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Expression of active barley seed ribosome-inactivating protein in transgenic wheat

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Abstract Phenotypically normal, transgenic wheat (*Triticum aestivum*, var. Frisal) plants expressing a barley seed ribosome-inactivating protein (RIP) were produced. Expression was controlled by an intron-enhanced cauliflower mosaic virus 35S promoter and has been completely stable over four generations so far, possibly due to matrix-associated regions (MARs) that flank the transgenes. An engineered fusion to a signal peptide derived from the barley seed ß-1,3-glucanase caused the transport of RIP to the apoplast. Activity of the accumulated protein could be shown by significant inhibition of a rabbit reticulocyte transcription/translation system. Plants expressing high levels of RIP were protected only moderately or not at all against infection by the fungal pathogen *Erysiphe graminis*.

Key words Wheat transformation · Ribosome-inactivating protein · Anti-fungal protein · Protein-targeting

Introduction

Ribosome-inactivating proteins (RIPs) are specific N-glycosidases that remove a conserved adenine residue from the large rRNA of the large ribosomal subunit. This inactivates the ribosome and blocks translation elongation. RIPs have been found in a wide range of plant families and in different plant organs (reviewed in Barbieri et al. 1993; Girbes et al. 1996; Hartley et al. 1996; Mehta and Boston 1998). Plant RIPs are subdivided into three classes (Mehta and Boston 1998). Most RIPs belong to type 1, i.e. monomeric proteins of approximately 30 kDa. Examples are pokeweed antiviral protein (PAP) and barley seed RIP (RIP30). Type-2 RIPs (e.g. ricin) are larger proteins that consist of two distinct sub-domains: the A-chain essentially resembles the type-1 RIPs and is linked by a disulfide bridge to the B-chain, which has lectin activity. Type-3 RIPs (e.g. maize b-32 and barley JIP60) are formed by two separate polypeptide chains that are formed from a larger inactive precursor by proteolytic cleavage. Their final structure resembles type-1 RIPs. RIPs are thought to be defense proteins, but they may also have other functions. Type-2 RIPs are among the most potent cell toxins. The B-chain mediates retrograde transport through the secretory pathway into the cytoplasm of intact cells, where the A-chain inactivates ribosomes (Lord and Roberts 1996). In contrast, type-1 RIPs are not toxic to intact cells, although their enzymatic activity can be several-fold higher than that of type-2 RIPs.

Most RIPs are active on a wide variety of ribosomes, including conspecific ones. Self-protection of RIP-producing plant cells is achieved by compartmentalisation to the extracellular space or the vacuole, so that RIPs are not able to reach the ribosome target in their own cytoplasm (Svinth et al. 1998). Some type-1 RIPs have antiviral activity. It has been hypothesized that they re-enter the cell with the virus and cause cell death. It is possible that, besides this suicide effect, these RIPs also have a more specific antiviral activity that is not linked to N-glycosidase activity (Tumer et al. 1997, 1998). Other RIPs, including cereal endosperm RIPs, have anti-fungal activity. Tritin and barley seed RIP do not have any signal peptide and are probably only weakly active or inactive on ribosomes of the producing cells. Barley RIP has been shown to be especially active on isolated fungal ribosomes of *Neurospora crassa* (Roberts and Selitrennikoff 1986). In an *in vitro* assay, Leah et al. (1991) tested the anti-fungal activity of the barley seed RIP on *Trichoderma reesei* and *Fusarium sporotrichioides* spores. Alone, it was ineffective, but in combinations with either a β-1,3-glucanase or a chitinase, or both, it synergistically increased anti-fungal effects. Transgenic tobacco plants expressing the barley seed RIP under a wound-inducible promoter led to some protection against *Rhizoctonia so-*

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lani (Logemann et al. 1992). Other experiments with transgenic tobacco have shown anti-fungal activity of the barley seed RIP also under a constitutive promoter (Jach et al. 1995). This anti-fungal activity could be enhanced by the addition of an N-terminal signal peptide for protein export, and a synergistic effect with barley seed chitinase was also demonstrated for the transgene situation (Jach et al. 1995).

Here we report the transformation of barley seed RIP (RIP30) into wheat as part of a project aiming at increasing the fungus resistance of wheat through the expression of several anti-fungal proteins (Bliffeld et al. 1999). RIP30 is expressed under the control of a strong, constitutive promoter, and the protein is targeted through the endoplasmic reticulum (ER) to the apoplast. We were able to regenerate several plant lines of normal appearance and fertility expressing the transgene at various levels. RIP30 was effectively localised to the intercellular space, and the intercellular wash fluids (IWF) of expressing lines strongly inhibited a rabbit reticulocyte lysate transcription/translation system. Anti-fungal effects of RIP alone, as assayed by infection of detached leaves with *Erysiphe graminis*, were small and of an uncertain significance.

Material and Methods

Oc cell preparation and transfection

Protoplasts from *Oryza sativa* (line Oc) were isolated from cell suspension culture and transfected as described previously (Chen et al. 1994) with modifications: suspension cells were cultured in MS salts and vitamins [(Murashige and Skoog, 1962), provided by Duchefa, Harlem, The Netherlands] plus 2 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D), 6% sucrose and 2.5 m*M* 2-morpholinoethanesulfonic acid (MES). Digestion was performed in 3% cellulase Onozuka R-10, 1% macerozyme R-10 (both Yakult Pharmaceutical Ind. Co, Tokyo, Japan), 8% mannitol and 1 m*M* $CaCl_2$ for 16 h with slight shaking at 30 C. The protoplasts were transformed by the PEG method with 10 µg plasmid DNA, incubated for 16 h with or without 20 µg/ml tunicamycin, and analysed for activity of β-glucuronidase (GUS) with 4-methylumbelliferyl-β-D-glucuronide (MUG) (Jefferson 1987).

Plasmid construction

Construct "l-GUS" contains a CaMV 35S promoter, an RTBV leader/intron fusion and a CaMV 35S terminator (Fütterer et al. 1994). The RTBV sequence used is a *Bgl*II/*Nde*I fusion of a splice donor part (7407 to *Bgl*II/7870; numbering of RTBV sequences according to Hay et al. 1991) and a splice acceptor part (*Nde*I/4850 to *Hind*III/5984). The splice donor is located in sORF1 of the RTBV leader sequence. Restriction sites *Bam*HI and *Spe*I have been engineered around sORF1 (see below). The GUS coding sequence including its initiation codon was fused 3' of the splice acceptor site to be in frame with sORF1 on the spliced mRNA. The plasmid backbone was derived from pUC19. "sp-l-GUS" was generated by replacement of the *Bam*HI/*Spe*I fragment containing the sORF1 with a polymerase chain reaction (PCR)-amplified *Bam*HI/*Spe*I fragment containing the barley seed ß-1,3-glucanase signal peptide (Leah et al. 1991) with primers no. 5 (5' GGGATCCACAATGGCTAGAAAAGATGTTGCC 3') and no. 6 (5' GGGACTAGTCACTGACCTGTACGCCGATGGACTGC 3'). The nucleotide sequence of l-GUS around the splice donor and splice acceptor is as follows: splice donor (7483) GCAAGG-**G**GATC**C***ATG* GCT.CAG.gtcagtga**c**tagtcgt (7520); splice acceptor (5963) ccactatacag.GGA.CAA.GCT.TC**C** ***ATG*** 3' (exon sequence in upper-case letters, introduced mutations in bold, start codons in italics, restriction sites for *Bam*HI, *Spe*I and *Hind*III are underlined). The deduced amino-acid sequence of l-GUS and sp-l-GUS is shown in Fig. 1a. s-GUS and sp-s-GUS are *Spe*I/*Spe*I (7515/5581) deletions of l-GUS and sp-l-GUS, respectively, resulting in a shorter intron (about 400 bp instead of 1500 bp). For the generation of the wheat transformation construct sp-l-GUS

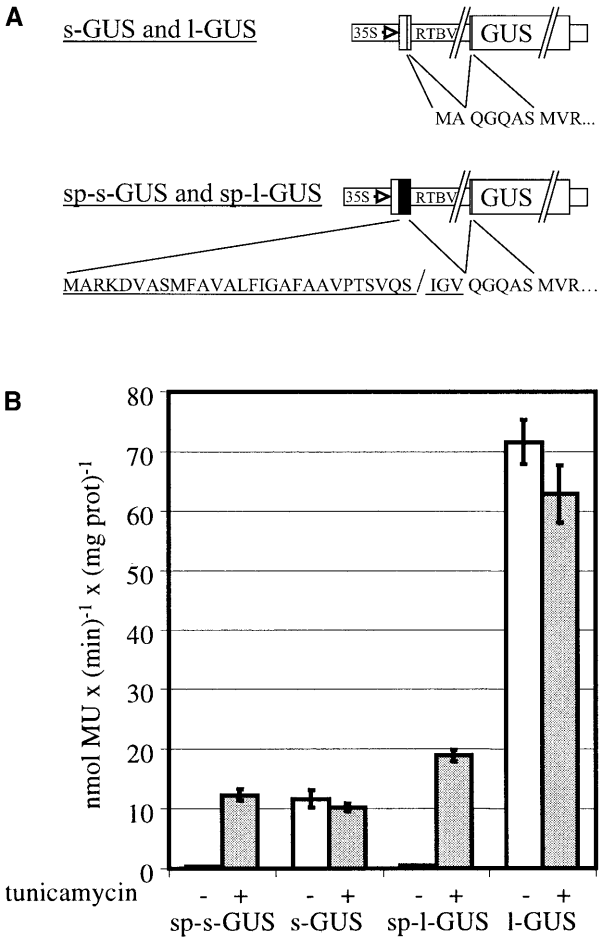

Fig. 1A, B ER-targeting of GUS. **A** Schematic presentation of the expression cassettes. All contain a CaMV 35S promoter (*narrow box with arrow*), the first exon of RTBV (*wide box*) with a short ORF spanning the splice donor site, varying lengths of an RTBV-derived intron (*narrow box labeled RTBV*), a β-glucuronidase (*GUS*) ORF in the second exon(*wide box*), in frame with the sORF on the resulting mRNA, and a CaMV transcription termination/polyadenylation signal (*narrow box at the end* of the expression unit). For ER-targeting, the RTBV sORF was replaced by the signal peptide (*sp*) of the barley seed ß-1,3-glucanase (*black box in sp-constructs*). The amino acids contributed to the GUS protein by the upstream sORFs are indicated *below* the respective constructs. The signal peptide is *underlined*, and the processing site is indicated by a *slash*. The RTBV intron was used in two variants, one of 400 bp and another of 1500 bp, resulting in two different vectors – with sp [sp-s-GUS and sp-l-GUS] and without [s-GUS and l-GUS]. **B** Transient transfection of rice protoplasts. The β-glucuronidase activity of protoplasts incubated with (+) or without (–) the *N*-glycosylation inhibitor tunicamycin (20 µg/ml) is shown. Results are the means of three independent experiments; *error bars* correspond to 1 SD

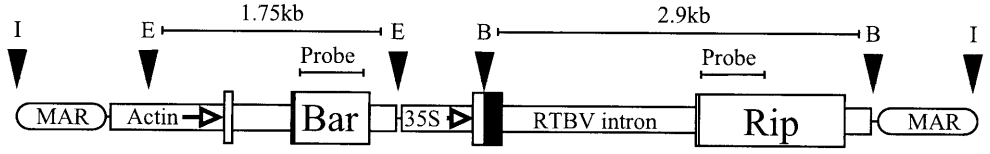

Fig. 2 Vector used for wheat transformation. The vector is derived from sp-l-GUS (Fig. 1a). The barley seed RIP30 coding sequence was introduced instead of GUS. For selection, a BAR gene driven by the rice actin 1 promoter and first intron was present on the same plasmid. Two different matrix attachment regions (*MAR*) flank the relevant sequences. Sites for I-*Sce*1 (*I*), *Eco*RI (*E*) and *Bam*HI (*B*) are indicated as well as the fragment sizes for *Bam*HI and *Eco*RI restriction and the location of the hybridization probes for Southern analysis (Fig. 4)

was introduced into a pUC19-derived plasmid containing two different matrix-associated regions [MARs: from tobacco (Breyne et al. 1992) and petunia (Dietz et al. 1994)] flanked by two I-*Sce*1 sites. The GUS coding sequence was replaced by the barley seed ribosome-inactivating protein (RIP30) coding sequence, and an actin promoter BAR cassette (pAB1, Bliffeld et al. 1999) was introduced upstream of the 35S promoter for selection against phosphinotricin (PPT) (Fig. 2).

Wheat transformation

Wheat transformation was performed as described previously (Bliffeld et al. 1999) with minor modifications. Wheat immature embryo scutellum derived callus (5–7 days after initiation) was used for particle-mediated direct gene transfer (Finer et al. 1992). Per shot, 0.5 mg gold particles were coated with 0.1 µg of excised DNA fragment (I-*Sce*1). Sixteen hours after bombardment, calli were transferred to solid SMS medium (MS salts and vitamins, 2 mg/l 2,4-D, 20 g/l maltose, 6 g/l agarose, 100 mg/l casein hydrolysate) containing 5 mg/l PPT. Developing calli were transferred to regeneration medium (MS salts and vitamins, 20 g/l maltose, 0.5 mg/l 1-Naphtylacetic acid (α), 2 mg/l N^6-benzyladenine, 6 g/l agarose, 5 mg/l PPT) after 4–6 weeks. Shoots (1.5–2 cm in length) were transferred to plastic containers containing half-strength MS salts and vitamins, 10 g/l maltose, 3 mg/l PPT and 2 g/l Gelrite. Plants longer than 5 cm, with green leaves and a developed root system were transferred to soil.

Southern blot

DNA was extracted with the Nucleon Phytopure Plant DNA extraction kit (Amersham Life Science, England). Southern blot analysis of phosphinotricin acetyltransferase (PAT)-assay positive lines was performed according to Boehringer Mannheim (The DIG System User's Guide for Filter Hybridization 1995). Between 15 and 20 µg of genomic DNA was cut with *Eco*RI or *Bam*HI and hybridized with a probe for the BAR or RIP30 coding region, respectively (see Fig. 2). As controls, digested genomic DNA of untransformed plants was loaded with and without 20 pg digested plasmid DNA. Primers to generate probes for BAR were: (1) 5' GTCTGCACCATCGTCAACC 3' and (2) 5' GTCCAGCTGCCAGAAAC 3'; and for RIP they were (1) 5' GCCACCTTCATCGGCCGGC 3' and (2) 5' GGGCGGTCACCGCGTCC 3'.

Western blot

Protein extracts were resolved by 12% SDS-Polyacrylamide gel electrophoresis (Mini-PROTEAN II Cell, Bio-Rad, Hercules, Calif.) and transferred to cellulose nitrate membrane (Optitran BA-S 83, Schleicher and Schuell, Dassel, Germany) according to Fütterer et al. (1995). Detection of the protein was performed by chemiluminescence using polyclonal rabbit antibodies (kindly provided by J. Mundy) and the ECL Western blotting analysis system (Amersham Pharmacia Biotech, UK). Intercellular wash fluid was extracted from leaf as described by Parent and Asselin (1984); the same buffer was used for total soluble leaf protein (frozen tissue was crushed in the presence of buffer and centrifuged in a table-top microfuge to remove cell debris) or total soluble barley seed protein (total seeds were ground and incubated in buffer for 30 min on ice, then cleared by centrifugation). Protein amounts loaded were approximately 40 µg for total soluble leaf extract and 6 µg for the IWF.

PAT assay

The enzyme assay for phosphinotricin acetyltransferase was performed as described (Fütterer et al. 1995). For lines producing several shoots, leaf material consisting of up to 5 shoots was collected and mixed for the assay. In cases of unusual segregation of the BAR gene in the progeny, as determined by chlorophenol-red assay, leaf material of individual shoots was tested, if available.

Chlorophenol-red assay

To test PPT tolerance of seed derived progeny, we grew plantlets aseptically and incubated shoot pieces (1 mm long) in modified chlorophenol-red (CR) assay medium (hormone-free SMS containing 0, 1, 10 and 100 mg/l PPT, 260 mg/l CR, pH 6.0) for 48 h at 25 °C under light (Bliffeld et al. 1999; Kramer et al. 1993).

Fungal infection with *E. graminis*

Infection of detached leaf pieces was performed as described previously (Bliffeld et al. 1999). The plant material used was cut from the end (omitting the tip) of the last or second-last leaf of plants at stage 9 or 10 (in "boot") in accordance to Feekes scale (Large 1954). The powdery mildew is a local field isolate that was maintained on wheat cv. Fidel.

Ribosome inactivating activity of IWF

The trancription/translation inhibition activity of IWF from transgenic and wild type plants was assessed in a rabbit reticulocyte lysate (TNT T7 Quick Coupled Transcription/Translation System, Promega Corp, Madison, Wis.). The luciferase activity generated by the control plasmid T7-luciferase was measured in intervals of 5 min, or after 35 min, at 30 °C, in reaction volumes of 25 µl containing 0.1 µg control DNA and 1 µl of IWF diluted in IWF buffer to reach a final IWF protein concentration of 0.3, 1, 3, 10 and 30 µg/ml reaction mix. IWF protein was added after 22 min of incubation.

Results

Development of an expression unit to target proteins to the endoplasmatic reticulum

Barley RIP30 is found naturally in the cytoplasm of endosperm cells. We designed a CaMV 35 S promoter with

a modified rice tungro bacilliform virus (RTBV) leader and intron capable of targeting proteins to the ER, and from there, by the default pathway, to the apoplastic space (Denecke et al. 1990). The targeting signal are the N-terminal 31 amino acids of the barley seed ß-1,3-glucanase, from which 28 are cleaved off upon translocation into the ER (Fig. 1a). The RTBV intron was chosen for two reasons. First, expression levels of the CaMV 35 S promoter are strongly enhanced by this intron, at least in rice (Klöti et al. 1999) and, second, the intron interrupts the cloned open reading frame (ORF). This inhibits the unwanted expression of possibly toxic proteins in *E. coli* from transcripts generated by the 35S promoter in bacteria (Assaad and Signer 1990). Four variants of this vector were compared using GUS as a reporter (Fig. 1a). These variants have a "short" or "long" RTBV-derived intron, and the first exons encode the signal peptide (constructs sp-s-GUS or sp-l-GUS, producing spGUS) or not (s-GUS or l-GUS, producing cytoplasmatic GUS). The vector with the long intron caused a sevenfold higher cytoplasmatic GUS expression than the one with the short intron (Fig. 1b). To prove the effective targeting of GUS to the ER, we used the fact, that bacterial ß-glucuronidase protein is inactivated in the ER by N-glycosylation and possibly also by miss-folding. N-glycosylation can be blocked by the specific inhibitor tunicamycin. For successful targeted spGUS only very little or no activity can be expected, but the activity should be restored in the presence of tunicamycin (Denecke et al. 1990). This is exactly the result obtained in transfected protoplasts: irrespective of the length of the intron, spGUS activity was drastically reduced (Fig. 1b). In the presence of tunicamycin, spGUS activity increased 36-fold, while expression from vectors for the cytoplasmatic GUS was reduced by about 10%, presumably due to the toxic effect of tunicamycin. It is noteworthy that for the gene with the short intron and signal peptide the expression was restored by tunicamycin to a level similar to that of the same gene without the signal peptide and without tunicamycin, whereas for the long intron the expression was restored only to about 25%. Possibly the capacity limit of the protein export or folding machinery was reached in this transient assay. These results show that protein expression and targeting to the ER were efficient with the designed vectors.

Wheat transformation

The Swiss spring wheat cultivar Frisal was chosen for transformation. Frisal shows reasonable embryogenesis from cultured scutellar tissue and an excellent regeneration frequency (M. Clausen and C. Sautter, unpublished results). The vector used for transformation was derived from sp-l-GUS, with GUS replaced by RIP30 (Fig. 2). To allow selection with PPT, we introduced upstream a *bar* gene driven by the rice actin 1 promoter/first intron that encodes PAT (Thompson et al. 1987). Two different matrix associated regions flank the cassette to alleviate any negative effects on the expression of the transgenes by the genome insertion site and/or to improve the transgene expression level and stability (Vain et al. 1999). Two I-*Sce*1 sites allow excision of the DNA fragment for transformation. From 5500 embryos isolated, 2500 showed sufficient embryogenesis and were bombarded (Table 1). After selection for growing and regenerating structures on PPT-containing medium, 97 rooting plantlets were transferred to soil. PPT selection at 5 mg/l appeared to be leaky with Frisal, and only 29 of these 97 plant lines expressed detectable PAT activity. The particular high number of escapes in this experiment may have resulted from the excellent regeneration frequency of the cultivar. Escapes in wheat transformation with PPT have also been reported by others; the number of escapes may depend on the cultivar and the concentration of PPT used (e.g. Becker et al. 1994; Nehra et al. 1994). In addition, in some cases PAT-expressing and non-expressing callus tissue seemed to be closely associated even under selection. Frisal cultures usually produce many shoots from a single regenerating callus. When the PAT activity of individual shoots derived from the same callus was analysed (Fig. 3), we observed in six cases that PAT expression was confined to some of them but lacking in others. This suggests that most likely the starting callus material must have been a chimera of expressing and non-expressing tissue. The overall transformation efficiency for the *bar* gene (PAT-expressing lines per bombarded embryos) was 1% and in the range of the ef-

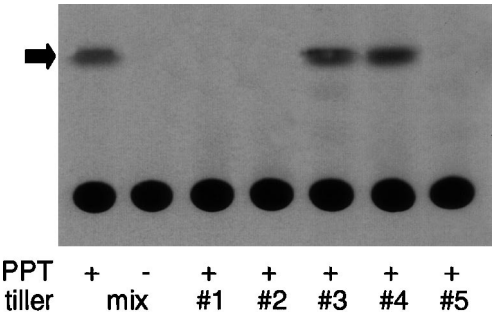

Fig. 3 PAT-assay from callus-derived shoots. Leaf material from five shoots regenerated from the same callus was used for the PAT assay either individually (nos. 1–5) or as mixture of all five (*mix*), with (+) or without (–) the substrate PPT. The reaction product Ac-PPT is indicated (*arrow*)

Table 1 Wheat transformation efficiency

Isolated embryos		5530[a]	193%
Bombarded embryos		2860	**100%**
Rooting plants		97	3.4%
PAT assay-positive		29	1.0%
Southern	BAR-positive	29	1.0%
	RIP[b]-positive	23	0.8%
Western	Expressing RIP[b]	11	0.4%

[a] All numbers indicate independent lines
[b] Barley seed RIP30

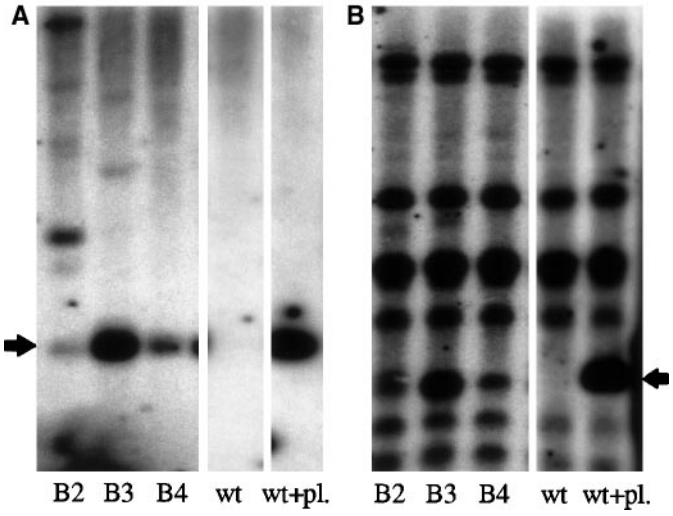

Fig. 4A, B Southern blot of transgenic wheat lines. Total DNA was extracted and (**A**) digested with *Eco*RI and probed for the BAR gene or (**B**) digested with *Bam*HI and probed for the RIP30 gene. Transgenic lines *B*2, *B*3 and *B*4 are shown, as well as the wild type (*wt*) and wild type with 20 pg digested plasmid DNA (wt+pl). *Arrows* indicate the positions of the respective plasmid-derived fragments. Lanes were cropped from one gel

ficiency reported by others (Becker et al. 1994; Blechl et al. 1998; Bliffeld et al. 1999; Lörz et al. 1998; Nehra et al. 1994; Vasil et al. 1993; Weeks et al. 1993; Witrzens et al. 1998). All regenerated plants had a normal appearance, when compared to non-transgenic regenerated plants (escapes), and were fully fertile. Seed-derived transgenic plants were phenotypically not distinguishable from the wild type. The segregation of *bar* was analysed with a modified chlorophenol-red assay of primary leaves of *in vitro*-grown seedlings. This assay monitors the survival of tissue under selective conditions, i.e. PAT expression, and revealed a 3:1 segregation in most cases. It was important to harvest the individual heads of regenerated plants with multiple shoot formation separately to avoid a distortion of the effective segregation ratio.

Molecular analysis

PAT assay-positive plants were further analysed by Southern and Western blotting. All plants positive in the PAT assay contained at least one copy of *bar* (Fig. 4, Table 2). Intact copies of the engineered RIP gene were present in 23 out of 29 lines. The RIP probe also hybridized to at least eight endogenous DNA fragments (Fig. 4). Some of these bands certainly represent the native wheat RIP genes, such as tritin (with 90% homology). An estimate of the copy number for *bar* and RIP30 of complete or rearranged genes is listed in Table 2. Western blotting was performed with total soluble protein and IWF of leaves. Eleven lines contained RIP30 above the detection limit, 5 at low levels and 6 at higher levels (Fig. 5a, Table 2). The RIP concentration in the IWF is 50-fold higher than in total soluble protein, indicating effective targeting to the apoplastic space (Fig. 5b). Total soluble barley seed protein was used as positive control. The electrophoretic mobility of transgene-derived RIP in wheat is the same as that of native barley seed RIP30,

Table 2 Transgenic lines expressing barley seed RIP protein

Transgenic plant line	Southern BAR probe[a]	Southern RIP probe[a]	Segregation BAR[b]	RIP30 expression (western)	Generations with stable expression[c]	Translation inhibition by IWF[d]	*E.graminis* growth reduction[e]
B2	1/6	1/4	3:1* (24)	+++	4/4	85%	
B3	>1/2	>1/1	3:1* (23)	+++	3/3	60–70%	
B4	1/1	1/0	3:1 (12)	++++	4/4	98%	0–20%
B12	1/2	1/1	≥3:1 (29)	++	3/3	30–40%	
B13	1/10	1/2	3:1* (11)	+	2/2	0–10%	
B15	0/5	0/–[f]	≥3:1 (8)	+	2/2	0%	
B18	>1/6	>1/6	≥15:1 (11)	++++	2/2	95%	
B21	>1/11	>1/4	≥15:1 (23)	+	3/3	0%	
B22	1/1	0/–[f]	≥3:1 (8)	+	2/2	0%	
B25	1/6	1/2	3:1 (23)	+++	4/4	90%	0–40%
B26	>1/9	1/4	≥3:1* (8)	+++	3/3	90%	

[a] Estimated number of complete/truncated copies of integrated transgene as observed on a Southern blot when the respective gene was excised. The number of intact copies could only be classified into one, or more than one (>1); the number of truncated RIP transgene copies may be higher than indicated, but was not clearly distinguishable due to many endogenous bands hybridising to the RIP probe

[b] The segregation analysis was performed using a modified chlorophenol-red assay. Segregation ratios are sorted in three classes: approximately 3:1 (3:1), 3:1 or 15:1 (≥3:1) and 15:1 or no segregation (≥15:1). An asterisk (*) indicates lines with observed chimeric calli (Fig. 3). In brackets, the number of progeny plants used to assess the segregation ratio

[c] Number of generations with stable transgenic RIP30 expression/tested generations

[d] Inhibitory activity of 3 µg/ml IWF protein in a rabbit reticulocyte lysate coupled transcription/translation assay. IWF extract from untransformed plants was defined as 0% inhibition

[e] The number indicates the reduction in the average number of growing *E. graminis* colonies compared to untransformed controls

[f] These lines do not have a complete copy of the barley RIP transgene, but due to the many endogenous bands hybridising to the RIP probe we can not exclude the presence of hidden truncated copies

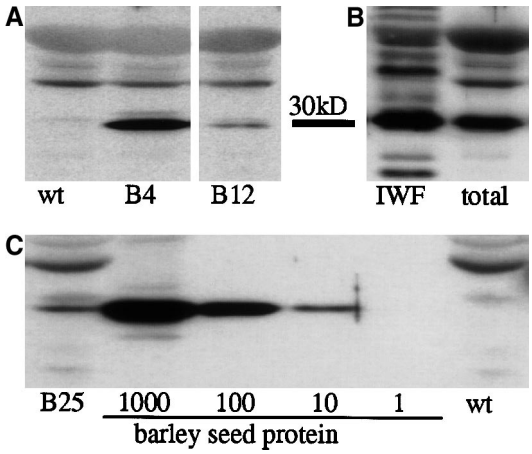

Fig. 5A–C Western blot with anti-RIP30 rabbit serum. **A** Total soluble leaf protein of wild type and transgenic lines expressing high (*B4*) and low (*B12*) levels of RIP30. **B** Leaf intercellular wash fluid protein (*IWF*, 6 µg) and total soluble leaf protein (40 µg) from the same transgenic wheat line (B25). **C** Total soluble leaf protein (40 µg) of line B25, the wild type (wt) and different amounts (ng) of total soluble barley seed protein

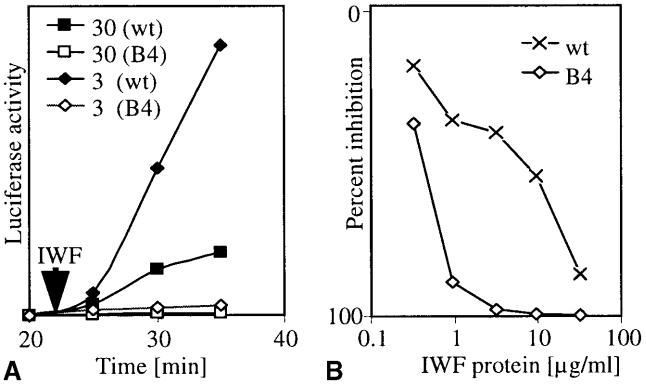

Fig. 6A, B Inhibition of a rabbit reticulocyte lysate-coupled transcription/translation system by wheat-leaf IWF. The lysate synthesizes luciferase from plasmid DNA, while IWF of the transgenic (line *B4*) or wild-type (*wt*) plants is added after 22 min. **A** Time course of luciferase activity (arbitrary units) for two different IWF concentrations (3 and 30 µg IWF protein per milliliter total reaction volume, **B** dose-response curve for different concentrations of IWF protein (inhibition of luciferase activity at 35 min compared to buffer only)

suggesting effective processing of the transported protein (Fig. 5c). Soluble barley seed protein contains approximately 1% RIP (Asano et al. 1984) or 11% (Coleman and Roberts, 1982). The intensity of the RIP30 signal from 40 µg total soluble wheat leaf protein was similar to that from 10 ng of barley seed protein. Therefore, in wheat lines expressing higher amounts of transgene, RIP30 constitutes approximately 0.0025% of the total soluble leaf protein or 0.1% of the IWF protein. In all cases so far analysed, the RIP-positive plants were also positive in a chlorophenol-red assay (data not shown); therefore, as expected, the two introduced transgenes appear to co-segregate. For some lines, the expression of RIP30 and the selectable marker has been monitored for up to four generations and found to be stable in all cases (Table 2).

Ribosome inactivating activity of IWF

As with GUS, the transport process or the modifications of the N-terminus of the engineered RIP (Fig. 1a) might have interfered with protein function. A cell-free rabbit reticulocyte lysate transcription/translation system was used to assay the ribosome-inactivating activity of apoplastic RIP30. The reticulocyte lysate was used to synthesize luciferase *in vitro* from plasmid DNA. Various amounts of IWF protein were added after 22 min of incubation (the earliest moment luciferase activity was detectable), and the kinetics of luciferase activity accumulation was followed (Fig. 6). The inactivating activity of IWF from line B4 was compared to that from untransformed plants at different concentrations of protein per reaction volume. At the same concentration, IWF of line B4 was always significantly more inhibiting than the control extract. At high protein concentrations, the inhibitory effect was noticeable after 3 min, and after 8 min, the activity of luciferase reached a steady level, indicating that active protein was no longer being synthesized. The inhibitory activity of the other expressing lines is shown in Table 2. Wheat lines expressing high levels of RIP30 also had high inhibitory activity, whereas lines with very low levels had no detectable activity. This correlation strongly argues for RIP as the cause of the observed inhibition, and the data are fully consistent with an inhibition of translation. The IWF of line B4 had no increased protease activity, since the luciferase activity leveled off, but did not decrease. Also, inhibition of *in vitro* transcription was unlikely because in this case the RNA already available at 22 min would still lead to a continuous increase of luciferase activity. For line B4, the 50% inhibition concentration was between 0.3 µg and 1 µg of IWF protein per milliliter of reaction volume (Fig. 6b). A 50% inhibition of rabbit reticulocyte ribosomes has been found at 15–25 ng/ml of purified barley RIP (Asano et al. 1984) and at 10 ng/ml (Roberts and Selitrennikoff 1986) or 50–100 ng/ml (Coleman and Roberts 1982) for Ascites cell ribosomes. Compared to these results, the activity of the line B4 IWF would correspond to a RIP30 content of at least 1%. This number is similar with that estimated from the immunological detection (Fig. 5). According to Western analysis, line B4 expressed about fourfold more RIP than line B25 (not shown). Taken together, these results show that the barley RIP targeted to the wheat apoplast retained full activity.

Infection of wheat with *E.graminis*

Two lines with high levels of RIP30 were chosen for a detached leaf infection assay with *E. graminis* spores, as described in Bliffeld et al. (1999). In this assay the num-

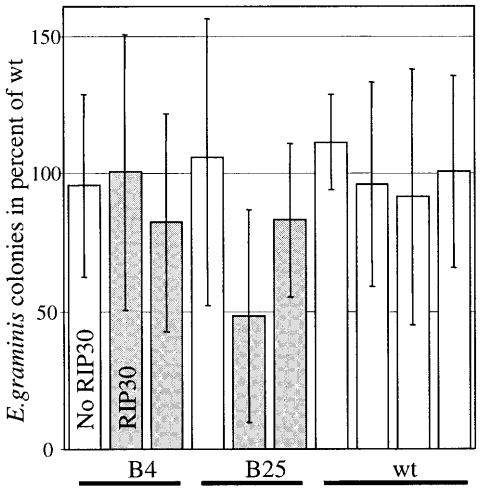

Fig. 7 Fungal infection assay of detached leaves with *E. graminis* spores. The number of growing fungal colonies on transgenic leaves was scored and compared to the average number on untransformed leaves (in percentages). Three plants per transgenic line were tested, two of them expressing RIP30 (*gray columns*), one not expressing due to loss of the transgene by segregation. Results shown are means of four individual experiments; *error bars* indicate 1 SD

ber of growing fungal colonies at a similar inoculation density was compared. Untransformed plants were inoculated as controls, as well as plants derived from the transgenic lines but lacking the transgenes due to segregation (Fig. 7). RIP30-expressing line B4 showed no or only slightly increased resistance, and for line B25 the susceptibility decreased by 20%–40% (Table 2). This small increment may be significant, since all the plants lacking a RIP gene showed a very uniform infection level with little variation of the mean.

Discussion

Wheat has been transformed with a gene for a barley seed RIP30 under the control of an enhanced CaMV 35S promoter. A leader peptide to direct the produced RIP to the ER and the apoplastic space was included in the expression construct for several reasons. First, Jach et al. (1995) have expressed RIP30 in transgenic tobacco as a cytoplasmic protein or linked to a N-terminal signal peptide (spRIP). The relative concentration of the RIP was higher in the intercellular fluid of spRIP compared to the cytoplasmic RIP in total protein, and the spRIP plants showed an increased resistance in tests with *R. solani* inoculation (50% disease index reduction versus 30%). Second, the localisation to the apoplast ensures the presence of RIP at the place where the initial interactions with the fungus occur. The apoplast is also the compartment to which two other proteins with supposed antifungal effects (a β-1,3-glucanase and a chitinase) are targeted in separately transformed wheat lines (Bliffeld et al. 1999 and unpublished results). Co-localisation should allow a full impact of the synergistic effects of these proteins. Third, the expression of barley RIP in wheat as a cytoplasmic protein has been attempted previously (Bliffeld et al. 1999), but no plants could be regenerated that expressed RIP. Transformation of the same cytoplasmic RIP into rice (cv. TP309) only yielded plants with truncated or inactive copies (H. Ding, M. Schrott, J. Fütterer, unpublished observations). This may indicate that cytoplasmic RIP30 is toxic enough to wheat and rice to prohibit the regeneration of expressing plants.

Most RIPs can also be active on the ribosomes of the RIP-synthesizing cells and, consequently, they have to be sequestered into the vacuole or the extracellular space, or their synthesis or activation has to be tightly regulated. Maize endosperm RIP b-32 is synthesised in the cytoplasm, but as inactive precursor that is proteolytically cleaved and activated during germination (Walsh et al. 1991). In barley (and probably wheat) RIP30 (tritin) is accumulated in the endosperm late during seed development (Leah et al. 1991). Tritin has been shown to be only weakly active on conspecific endosperm ribosomes and not active on tobacco leaf ribosomes (Massiah and Hartley 1995). The transformation of barley RIP into tobacco led to normal plants with a normal transformation efficiency (Jach et al. 1995; Logemann et al. 1992). The transformation of maize b-32 into tobacco (Maddaloni et al. 1997) and rice (Kim et al. 1999) seemed not to cause problems as well, but at least in rice leaves, the RIP was not processed properly. Many other reports describe abnormal phenotypes linked to RIP expression, e.g. for PAP (Lodge et al. 1993), PAPII (Wang et al. 1998), JIP60 (Gorschen et al. 1997) and trichosanthin (Lam et al. 1996), all in tobacco. With a targeted RIP30, we were able to regenerate transgene-expressing, normal and fully fertile wheat plants at a good transformation efficiency of approximately 0.4%. We could show that this RIP30 is effectively located to the extracellular space, since the concentration of RIP in the IWF was 50-fold higher than in total soluble protein extracts from the same plant (Fig. 5b). RIP30 produced in wheat seems to be comparable with its natural counterpart in barley seed extract, with respect to its electrophoretic mobility (Fig. 5c) and its specific activity, as tested in a coupled transcription/translation rabbit reticulocyte lysate (Fig. 6). The inhibition activity of IWF from different lines correlates very well with the approximate amount of RIP30 detected by Western blotting (Table 2).

Transgenic plants expressing RIPs have been used to test defense properties attributed to this group of proteins. In tobacco, increased virus resistance was achieved with the expression of trichosanthin (Lam et al. 1996), PAP (Lodge et al. 1993), PAPII (Wang et al. 1998), virus-induced dianthin (Hong et al. 1996) and C-terminally deleted, inactive PAP (Tumer et al. 1997) (which infers that the resistance may not necessarily be linked to N-glycosidase activity on "self" ribosomes), as well as increased fungal resistance against *R. solani* with PAPII (Wang et al. 1998), truncated PAP (Zoubenko et al. 1997), maize b-32 (Maddaloni et al. 1997) and barley RIP30 (Jach et al. 1995; Logemann et al. 1992). Rice ex-

pressing, but not properly processing, the maize b-32 type 3 RIP was not resistant against *R. solani* or *Magnaporthe grisea*. In our case, the expression of functional barley RIP in wheat had at best a small effect on the infection of detached leaves by *E. graminis* spores. Leah et al. (1991) have demonstrated that RIP30 alone has no activity on the growth of fungal spores *in vitro* but that RIP30 has an anti-fungal effect in combination with cell-wall degrading enzymes, which may generate an entry route for the RIPs to the fungal cytoplasm. The anti-fungal effect of RIPs in the other cases reported so far may be due to the presence of sufficient amounts of synergistic anti-fungal proteins in tobacco that are lacking in wheat, at least in the cultivar used, which is naturally very susceptible to *E. graminis*.

Transgenic Frisal lines expressing chitinase and ß-1,3-glucanase have been produced (our unpublished results). The different transgenes will be combined by crossing, and the anti-fungal effect will be analysed. Our aim is to generate by genetic engineering a type of resistance that mimics quantitative resistance, which is regarded as the most durable form of resistance against powdery mildew but one difficult to breed for (Keller et al. 1999). The generation of wheat plants with an active RIP in the apoplast is an important step towards this goal.

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