity and standard error is shown

activity was high in cultured cells. Nevertheless, GUS activity was higher in elicitor-treated cells than in control cells; GUS expression increased twofold within 2 h after treatment (Fig. 6).

A 120-bp *Rtlp1* promoter fragment confers strong elicitor responsiveness

Elicitor-responsive regions in the *Rtlp1* promoter were identified by transiently expressing promoter deletion constructs in cultured rice cells. Four truncated forms of the *Rtlp1* promoter were prepared and inserted upstream of the TATA-box in pTATAintGUS. Five constructs (one full-length and four truncated) were transfected into rice cells and transfected cells were tested for transient GUS-expression in response to elicitor (Fig. 7a). A truncated construct including region M3 (262 bp) (pRtlp1M3) was insensitive to elicitor-treatment (no increase in GUS activity); this truncated promoter lacks W-boxes. Truncated promoters including regions M2 + M3 (647 bp; pRtlp1M4) or M2 (385 bp; pRtlp1M2) had two- to threefold stronger GUS activity in elicitor-treated cells than the TATA-only promoter; these truncated promoters have two W-boxes and one GCC-box. A promoter construct including region M1 (120 bp) (pRtlp1M1) had the highest induction level and had sixfold higher GUS activity in elicitor-treated cells than the TATA-only promoter; the 120 bp region M1 includes six W-boxes. This truncated promoter construct responded to elicitor in a dose-dependent manner (Fig. 7b). In this expression system, the construct including the full-length 767 bp *Rtlp1* promoter (eight W-boxes and one GCC-box; pRtlp1M0) supported a threefold increase in GUS expression after elicitor-treatment, which was a smaller induction than pRtlp1M1 (120 bp promoter construct).

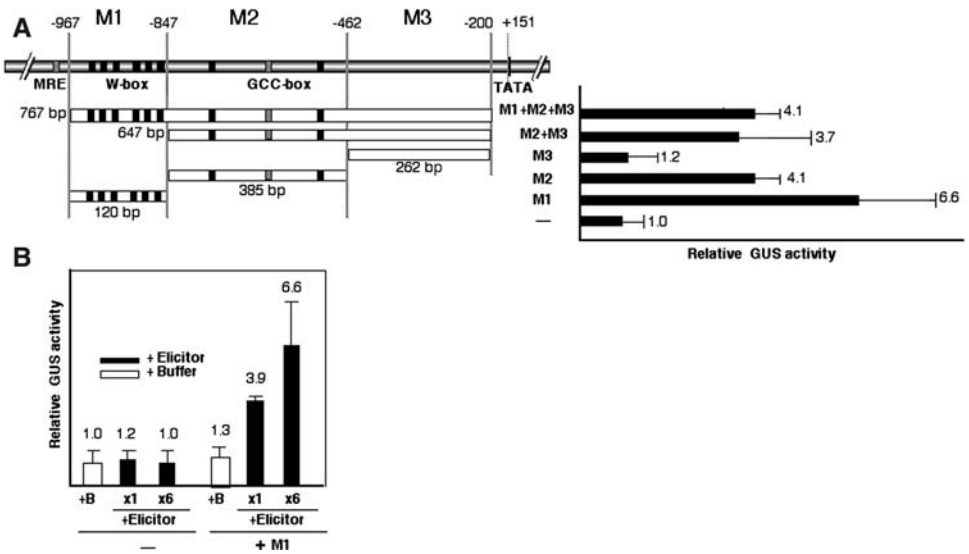

Fig. 7 Determination of elicitor-responsive regions in the *Rtlp1* promoter. **a** Schematic representation of the *Rtlp1* promoter region with W-boxes (*left*) and transient GUS activity of each region of *Rtlp1* promoter after particle bombardment delivery of promoter-GUS fusion into elicitor-treated cells. Nucleotide sequence numbers are coordinated from +1 at the translation start site. **b** Elicitor inducibility of M1 fragments (pRtlp1M1intGUS). *Bar* indicates elicitor inducibility of TATAintGUS. Mean specific activity and standard error is shown

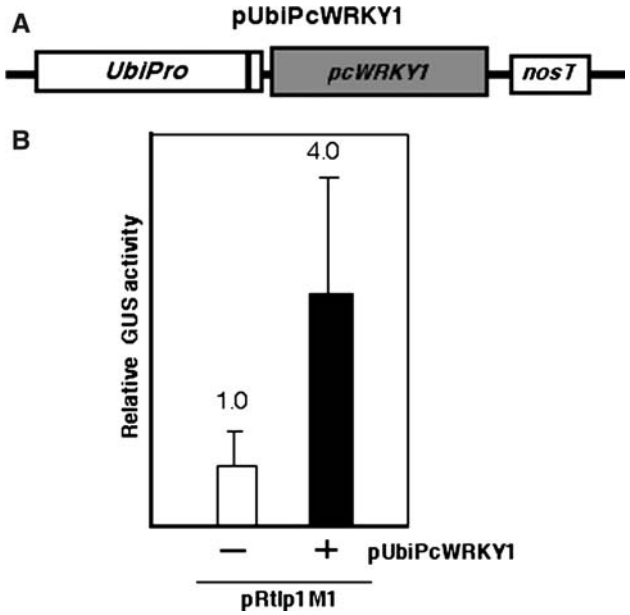

Fig. 8 Transactivation capacity of M1 fragments (pRtlp1M1intGUS) during co-transformation with parsley WRKY1 cDNA. WRKY1 expression was driven by the maize ubiquitin1 promoter. GUS expression was measured in transformed cultured cells. **a** Constructs used for co-transformation. *H Hin*dIII; *E Eco*RI; *S Sac*I., **b** GUS activity was measured in at least three independent experiments. Mean specific activity and standard error is shown

WRKY transcription factor confers elicitor-responsiveness to the 120 bp *Rtlp1* promoter fragment

WRKY is a transactivation factor that bind to W-box sequences and activates transcription of the gene downstream of the W-box. Thus, it is possible that WRKY stimulates transcription from the *Rtlp1* promoter. This idea was tested using WRKY1 cDNA from parsley and transient transactivation of pRtlp1GUS in rice cells. WRKY1 cDNA (Rushton et al. 1996) was fused with maize *Ubi-1* promoter (Fig. 8a), and the construct was co-transformed with pRtlp1M1intGUS into rice calli using particle bombardment. The transient assay indicated that GUS expression was fourfold higher in cells co-expressing parsley WRKY1 than in cells without WRKY1 (Fig. 8b).

Discussion

This study presents a functional analysis of the promoter of *Rtlp1* in wild type rice, transgenic rice and in cultured rice cells. The results indicate that the *Rtlp1* promoter is rapidly induced by several agents including rice blast fungus and that W-box motifs in the *Rtlp1* promoter are required for regulation of *Rtlp1* expression.

The *Rtlp1* promoter induces expression of *Rtlp1* or a Rtlp1GUS fusion gene within 6 h after infection with rice blast fungus (Figs. 1, 4). Previous studies report that rice blast infection causes hyphal invasion of rice epidermal cells 19 h after infection (Koga et al. 1998) indicating that *Rtlp1* is induced before hyphae invade and overgrow leaf cells. It is possible that plant wounding or an elicitor generates a signal that flows from the penetration hole of the rice blast appressorium to the host cells; these signals could be transmitted downstream to activate transcription of PR genes including *Rtlp1*.

Promoter regions have been isolated by several methods including inverse-PCR (Ochman et al. 1988), TAIL-PCR (Liu et al. 1995; Terauchi and Kahl 2000) or conventional screening of genomic DNA libraries. The 5′-flanking regions of plant genes are usually AT-rich. Therefore, the 5′-flanking region of *Rtlp1* was amplified by inverse-PCR using template genomic DNA digested with restriction enzymes that recognize AT-rich regions. This modified inverse-PCR method was highly efficient for recovery of the target promoter sequence, and this method may be generally useful for isolation of promoter regions of plant genes.

SA and JA may play key roles in signal transduction cascades that contribute to plant defense mechanisms. SA and JA may be antagonistic to each other (Niki et al. 1998; Pieterse and van Loon 1999). Schweitzer et al. (1997) indicated that PR-proteins accumulate in plants treated with JA, but pathogen attack does not enhance the level of endogenous JA. The role of SA in host defense is not clear, in part because the endogenous level of SA is very high in rice, especially in leaves (Silverman et al. 1995; Yang and Qi 2000). Results presented here indicate that the activity of the *Rtlp1* promoter increases 1 h after SA-treatment and 2 h after MeJA-treatment (Fig. 5). Transcription of *Pbz1*, a rice PR-protein gene, is also enhanced 2 h after SA-treatment and 4 h after MeJA-treatment (Lee et al. 2001). These results suggest that rice PR-proteins can be induced by SA or JA. Silverman et al. (1995) found a correlation between the level of leaf SA and generalized blast resistance in 28 varieties and suggested that SA plays a role as a constitutive defense compound. Rice *Pbz1* was also induced by blast fungus even in SA-deficient transgenic plants harboring the NahG gene (Lee et al. 2001). However, SA may not play a role in signaling during the defense response in rice. Alternatively, exogenous SA may enhance production of H_2O_2 despite the high level of endogenous SA in rice (Rao et al. 1997; Ganesan and Thomas 2001); thus, *Rtlp1* and *Pbz1* could be induced by H_2O_2 that accumulates in plants treated with exogenous SA. A recent microarray gene expression study examined 2,375 *Arabidopsis thaliana* genes and showed that 25% of MeJA-inducible genes are also induced by SA (Schenk et al. 2000). This observation is consistent with the hypothesis that defense signaling in plants may have a different transcriptional

network from wounding and JA or pathogen attack and SA. Brown et al. indicated that in addition to the roles in regulating ethylene-responsiveness, ethylene response factors (ERFs) also play important roles in Jasmonate-responsiveness, possibly by interaction with the GCC-box in in *A. thaliana* (2003). One GCC-box sequence founnd in M2 region of *Rtlp1* promoter may interact with transcriptional factor ERFs and lead to Jasmonate-responsiveness of *Rtlp1* promoter.

The *Rtlp1* promoter was also induced 1 h after leaf wounding and 2 h after elicitor-treatment of cultured cells (Figs. 5, 6). Basal *Rtlp1* promoter activity was high in cultured cells even in the absence of elicitor; this may indicate mechanical wounding of cells during suspension culture. However, *Rtlp1* promoter activity increased in cultured cells exposed to elicitor, suggesting that the fungal elicitor might induce *Rtlp1* in infected cells. Rushton et al. (1996) and Euglem et al. (1999) indicated that the W-box (TGAC core motif) is a key element in the mechanism of fungal-elicitor stimulated transcription in parsley. There are eight W-boxes in the 1.2 kb 5′-flanking region of *Rtlp1*. In addition, the W-boxes are required for inducible transcription from the *Rtlp1* promoter. Transient expression using a truncated promoter–reporter fusion construct showed that a 262 bp fragment (pRtlp1M3) without W-boxes does not respond to elicitor. Furthermore, the highest elicitor-response was achieved with a 120 bp fragment (pRtlp1M1) containing six W-boxes and the response was dose-dependent. These results strongly suggest that the W-box element is essential to the fungal elicitor responsiveness of the rice *Rtlp1* promoter.

Rushton et al. (1996) and Eulgem et al. (1999) showed that the WRKY zinc-finger transcription factor binds to the W-box element. WRKY mRNA rapidly accumulates near sites of fungal infection and WRKY activates expression of downstream genes by binding to promoters containing W-box elements. Results presented here indicate that the 120 bp fragment (pRtlp1M1) containing six W-boxes was activated by co-expression of parsley WRKY1 (Fig. 8). These data suggests that a WRKY may be one of trans-acting factors which activate the W-box elements in the promoter region of rice PR-protein genes including *Rtlp1*. In *A. thaliana*, genes encoding WRKY proteins form a superfamily with approximately 100 members (Eulgem et al. 2000). Recently, rice WRKY has begun to be studied in detail. In rice, 98 WRKY genes in *japonica* and 102 in *indica* rice were identified (Ross et al. 2007). An elicitor-inducible WRKY gene, OsWRKY1, was isolated using cDNA differential display in cells exposed to blast fungal elicitor (Kim et al. 2000). Gene expression of OsWRKY10 and OsWRKY82 increased by SA- and JA-treatments, similaly to Fig. 5 in this paper (Ryu et al. 2006). Over-expressing the elicitor-induced OsWRKY71 upregulated defense-regulated genes (Chujo et al. 2008). In addition, rice WRKY45 is suggested to play a role in BTH-induced and SA-mediated defense signaling based on RNAi and overexpression experiments (Shimono et al. 2007). Among these OsWRKYs, OsWRKY1 and OsWRKY82 belong to WRKY group I-a as well as parsley WRKY1. In addition, OsWRKY1 has the highest sequence homology to parsley WRKY1. Therefore, OsWRKY1 may be one of transcriptinal factor candidates, which activate transcription of *Rtlp1* in response to elicitor.

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