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Introduction and constitutive expression of a rice chitinase gene in bread wheat using biolistic bombardment and the *bar* gene as a selectable marker

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Abstract Our long-term goal is to control wheat diseases through the enhancement of host plant resistance. The constitutive expression of plant defense genes to control fungal diseases can be engineered by genetic transformation. Our experimental strategy was to biolistically transform wheat with a vector DNA containing a rice chitinase gene under the control of the CaMV 35 S promoter and the *bar* gene under control of the ubiquitin promoter as a selectable marker. Immature embryos of wheat cv 'Bobwhite' were bombarded with plasmid pAHG11 containing the rice chitinase gene *chi11* and the *bar* gene. The embryos were subcultured on MS2 medium containing the herbicide bialaphos. Calli were then transferred to a regeneration medium, also containing bialaphos. Seventeen herbicide-resistant putative transformants (T_0) were selected after spraying with 0.2% Liberty, of which 16 showed *bar* gene expression as determined by the phosphinothricin acetyltransferase (PAT) assay. Of the 17 plants, 12 showed the expected 35-kDa rice chitinase as revealed by Western blot analysis. The majority of transgenic plants were morphologically normal and self-fertile. The integration, inheritance and expression of the *chi11* and *bar* genes were confirmed by Southern hybridization, PAT and Western blot analysis of T_0 and T_1 transgenic plants. Mendelian segregation of herbicide resistance was observed in some T_1 progenies. Interestingly, a majority of the T_1 progeny had very little or no chitinase expression even though the chitinase transgene was intact. Because PAT gene expression under control of the ubiquitin promoter was unaffected, we conclude that the CaMV 35 S promoter is selectively inactivated in T_1 transgenic wheat plants.

Key words *Triticum aestivum* · Transformation · Microprojectile bombardment · Chitinase gene · *bar* gene

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Introduction

Bread wheat (*Triticum aestivum* L. em. Thell) is a worldwide cereal crop and a staple food source for billions of people. Many fungal pathogens which infect the spikes, leaves and roots of wheat plants are responsible for substantial yield loss. Application of chemical fungicides is not economical and is also detrimental to the environment. Worldwide, approximately US$8.7 billion are spent annually for protecting crops from diseases and pests (see Shah et al. 1995). The protection of plants by breeding for specific resistance has not always been effective, especially for wheat scab (head blight), which is a major disease of wheat in South China. In response to pathogen invasion, plants synthesize and accumulate pathogenesis-related (PR) proteins, such as chitinases (Collinge and Sluzarenko 1987; Linthorst 1991). However, the inducible defense mechanisms of plants are often too weak or appear too late to be effective for protection.

With the development of techniques for plant transformation, transgenic wheat incorporating a variety of genes, including those encoding β-glucuronidase, neomycin phosphotransferase II, hygromycin phosphotransferase, herbicide resistance, high-molecular-weight glutenin and nuclear male sterility, have been produced by biolistic processes (Altpeter et al. 1996a,b; Blechl and Anderson 1996; Ortiz et al. 1996; Srivastava et al. 1996; De Block et al. 1997; Luthra et al. 1997, review) and recently by *Agrobacterium*-mediated gene delivery (Cheng et al. 1997). Wheat transformation has progressed to a point where the transfer of agronomically useful genes such as PR protein genes can now be attempted. Chitinases, a subgroup of PR proteins with a role in plant defense, catalyze the degradation of chitin, which is a component of the cell wall of many filamentous fungi. The in vitro antifungal potential of chitinases has been reported (Collinge et al. 1993; Ji and Kuc 1996), and the introduction of chitinase genes into plants under the control of a constitutive promoter has been found to increase plant resistance to fungal pathogens in greenhouse studies (Broglie et al. 1991; Lin et al. 1995; Tabei et al. 1998) and field trials (Grison et al. 1996). Recently, Lin et al. (1995) reported the production of transgenic rice plants with constitutive expression of a rice chitinase gene, *chi11*, under control of the cauliflower mosaic virus (CaMV) 35S promoter. The transgenic rice plants and their progeny showed a high expression of chitinase and had increased resistance to sheath blight (Lin et al. 1995).

Here we report the biolistic-mediated production of transgenic wheat plants with constitutive expression of the rice *chi11* chitinase gene regulated by the CaMV 35S promoter. The *bar* gene driven by the maize ubiquitin promoter (Christensen and Quail 1996) was used as the selectable marker. It confers resistance to the herbicides bialaphos (Meiji Seika Kasha, Tokyo, Japan) and Liberty (AgrEvo™, Wilmington, USA) which contain phosphinothricin (PPT).

Materials and methods

Plant materials and tissue culture

The spring wheat, 'Bobwhite', and the Chinese winter wheat, 'Yangmai 158', were grown in a greenhouse (25°C, 16/8 h light/dark). At 12–14 days post-anthesis, seeds were collected, surface-sterilized in 70% ethanol for 1–3 min and 33% Clorox for 15 min and then rinsed three times with sterile distilled water. Immature embryos (0.5–1.5 mm long) were aseptically removed and cultured scutellum up on callus-induction medium, MS2 (MS medium supplemented with 2 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D), 100 mg/l casein hydrolysate and 30 g/l sucrose) (Murashige and Skoog 1962). After 2 days of culture, the embryos were subjected to microprojectile bombardment with a biolistic gun. After a second 2 days, the bombarded embryos were transferred to MS2 medium with 1 mg/l bialaphos. After 1 week of culture, the calli were transferred to fresh MS2 medium with 3 mg/l bialaphos for 2–3 weeks. All of the calli were transferred to half-strength MS (1/2 MS with 20 g/l sucrose) or MS medium supplemented with 5 mg/l bialaphos to induce shoot formation. After 1 month of culture, the regenerated plantlets were reselected for resistance to bialaphos on 1/2 MS medium containing 5 mg/l bialaphos. Surviving plants with well-developed roots were grown in a greenhouse. Regenerated plants were evaluated for herbicide resistance by painting and/or spraying with a 0.2% solution of Liberty (containing: 18.19% DL-phosphinothricin). All of the T_1 plants were grown in a growth chamber (25°C, 16/8 h light/dark).

Plasmid

The plasmid pAHG11 used in this experiment contains a rice chitinase gene (*chi11*) and the *bar* gene under the control of the CaMV 35S and maize ubiquitin promoters, respectively. The selectable *bar* gene of *Streptomyces hygroscopicus* encodes phosphinothricin acetyltransferase (PAT), which inactivates phosphinothricin (PPT), the active component of bialaphos, by acetylation (Murakami et al. 1986; Thompson et al. 1987). The rice chitinase gene, *chi11*, was obtained from a rice genomic clone (Huang et al. 1991) and reconstructed by Lin et al. (1995) in the pGL2 vector. Gu (1996) introduced the 1.5-kb *Hin*dIII fragment containing the *chi11* gene and the CaMV 35S promoter from pGL2 into pAHC20 (Christensen and Quail 1996) to obtain pAHG11 (Fig. 1).

Particle bombardment

A biolistic gun, PDS-1000 He (Bio-Rad), was used in this experiment. The coating of DNA on 1.0-μm gold particles was according to Nehra et al. (1994). Before bombardment, the immature embryos were arranged side by side with the scutellum exposed at the center of a 100 × 15-mm petri dish. All bombardments were performed at a pressure of 1100 psi. Each plate was consecutively bombarded at particle travel distances of 7 and 10 cm. The distance between the rupture disk and the macrocarrier, and that of macrocarrier travel, were 12 and 8 mm, respectively.

Protein extraction

Wheat flag leaf pieces (100–300 mg) were ground into a powder in liquid nitrogen. Protein was extracted by mixing the powder and 300 μl of 0.1 *M* KH_2PO_4 buffer (pH 6.5) with 1 m*M* phenylmethylsufonylfluoride (PMSF) and centrifuging at 15 000 rpm for 5 min. The protein concentrations of the extracts were determined using the bicinchoninic acid microtiter plate assay (Pierce, Rockford, Ill.). Bovine serum albumin was used as a standard.

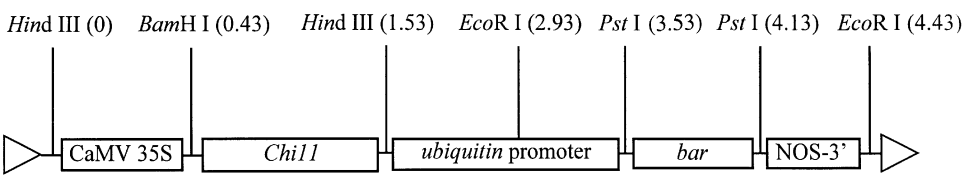

Fig. 1 Schematic representation of plasmid pAHG11 used for transformation. The size of the plasmid is 7.3 kbp. The *numbers* in *parenthesis* indicate distances in kilobasepairs

PAT activity

PAT activity was analyzed according to De Block et al. (1987) with modifications. Two microliters of 3 m*M* DL-PPT and 2 µl of [^{14}C]-acetyl coenzyme A (2.2 GBq/mmol, New England Nuclear) were mixed with 16 µl of protein extracts containing 25 µg of protein. After incubation at 37°C for 30 min, 6-µl aliquots of the samples were spotted onto a silica gel thin-layer chromatographic plate (EM Science, Gibbstown, N.J.). Ascending chromatography was carried out in a 3 : 2 (v/v) mixture of 1-propanol and concentrated NH_4OH (28–30% NH_3) for about 2 h. Acetylated-PPT was visualized by autoradiography.

SDS-polyacrylamide gel electrophoresis and Western blotting

Aliquots containing 50 µg or 200 µg of proteins in 1 × sample buffer (0.75% TRIS-HCl, pH 6.8, 10% glycerol, 2% SDS, 5% β-mercaptoethanol and 0.001% bromophenol blue) were boiled for 5 min in a water bath and then chilled on ice. Samples were separated on 12% SDS-polyacrylamide gels (Blackshear 1984). A Rainbow marker (Amersham, Arlington Heights, Ill.) was used as the molecular weight standard. A Hoefer Scientific Dual Mini Gel Apparatus was used for gel electrophoresis. Samples were electrophoresed at 15 or 20 mA per gel until the dye front reached the bottom of the gel.

Western blotting was according to Winston et al. (1987). After electrophoresis, the proteins were transferred from the gel to an Immobilon™-P Transfer Membrane (Millipore, Bedford, Mass.) using a semi-dry blotting apparatus (Bio-Rad, Hercules, Calif.) according to the manufacturer's instructions. The membranes were blocked with 2.5% (w/v) gelatin. The chitinase antibody (first antibody) was a polyclonal antibody raised in rabbits against a barley chitinase (Swegle et al. 1992). The antibody was diluted to 1 : 1000 (v/v). Goat anti-rabbit IgG (H + L) horseradish peroxidase (HRP) conjugate (Bio-Rad) was used as the second antibody at a dilution of 1 : 2000. The protein polypeptides recognized by the antibody were visualized with a HRP color development reagent from Bio-Rad.

Southern blot analysis

Total DNA was isolated from leaves of wheat according to the method of Riede and Anderson (1996). The DNA (30 µg per sample) was digested with 80 to approximately 160 units of restriction enzymes overnight and electrophoresed in a 1% agarose gel. DNA fragments were transferred onto a nylon membrane (Hybond-N$^+$, Amersham, Arlington Heights, Ill.), and then hybridized with α-[^{32}P]-dCTP-labelled DNA fragments derived from the coding region of the *bar* (603 bp) or *chi11* (1.1 kb) genes.

Results

The effect of bialaphos on callus formation and plant regeneration

Different concentrations of bialaphos were evaluated for their effects on callus formation from precultured embryos and on plant regeneration from calli. When the immature embryos were precultured on MS2 medium for 6 days, the efficiency of callus formation on media containing 1 and 3 mg/l bialaphos was similar to that on the control medium without bialaphos, indicating that this concentration of bialaphos may not be useful in identifying transgenic calli (Table 1). After being cultured on callus induction medium with 1–3 mg/l bialaphos, the calli differentiated shoots at a much lower efficiency on the differentiation medium without bialaphos compared to control calli (Table 1). Complete inhibition of plant regeneration from non-transformed calli occurred at 3 and 5 mg/l bialaphos in differentiation media (1/2 MS, MS with 2 mg/l kinetin), but 1 mg/l was ineffective (Table 2). Consequently, 5 mg/l bialaphos was used in the differentiation medium in subsequent experiments to ensure complete inhibition of shoot induction from non-transformed calli.

The effect of differentiation medium on the production of putative transgenic plants

Three differentiation media, 1/2 MS, MS and MS with 2 mg/l kinetin (MS + 2Kt), were tested in this experiment. The frequency of differentiation of shoots from non-bombarded calli on 1/2 MS medium was equal to that on MS + 2Kt medium (Table 2). For differentiation of the bombarded calli, the 1/2 MS medium was superior to MS with or without 5 mg/l bialaphos in the medium (Table 3). The 1/2 MS medium supplemented with 5 mg/l bialaphos yielded Liberty-resistant plants (15 plants/175 embryos) with a transformation efficiency of 8.6% (Table 3).

Table 1 Effect of bialaphos on callus formation and plantlet regeneration of immature embryos precultured on MS2 medium for 6 days (Chinese winter cv 'Yangmai 158') (*kt* kinetin)

MS2 + bialaphos (mg/l)	Number of embryos	Calli (%)	Regeneration medium (without bialaphos)	Number of Calli	Calli with shoots[a] (%)
0	125	100	MS + 2Kt	113	36.3
1	115	100[b]	MS + 2Kt	110	4.5
3	164	94.5[c]	MS + 2Kt	155	4.5

[a] Shoot length > 0.5 cm after the calli were cultured for 4 weeks in regeneration medium
[b] Some scutella did not produce obvious calli, and callus size was similar to that of control
[c] Callus size was slightly smaller than that of control

Table 2 Effect of bialaphos on shoot regeneration from non-transgenic calli in various regeneration media (cv 'Bobwhite')[a]

Differentiation medium + bialaphos (mg/l)	Number of calli	Differentiated calli with shoots	
		Number	%
1/2 MS	105	48	45.7
MS + 2Kt	125	50	40.0
1/2 MS + 1	125	59	47.2
1/2 MS + 3	167	0	0.0
1/2 MS + 5	140	1[b]	0.7
MS + 2Kt + 3	100	0	0.0

[a] The immature embryos were cultured on MS2 medium for 4 weeks, and then the calli were cultured on regeneration medium for 4 weeks
[b] A very thin and yellow leaf

Selection and PAT activity assay

All of the calli produced on MS2 + 3 mg/l bialaphos medium were transferred to differentiation medium containing 5 mg/l bialaphos. The shoot differentiation efficiency was very high (up to 6.3%) for 'Bobwhite' (Table 3). Sometimes, more than one shoot was derived from a single callus. The shoots regenerated on selection medium elongated slowly in comparison to the shoots of control calli on the non-selective medium. After 3 weeks, some of the regenerated plants retained a green color, grew quickly, and developed strong roots; some grew slowly and produced short roots, others became yellow and necrotic. Only well-rooted plants were transferred to the greenhouse.

All T_0 plants were painted and/or sprayed with 0.2% Liberty sequentially at the tillering, stem elongation and blooming stages. Sixteen Liberty-resistant plants were obtained from a total of 38 regenerated plants. Of those, 15 Liberty-resistant plants were identified from the 1/2 MS medium with 5 mg/l bialaphos and 1 from MS medium without bialaphos (Table 3). One additional Liberty-resistant plant out of 10 regenerated plants came from a separate preliminary experiment in which calli were bombarded with a mixture of plasmid pAHG11 and another containing a rice thaumatin-like protein (TLP) gene (Velazhahan et al. 1998).

A PAT activity assay was used to confirm the expression of the selectable *bar* gene in Liberty-resistant plants by thin-layer chromatography. Of the 17 plants tested 16 showed PAT activity in this assay. Data for 7 T_0 plants are shown in Fig. 2A. The expression level varied among the T_0 plants.

The T_1 and backcross progenies from the 17 T_0 plants were planted in the growth chamber and analyzed for genetic segregation to herbicide resistance (Table 4). T_1-14 and T_1-36 populations showed the expected 3 : 1 ratio for resistance to 0.2% Liberty on the basis of the χ^2 test. The transgenes in these plants are probably clustered at one locus. The segregation ratio of T_1-12 deviated from 3 : 1 ($\chi^2 = 4.24$, $P = 0.025$–0.05) but fit the ratio of 15 : 1 ($\chi^2 = 1.15$, $P = 0.25$–0.5) expected for Mendelian segregation of transgenes at two unlinked loci. The segregation ratios for the herbicide resistance of the other T_1 and backcross populations could not be ascertained accurately because of small sample sizes.

PAT activity assays of extracts of T_1 plants confirmed that the *bar* gene was active in the T_1 plants (Fig. 2B). The results shown in Fig. 2B indicate that the PAT activity of T_1 plants was comparable to that in T_0 plants (Fig. 2A).

The 16-day-old immature T_2 embryos from some T_1 plants were cultured on half-strength MS medium (1/2 MS) supplemented with 3 mg/l bialaphos to quickly obtain T_2 plants and to rapidly identify homozygous transgenic plants among them. The embryos from non-transformed Bobwhite plants did not germinate in this selection medium. After 4 days in culture, most of the seedlings were longer than 3 cm. The data from the germination tests indicated that some T_2 plants did not segregate for bialaphos sensitivity and were therefore, homozygous for the *bar* gene (data not shown). All of the plants derived from the embryo germination test

Table 3 Effect of differentiation medium on shoot formation from immature embryos (cv 'Bobwhite'), either bombarded[a] (B) with particles carrying the *bar* gene or not bombarded (NB)

Differentiation medium + bialaphos (mg/l)	Number of calli	Calli with shoots		Number of plants in greenhouse[b]	Number of plants in Liberty[R]
		Number	%		
1/2 MS(B)	150	26	17.3	7	0
1/2 MS + 5(B)	175	11	6.3	28	15
MS(B)	75	7	9.3	3	1
MS + 5(B)	105	1	0.9	0	0
MS(NB)	257	226	87.9	0	–
MS + 5(NB)	187	0	0.0	0	–
Total	505(B) + 444(NB)	–	–	38	16

[a] Bombarded embryos were cultured on 1 mg/l bialaphos MS2 medium for the first week, 3 mg/l bialaphos MS2 medium for the next 3 weeks, and then transferred to differentiation media
[b] All regenerated plantlets were cultured on 1/2 MS medium containing 5 mg/l bialaphos for 3 weeks before transplanting to the greenhouse

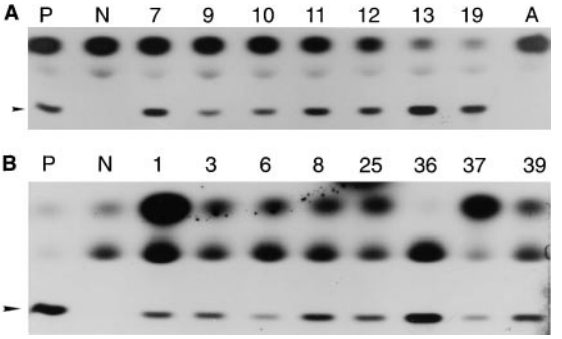

Fig. 2A, B PAT activity in protein extracts of leaf tissue for T_0 (**A**) and T_1 (**B**) plants. *P* Positive control, transgenic wheat callus expressing *bar* gene, *N* non-transformed 'Bobwhite', *A* [^{14}C]-acetyl coenzyme A, *other lanes* are extracts from plants that were resistant to the herbicide. The position of acetylated phosphinothricin is indicated with the *arrowhead*

Table 4 Segregation of resistance to 0.2% Liberty of T_1 or backcross plants

Number of progeny[a]	T_1 progeny		
	Total	Resistant (R)	Sensitive (S)
3, 6, 8, 9, 10, 11, 13, 19, 25, 39[b]	91	76	15
12[c]	49	43	6
14[d]	30	23	7
36[e]	50	38	12
1B, 7B, 25B, 28B, 37B[f]	36	16	20

[a] B, Backcrossing with nontransformed 'Bobwhite'; other plants were produced by selfing
[b] Pooled data for 10 T_1 plant lines
[c] Fits 15R : 1S ratio ($\chi = 1.15$, $P = 0.25$–0.5)
[d] Fits 3R : 1S ratio ($\chi = 0.04$, $P = 0.75$–0.9)
[e] Fits 3R : 1S ratio ($\chi = 0.03$, $P = 0.75$–0.9)
[f] Pooled data for backcross progenies
[b,f] Pooling of data was done because of the small sample sizes for these plants

also survived spraying with 0.2% Liberty at the tillering stage, indicating that the *bar* gene was still functional in the T_2-generation plants.

Expression of the rice chitinase gene *chi11* in transgenic wheat

The 17 Liberty-resistant plants were analyzed for chitinase expression. Extracts of 12 T_0 plants (1, 3, 6, 8, 9, 13, 14, 19, 25, 28, 36 and 37) had protein bands that reacted with a barley chitinase antibody and had the same size (35 kDa) as the positive control from a transgenic rice plant (data shown for 9 of these 12 T_0 plants in Fig. 3). The other 5 plants had no detectable chitinase (data not shown). The level of chitinase expression varied among the transgenic plants. The highest levels were seen in plants T_0-1 and -25. In addition to the

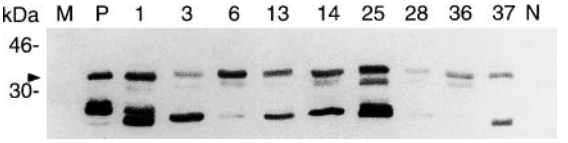

Fig. 3 Western blot analysis of leaf protein extracts of putative transgenic wheat plants. *M* Size markers in kilodaltons (kDa) are indicated on the *left*, *P* positive control, protein extract from a leaf of a homozygous transgenic rice plant transformed with plasmid pGL2Chi11 (Lin et al. 1995), *N* negative control, protein was extracted from leaves of a non-transformed 'Bobwhite' plant. The *numbers* indicate individual putative T_0 transgenic plants. Each lane contained 200 μg of protein except for the positive control, which had 50 μg of protein. The *arrowhead* indicates the expected 35-kDa chitinase band encoded by *chi11*

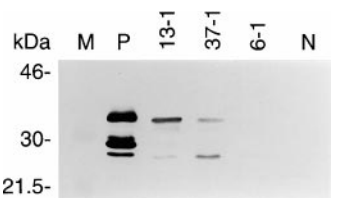

Fig. 4 *chi11* expression in T_1 plants. All lanes including a negative control (*N*) were loaded with 200 μg protein except for the positive control (*P*) which was loaded with 50 μg. *M* Protein size marker at 21.5 kDa, 30 kDa and 46 kDa

35-kDa band, other chitinase bands were also found in putative transgenic plants and in the positive control. Lin et al. (1995) presumed that these bands were derived from the 35-kDa chitinase by proteolytic processing. In a majority of the plants selected for Liberty resistance, the unselected rice chitinase gene appeared to be expressed at variable levels. These levels are comparable to those reported by Lin et al. (1995) for transgenic rice plants expressing the same chitinase gene.

Herbicide-resistant T_1 plants were grown in a growth chamber, and 124 plants were assayed for the expression level of the chitinase by Western blot analysis. A vast majority of them (103 plants) showed no expression, and 21 plants showed a very low level of expression of the 35-kDa rice chitinase band. Of these, only 2 were easily detectable (Fig. 4). However, this level was significantly lower than the level in T_0 plants or positive controls.

Southern blot analysis

DNA was extracted from 13 T_0 plants with herbicide resistance and 2 lacking resistance to check for the integration of the rice chitinase and *bar* genes in transgenic plants. A mixture of *Hin*dIII and *Bam*HI restriction enzymes was used to digest the DNA and release a 1.1-kb fragment containing the coding region of the

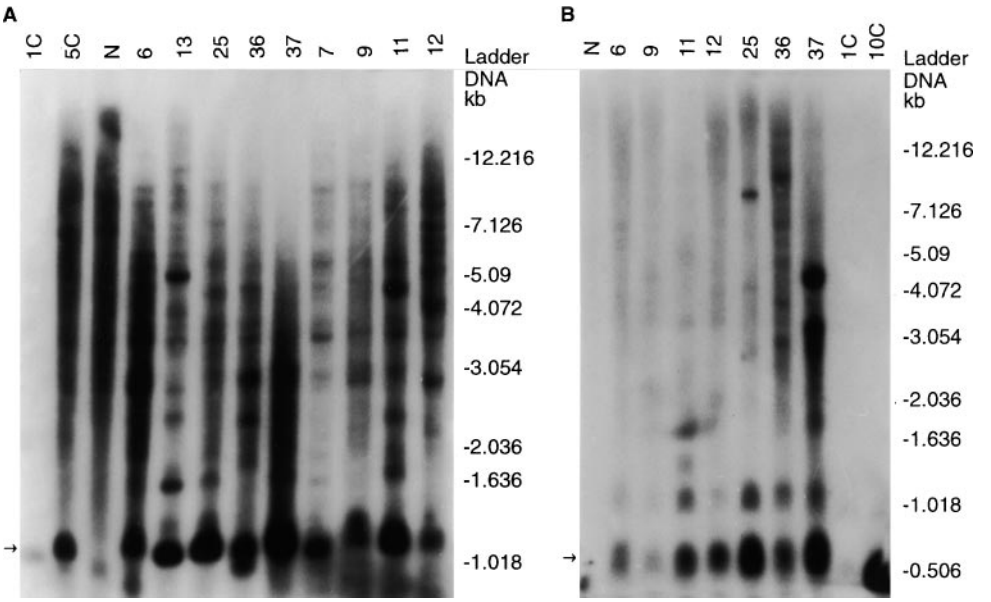

Fig. 5A, B Southern blot analysis of T_0 plants. **A** DNA double-digested with *Hin*dIII and *Bam*HI and probed with the 1.1-kb *chi11* coding region DNA, *1C*, (*5C*) one-copy (3 pg) and five-copy reconstruction with the 1.1-kb *chi11* fragment, respectively mixed with *Hin*dIII-*Bam*HI-digested 'Bobwhite' DNA, *N* non-transformed digested 'Bobwhite' DNA; the *arrow* indicates the 1.1-kb chitinase band. The *remaining lanes* show DNA profiles of transgenic plants. **B** DNA was digested with *Pst*I and probed with the 0.6-kb *bar* gene coding region fragment. *1C*, (*10C*) one-copy (1.5 pg) and ten-copy reconstruction, respectively, with the 0.6-kb *bar* gene fragment, *N* non-transformed digested 'Bobwhite' DNA; the *arrow* indicates the 0.6-kb *bar* gene band. The sizes of marker bands are indicated on the *right*

chitinase transgene. Results from 9 transgenic plants and 1 control plant (lane N) are shown in Fig. 5A. All of the herbicide-resistant plants analyzed (T_0-6, -7, -9, -11, -12, -13, -25, -36, -37) contained the expected 1.1-kb chitinase transgene fragment. The 1.1-kb band was missing in 2 herbicide-sensitive plants (data not shown) and the non-transformed control (lane N).

DNA from 9 herbicide-resistant plants (T_0-6, -7, -9, -11, -12, -13, -25, -36, -37) and 2 sensitive plants (T_0-2, -15) was digested with *Pst*I and used to check for the presence of the *bar* gene. DNA from non-transformed plants and herbicide-sensitive plants exhibited no hybridization to the *bar* coding region fragment (lane N, Fig. 5B and data not shown). The blots from herbicide-resistant plants contained the expected 0.6-kb band detectable by the *bar* probe.

A comparison of the intensities of the 1.1-kb *chi11* and 0.6-kb *bar* gene bands of Fig. 5A and Fig. 5B with the intensities of corresponding bands in copy-number reconstruction lanes (lane 1C and 5C of Fig. 5A and 1C and 10C of Fig. 5B) gave an estimated range for the copy numbers of intact fragments. These ranged from 2 to 10 in different transgenic plants.

To determine whether the intact transgenes were inherited by the T_1 progeny, we digested DNA from single representative progeny of several T_0 plants with *Hin*dIII or *Eco*RI and probed it with *chi11* or *bar* coding region fragments. Digestion with *Hin*dIII and *Eco*RI releases the intact promoter-coding region fragments of the *chi11* and *bar* genes, respectively. However, digestion with *Hin*dIII, which cuts outside of the *bar* transgene, and digestion with *Eco*RI, which cuts outside of the *chi11* transgene, could provide a diagnostic way of identifying independent transformations, because they result in distinct band patterns (see Fig. 6B and C). From these results, we conclude that T_1-1B and T_1-7B, may have originated from the same transformation event. Similarly, T_1-3 and T_1-9 also may be siblings (6C, D). Figure 6A also indicated that 14 of the transgenic plants examined in this experiment contained an intact chitinase gene, including the CaMV 35S promoter, as they all contained the expected 1.53-kb *Hin*dIII fragment detected by the *chi11* probe. All 15 plants had the *bar* gene (Fig. 6D). Several herbicide-sensitive T_1 plants derived from T_0 transgenic plants did not have the *chi11* or *bar* genes, as determined by Southern blot analysis.

If the T_0 plants contained two or more transgene loci, segregation of transgenic loci among T_1 progeny could be detected by Southern blot analysis. To investigate this possibility, we extracted DNA samples from 121 herbicide-resistant T_1 plants derived from 15 T_0 plants and digested these with *Hin*dIII; the Southern blot was hybridized with the 1.1-kb *chi11* coding region probe. In nearly all cases, T_1 progeny from a single T_0 plant had the same pattern (data not shown). These results indicate that in most cases, all the transgenes (*bar* or *chi11*) were clustered at a single locus or linked closely in the wheat chromosome. The only exception were T_1 plants from T_0-12 that showed segregation for

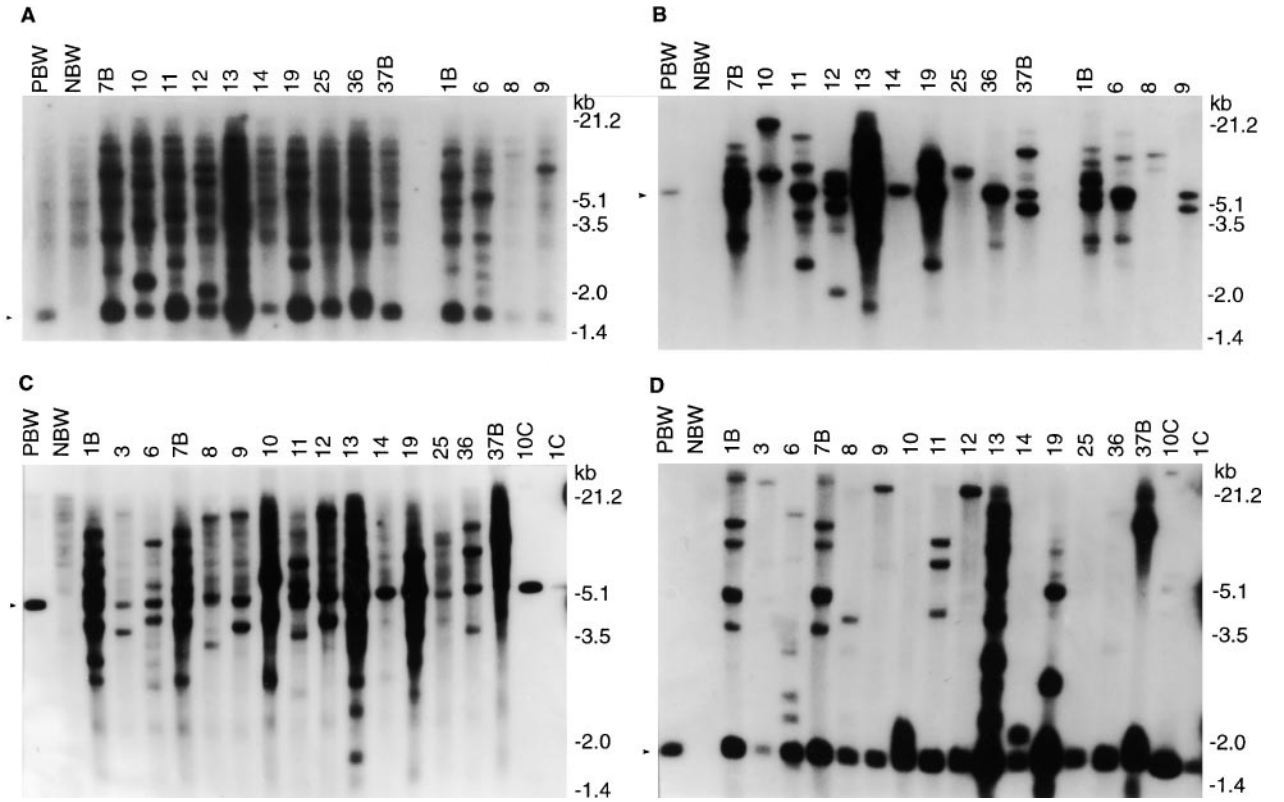

Fig. 6A–D Southern blot analysis of T_1 plants. The DNAs were digested with *Hin*dIII (**A**,**B**) or *Eco*RI (**C**,**D**). The membranes were probed with the 1.1-kb *chi11* gene (**A**,**B**) or the 0.6-kb *bar* gene (**C**,**D**). *PBW* 'Bobwhite' genomic DNA was mixed with the 1.53-kb *chi-11* fragment after digestion (**A**), the 5.8-kb vector DNA fragment containing the *bar* gene (**B**), the 5.8-kb vector fragment containing the *chi11* gene (**C**), or the 1.5-kb fragment containing the *bar* gene (**D**). *NBW* Non-transformed 'Bobwhite'. The expected *chi11* or *bar* DNA bands are marked with *arrowheads*. Reconstruction samples containing one-(*1C*) and ten-copy equivalents (*10C*) of *Eco*RI-digested pAHG11 also are included in **6C** and **6D**. Transgenic plant numbers are indicated on the *top* of the DNA lanes

the transgenic *bar* gene (Fig. 7A) and chitinase gene (Fig. 7B). Two distinct patterns of transgene bands (for both *chi11* and *bar*) were detected among progeny of plants of T_0-12, suggesting the presence of two independent loci. These findings are in agreement with the results of segregation shown in Table 4.

Morphology and fertility of transgenic plants

All of the T_0 transgenic plants were weaker and shorter than seed-derived plants. The T_1 plants were normal in appearance and resembled control wheat plants. However, some plants had a low seedset. Five of the 17 T_0 plants and 15 of the 124 T_1 transgenic plants showed reduced fertility.

Discussion

The biolistic procedure for plant transformation has been optimized for transformation efficiency (Vasil et al. 1993; Yang and Christou 1994; Casas 1995; Jain et al. 1996; Heiser 1992; Kemper et al. 1996) and for methods of screening putative transformants using bialaphos or PPT as a selective agent (Nehra et al. 1994; Becker et al. 1994; Altpeter et al. 1996b; Jain et al. 1996). The biolistic approach was a reliable method of obtaining wheat transformants in our studies, with a transformation efficiency as high as 8.6% (15/175, Table 3). This compares favorably with previous reports (Altpeter et al. 1996b).

Plant regeneration efficiency is a critical component of overall transformation efficiency. Plant genotype and composition of the shoot induction medium are the two main elements that affect plant regeneration efficiency. The wheat cultivar 'Bobwhite' is widely used for transformation because of its high regeneration efficiency (Weeks et al. 1993; Vasil et al. 1993; Zhou et al. 1995; Altpeter et al. 1996a,b; Blechl and Anderson 1996; Gu 1996; Cheng et al. 1997). The transformation frequency was usually 0.1–2.0% when MS media containing various hormones or plant growth regulators were used for plant regeneration (see Altpeter et al. 1996b). In an effort to improve the efficiency of wheat transformation, 1/2 MS medium was tested in this study. Immonen (1996) reported that using 1/2 MS as

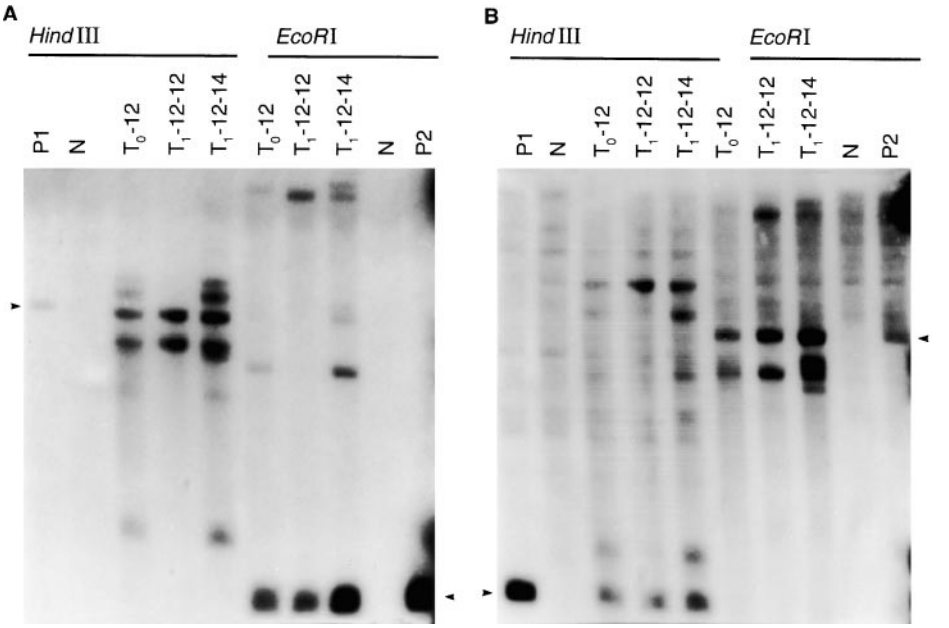

Fig. 7 The pattern of segregation of transgene bands in a Southern blot analysis of progeny of T_1-12. The membrane was probed with the *bar* gene (**A**) or the *chi11* coding region (**B**). *P* pAHG11 DNA digested with *Hin*dIII (*P1*) or *Eco*RI (*P2*), *N* non-transformed 'Bobwhite' DNA digested with *Hin*dIII or *Eco*RI. Positive control bands are indicated with *arrowheads*

a differentiation medium gave results similar to those using MS medium with various combinations of hormones for triticale plant regeneration when the calli were cold-treated for 12 days at 8°C. Our results indicate that 1/2 MS medium was comparable to MS + 2Kt with respect to frequency of plant regeneration from 'Bobwhite' calli (Table 2). We compared the regeneration frequencies of bombarded calli with and without cold treatment (6°C, 14 days). No obvious difference in regeneration frequency was observed (data not shown). Therefore, 1/2 MS containing 5 mg/l bialaphos was used as a differentiation medium for bombarded 'Bobwhite' calli without cold treatment. The use of such a medium led to a high transformation frequency (Table 3).

We used a protocol with bialaphos in both the callus induction medium and regeneration medium to minimize the regeneration of "escape" plants. Various effects of this herbicide on callus differentiation have been reported. Bialaphos at 0.01–0.1 mg/l promoted plantlet production from calli and suspension cultures in Gladiolus (Kamo and Vaneck 1997). At 3 mg/l, this herbicide decreased embryoid formation on the calli of 'Chinese Spring' wheat by tenfold (W. Chen et al. unpublished) and inhibited wheat plant regeneration (Nehra et al. 1994). Even though the MS2 medium used for callus induction contained 100 mg/l casein hydrolysate, which possibly weakened the role of bialaphos as a selective agent during embryoid formation (R. Chibbar, personal communication), 1–3 mg/l bialaphos inhibited the subsequent regeneration of control plants (Table 1). A concentration of 5 mg/l bialaphos in plant regeneration medium was high enough to inhibit shoot formation from nontransformed calli (Tables 2 and 3, Altpeter et al. 1996b). However, if the callus size was too large (diameter bigger than 5 mm) or if too many calli (more than 36 pieces) were allowed to differentiate in one petri dish (100 × 15 mm), shoots were also produced from a few nontransformed calli. However, the roots from non-transgenic plants on differentiated calli grew very slow and rarely reached 2 cm in 1 month. Therefore, our suggested procedure for obtaining putative transgenic plants and minimizing the number of "escape" plants includes the following steps: (1) transfer the bombarded immature embryo [precultured 2–5 days before bombardment (Nehra et al. 1994; Altpeter et al. 1996b)] to MS2 medium and culture for 2 days; (2) subculture on MS2 medium with 1 mg/l bialaphos for 1 week; (3) subculture calli on MS2 medium with 3 mg/l bialaphos for 2–3 weeks; and (4) transfer all of the calli to regeneration medium with 5 mg/l bialaphos to allow shoot formation.

The 17 plants studied extensively in the experiments were selected for resistance to spraying with 0.2% Liberty. Even though Southern blot data indicated that multiple copies of the *bar* and *chi11* genes were present, the genes segregated in a Mendelian fashion for a single locus with the exception of plant no. 12 which appeared to have two transgene loci. This observation is consistant with other reports that multiple copies of the transgenes carried in the same or different plasmids tend to be clustered at a single locus (Christou 1996).

A potentially serious problem in the use of transgenic plants in plant breeding is the instability of transgene expression (Stam et al. 1997). Silencing of the expression of the introduced transgene was reported even in

T_0 transgenic potato plants following selection for a linked selectable marker gene (Ottaviani et al. 1993). The loss of expression by the inactivation of the transgene has been studied extensively in other systems, and several mechanisms for gene silencing have been proposed (Register et al. 1994; Matzke et al. 1994; Meins and Kunz 1994). The results of our study also indicate that the rice chitinase gene undergoes silencing in transgenic wheat plants. Only 12 among 17 herbicide-resistant T_0 plants showed detectable *chi11* expression even though all of them had the chitinase transgene with an intact chitinase promoter-coding region fragment(s) as shown by Southern blot analysis. The failure to detect chitinase expression in 5 plants may have been due to the inactivation of *chi11* early in the development of these plants, although other explanations such as position effects cannot be excluded. The 12 plants with good expression of chitinase transmitted their transgenes to the T_1-generation plants, but the chitinase was not detected in most of the progeny. In many cases, none of the progeny expressed the transgenic chitinase, although T_1 plants showed a high level expression of the *bar* gene. In fact, we have not found any evidence for *bar* gene silencing in T_1 plants, either by PAT assays or by spraying with 0.2% Liberty. The finding that the expression of the chitinase gene driven by the CaMV 35S promoter was silenced, but not the expression of the *bar* gene under the control of ubiquitin promoter, suggests that a difference between the two promoters may be responsible. In support of this hypothesis, we found that the expression of a rice TLP gene under the control of the ubiquitin promoter is not silenced in T_1 and T_2 plants (W. Chen et al. unpublished).

Several groups have reported that the level of expression of the transgene is greater with the ubiquitin promoter than with CaMV 35S promoter in transient expression in monocotyledonous plants (Taylor et al. 1993; Gallo-Meagher and Irvine 1993; Li et al. 1997). In an analysis of transgenic rice plants with the same CaMV 35S promoter-*chi11* used in the present study, we estimated that the rice chitinase accumulated to a level of 0.05–0.1% of total protein in some plants with the transgene locus (Lin et al. 1995 and unpublished data). This value compares favorably with the level of expression of other transgene products in rice. Data from Western blot analysis of some of the hemizygous T_0 wheat plants (Fig. 3) indicate that the chitinase levels in these plants were only about one-half to one-fourth of the levels in homozygous rice plants used as a positive control, indicating that the CaMV 35S promoter was active in wheat plants as well. However, we have not yet directly compared the levels of expression of chitinase genes under the control of the ubiquitin promoter and the CaMV 35S promoter to determine whether the ubiquitin promoter is indeed more effective. The levels of chitinase were significantly lower in T_1 transgenic wheat plants than in T_0 plants (one-tenth or less), suggesting that transgene expression is attenuated presumably due to gene silencing. We have not analyzed the timing of this silencing. In transgenic rice with the same promoter-rice chitinase gene combination, silencing of the transgene was detected in about 20% of the T_3- and T_4-generation plants, begining 8 weeks after germination. Silencing in this case was shown to be transcriptional as no chitinase transcripts (sense or antisense) were observed in nuclear run-on transcription experiments (Thara et al. personal communication). The nearly complete loss of chitinase gene expression in T_1-generation wheat plants indicates that the CaMV 35S promoter is more prone to silencing in wheat than in rice. We are currently analyzing the T_2-generation plants to determine if the silencing is reversible.

Several mechanisms have been proposed to explain alien gene inactivation: methylation of transgenes, anti-sense RNA production and heterochromatinization of the transgene locus. Related phenomena include co-suppression of alien genes and endogenous genes, and position effects (Finnegan and McElroy 1994; McElroy and Brettel 1994; Matzke and Matzke 1995; Meyer 1995; Meyer and Saedler 1996; Stam et al. 1997). Silencing also may be influenced by environmental factors (Hart et al. 1992). The basis of the silencing of *chi11* gene expression in some T_0 and T_1 transgenic plants in this study is unknown. A more detailed molecular analysis is in progress.

Our original objective was to test transgenic plants constitutively expressing chitinase in assays using plant pathogenic fungi. However, because of the loss of chitinase expression in T_1 plants, this is not feasible at present. Fortunately, the use of the ubiquitin promoter resulted in transgenic wheat plants expressing the rice TLP gene (W. Chen et al. unpublished). We will attempt to generate transgenic plants with a high level of chitinase expression using the ubiquitin promoter. These plants expressing chitinase and/or TLP will be tested for enhanced resistance to fungal diseases of wheat.

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