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Stable transgene expression and random gene silencing in wheat

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Summary

Wheat genes for pathogenesis-related (PR-)proteins, chitinase and β-1,3-glucanase, under the control of maize ubiquitin promoter-intron were used for transforming the spring wheat 'Bobwhite', using a biolistic approach. Twenty of the 24 primary transgenic lines expressing the PR-protein genes in the T_0 generation were silenced in either the T_1 or T_2 generations. Two apparently genetically identical regenerants arising from a single callus co-bombarded with chitinase and β-1,3-glucanase transgene combinations, but differing in the expression of the transgenes were selected for further characterization. In one homozygous line, transgene silencing was observed in the T_3 plants, while the other line homozygous for the transgene loci stably expressed and inherited the transgenes to at least the T_4 generation. Southern blot analyses of genomic DNA from the two lines using the isoschizomeric methylation-sensitive enzymes, *Msp*I and *Hpa*II, revealed a higher degree of methylation of CCGG sequences in the line with the silenced transgene locus. Analysis by reverse transcriptase-polymerase chain reaction, Northern blotting and Western blotting detected stable expression of the transgenes in the line with a lesser extent of methylation, whereas the line with a higher level of CCGG methylation had no transgene expression by the T_3 generation. The germination of seeds from the silenced plants in the presence of a cytidine analogue, 5-azacytidine (azaC), did not lead to a reversion of this phenotype.

Introduction

Wheat (*Triticum aestivum* L.) is one of the most important grain crops of the world, ranking next to rice in human consumption, and is a major source of energy. Because of its economic importance, over the last decade wheat has been a primary world-wide target for the application of genetic engineering to improve its agronomic traits and disease resistance. The large size of the wheat genome (17 000 Mb) makes it a challenging crop for any genetic manipulation (Patnaik and Khurana, 2001).

The efficiency of genetic engineering depends on the stable and predictable expression of the inserted genes. The major obstacle to the genetic engineering of crops appears to be gene silencing. While transgene silencing in dicotyledonous plants has been studied extensively, random gene silencing in monocots is not completely understood, but is known to occur at both transcriptional and post-transcriptional levels (Iyer *et al*., 2000). Only a few groups have studied transgene silencing in wheat (Alvarez *et al*., 2000; Chen *et al*., 1998; Demeke *et al*., 1999; Muller *et al*., 1996), and there is a need to understand this mechanism, especially in the context of polyploid genomes.

Silencing of a foreign gene after integration into the genome within a few generations illustrates the inherent defence mechanisms of plants against foreign DNA invasion and expression (Demeke *et al*., 1999; Kumpatla *et al*., 1997; Matzke *et al*., 1996). The over-expression of homologous genes in transgenic plants often leads to homology-dependent silencing of both the endogenous genes as well as the transgenes (Alvarez *et al*., 2000; Meyer and Saedler, 1996). Unstable gene expression is often related to the integration of multiple copies of the transgene in the plant genome (Muller *et al*., 1996), the position effect (Weiler and Wakimoto, 1995) and to the extent of methylation in the transgene loci (Srivastava *et al*., 1996).

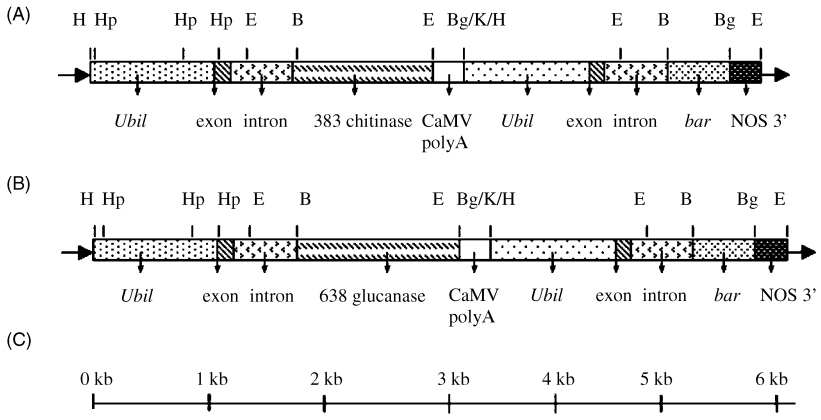

Figure 1 The structure of the plant transformation plasmids used in this study. (A) pAHC20ubi383 chitinase and (B) pAHC20ubi638 β-1,3-glucanase used for transforming the wheat embryos. The expression of the gene of interest and the selectable marker gene, *bar*, was driven by the maize ubiquitin promoter-exon1-intron1 (Ubi1). The gene cassettes for the promoter, transgenes(s), cauliflower mosaic virus (CaMV polyA) terminator region and nopaline synthetase (NOS) terminator are shown as boxes drawn approximately to scale. The plasmid vector backbone (pUC9, 3.0 kb) is indicated by the arrowheads at the left ends. Relevant restrictions sites are indicated. H, *Hin*dIII; E, *Eco*RI; B, *Bam*HI; K, *Kpn*1; Bg, *Bgl*II; and H, *Hpa*II. (C) Scale in kilobases.

The role of pathogenesis-related (PR-) proteins in protection against diseases in transgenic wheat has recently been demonstrated (Bieri *et al*., 2000; Bliffeld *et al*., 1999; Chen *et al*., 1999; Oldach *et al*., 2001; Schweizer *et al*., 1999). Chen *et al*. (1999) were the first to demonstrate the importance of the choice of the promoter driving transgene expression in the recovery, stable inheritance, and expression of transgenes in wheat. In a continuation of this work, we attempted to generate transgenic wheat plants with enhanced levels of resistance to various fungal diseases deploying genes for PR-proteins. The spring wheat, 'Bobwhite' was co-transformed with the PR-protein genes, encoding chitinases and β-1,3-glucanases singly or in combination. These genes were placed under the control of a constitutive promoter, maize ubiquitin promoter-intron (*Ubi1*), and were used for the co-transformation of wheat with the selectable marker gene, *bar*. In this paper, we describe our analyses of transgene expression in 24 transgenic plants and the occurrence of random and progressive gene-silencing in wheat. We studied the DNA methylation patterns using two different transgenic lines arising from the same callus, which were identified as being apparently genetically identical but differing in the expression of the transgenes. The analysis revealed that transgenic plants were methylated in the CCGG sequences, and the silenced line with a higher degree of methylation in the CCGG sequences did not accumulate the transgene-encoded proteins. The transgene-silencing phenomenon in this case appeared to be irreversible.

Results

Characterization of putative transgenic plants

Twenty-four independent primary transgenic lines with the functional selectable marker, *bar* gene, were identified by painting the putative transgenic plants with a 0.2% w/v Liberty solution. PCR analysis using gene-specific primers was performed to confirm the presence of the *bar* gene and the expected PR-gene(s). Western blot analysis was used to detect the presence of chitinases and/or β-glucanases in these plants (Figure 2A,B). All 24 plants were selfed to obtain seeds for further propagation.

The leaves of T_1 progenies from all of the 24 T_0 plants were painted with Liberty and analysed by PCR to detect the segregation of transgene(s) in each transgenic line. All T_1 progenies of 18 of the 24 lines were susceptible to Liberty, even though a majority of them (about 75%) had the *bar* gene (and the other transgenes), as revealed by PCR. Progenies from the remaining six T_1 lines survived painting with this herbicide. These T_1 plants accumulated PR-proteins as revealed by Western blot analysis (Figure 2C) and were PCR-positive for the gene of interest. The segregation patterns for resistance/susceptibility to Liberty of T_1 plants were consistent with one or two transgene loci. Segregation ratios were close to 3 : 1 or 15 : 1 (data not presented). The T_1 progenies from the 18 Liberty-sensitive lines also had no detectable transgene-encoded proteins (data not shown), presumably resulting from the silencing of both the *bar* and PR-protein transgenes. Additional evidence for progressive transgene silencing was observed in the T_2 generation in two of the six expressing lines (lanes 1 and 12, see Figure 2D) (Anand *et al*., 2003). The levels of transgene-encoded proteins in the T_1 and T_2 generation plants of these two lines were lower than the levels in the T_0 parents, as detected by Western blot analysis (Figure 2D) suggesting additional transgene silencing had occurred in these T_2 generation plants. No evidence of functional transgenes was found in T_3 generation plants of the above two lines (data not shown). The remaining four lines (#3, #8, #11 and #16) continued to stably express the *bar* gene and the genes of interest in the T_3 and T_4 generations (data not

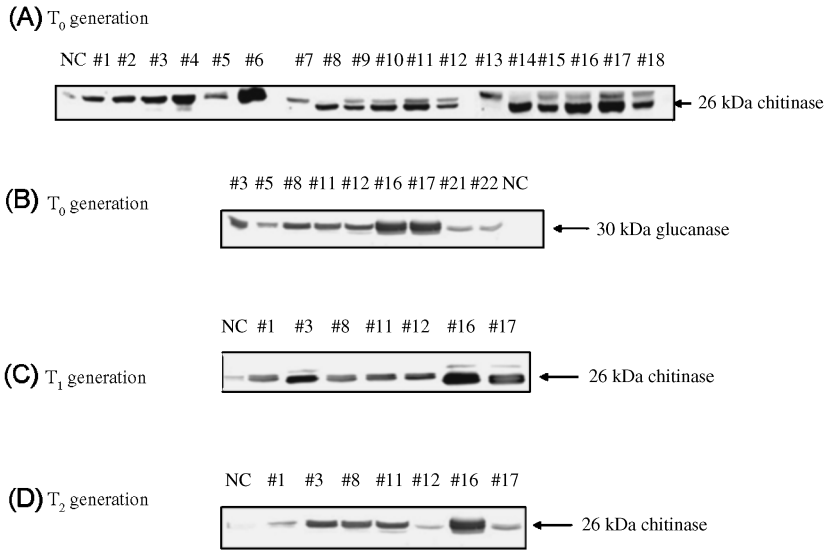

Figure 2 Progressive transgene silencing in transgenic wheat lines in different generations. Aliquots containing 250 μg of total leaf protein extract were analysed by Western blotting for chitinase and glucanase (as indicated on the left) levels in different lines of Liberty-resistant plants. (A) and (B): T_0 plants. (C) and (D), T_1 and T_2 plants, respectively. Lanes numbered 1–22 indicate different transgenic lines. Lanes marked 16 and 17 were derived from two tillers of a single T_0 plant (32) which exhibited differences in transgene expression in subsequent generations and are referred to as lines 32A2#3 and 32C3#4, respectively. NC: non-transgenic, 'Bobwhite' control plants. The expected sizes of the transgene-encoded proteins are indicated by arrows. Lines with the numbers, 3, 5, 8, 11, 12, 16, 17, 21 and 22 were co-bombarded with two different transformation plasmids, one containing chitinase gene and the other a glucanase gene. Others were bombarded with either chitinase or glucanase vectors as indicated.

shown). Interestingly, there was a difference in transgene expression levels between lines, derived from two tillers of plant #32 (tillers 32A and 32C; compare lanes 16 and 17, Figure 2C,D) suggestive of selective transgene silencing in the T_2 progeny of tiller 32C (line 32C3#4) but not in the line derived from the tiller 32 A (line 32A2#3).

Transgene silencing is random and progressive

In order to be able to understand the phenomenon of gene silencing in wheat, we focused on two transgenic lines derived from two T2 plants, 32A2#3 (expressing the 383 chitinase and 638 glucanase transgenes, lane 16, Figure 2D) and 32C3#4 (silenced for 383 chitinase and 638 glucanase expression, lane 17, Figure 2D). The 32A2#3 and 32C3#4 plants came from two different T_0 tillers, 32A and 32C of a single transgenic event arising from the same primary callus. Southern blot analysis with four different restriction enzymes and four transgene probes (data for the *Ubi1* and *bar* probes shown) confirmed that the lines derived from the two T_2 parents, 32A2#3 and 32C3#4, were apparently genetically identical (see Figure 7), except for the differences in the banding pattern in *Eco*RI-digested DNA.

The T_2 parents, 32A2#3 and 32C3#4 were resistant to Liberty in the leaf-painting assay, but differed in the expression levels for the transgenes. The T_2 plant, 32C3#4, showed evidence of lower transgene activity as revealed by considerably lower levels of both chitinase and glucanase transgene transcripts in leaf RNA compared to the 32A2#3 plant by Northern blot and RT-PCR analyses (Figures 3 and 4). These

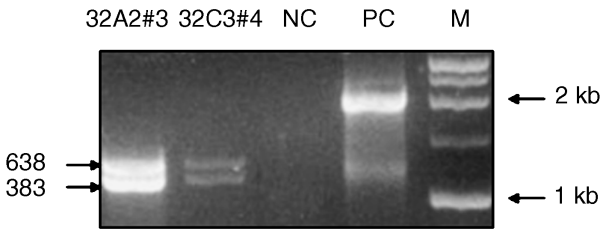

Figure 3 Reverse transcriptase (RT-)PCR for detecting the presence of the transgene RNA transcripts in the different transgenic lines. Ten micrograms of total RNA was used to carry out the RT-reaction, followed by 20 PCR cycles using primers specifically designed for detecting the transgene transcripts. Lanes: 1, 32A2#3 (high-levels of expression); 2, 32C3#4 (undergoing transgene silencing); NC, non-transgenic control; PC, pAHCUbi383 plasmid DNA as positive control; and M, 1 kb DNA ladder. Arrows indicate the migration positions of indicated size fragments of 1 kb DNA ladder (right arrows) and the RT-PCR amplification products corresponding to 638 glucanase and 383 chitinase mRNA (left arrows).

results are consistent with the results of Western blot analyses for chitinase shown in Figure 2D and for glucanase (Anand *et al*., 2003).

The T_2 parents 32A2#3 and 32C3#4 were identified as homozygous through fluorescent *in situ* hybridization (FISH) analysis with labelled 383 chitinase and 638 glucanase DNA probes. Both lines gave identical FISH patterns, indicating a single transgene locus near the telomere of homologous chromosomes tentatively identified as the long arm of chromosome 6A (Figure 5) and identified them as homozygous for the transgene locus. These plants were selfed and the seeds from them were collected to obtain T_3 progenies. Twenty T_3 generation plants

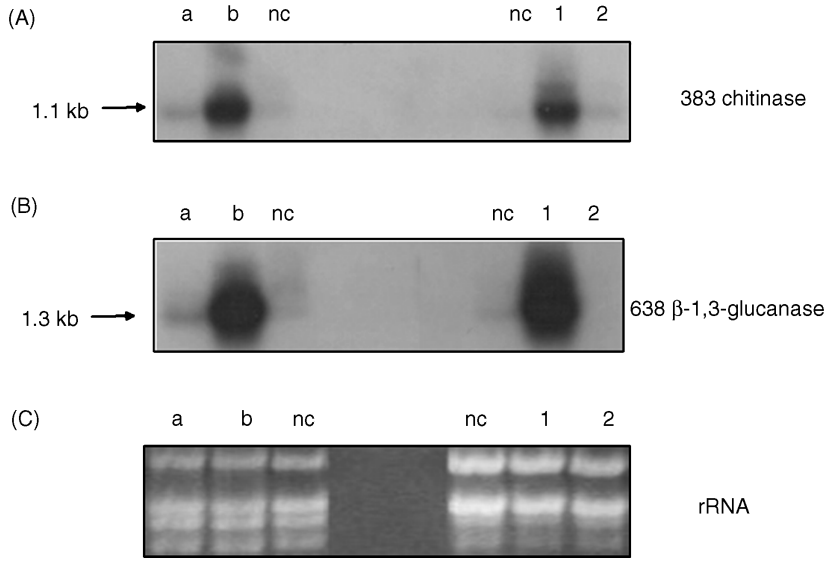

Figure 4 Northern blot analyses of the T_2 plants (left set of lanes) and T_3 plants (right set of lanes) of the different transgenic lines. Ten micrograms of RNA extracted from the leaves of similar physiological growth at 4 weeks was fractionated on a formaldehyde–agarose gel, blotted on to a nylon membrane and probed with ^{32}P-labelled 383 chitinase or 638 β-1,3 glucanase DNA. (A) Detection of the 383 chitinase transcripts in T_2 and T_3 plants. Lanes: a, progeny from 32C3 plant; b, progeny from 32A2 plant; nc, non-transgenic control; 1, 32A2#3 and 2, 32C3#4. (B) Detection of the 638 glucanase transcripts in T_2 and T_3 plants. Lane designations are as in (A). (C) Ethidium bromide stained gel showing that equal amounts of total RNA were loaded in each lane, in each set.

derived from each of the above lines were painted with Liberty and all were found to be resistant to the herbicide. They were further characterized by PCR analysis for the presence of the *bar* gene and PR-protein gene(s) using the transgene-specific primer combinations. These analyses confirmed that all progenies were homozygous for the transgene loci (Anand *et al*., 2003). Northern blotting (Figure 4) and Western blotting (Figure 6) were carried out on the homozygous plants from each line to determine the expression level of the transgenes. The progenies derived from line 32A2#3 had much higher levels of the transcripts and transgene-encoded proteins in the T_3 plants compared to line 32C3#4, which had levels similar or comparable to non-transgenic control plants. The line 32C3#4 was thus classified as a silenced line.

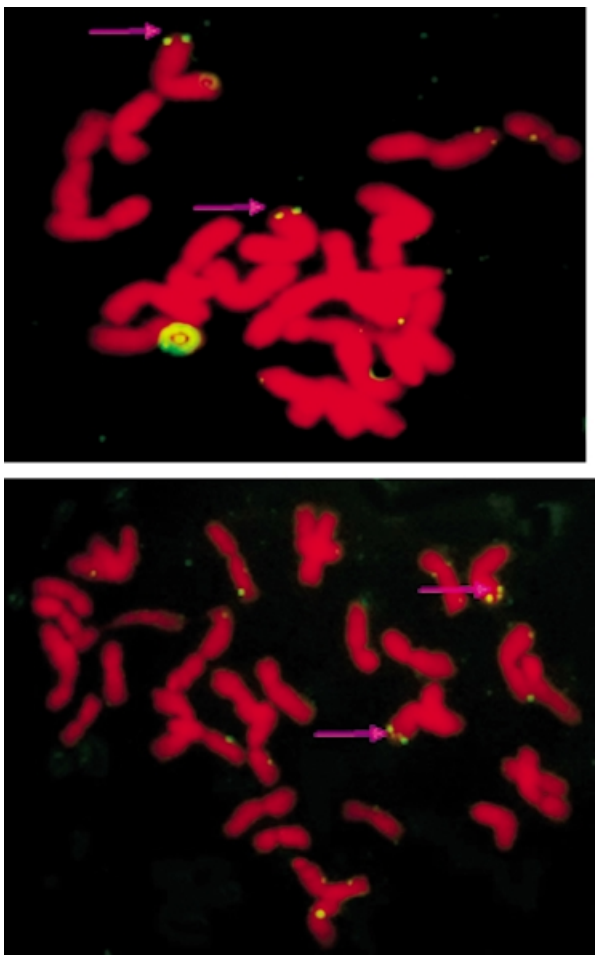

Figure 5 Detection of transgenes by FISH. The signals for the transgenes were seen in the telomeric region of a pair of chromosomes in homozygous T_3 plants of #32A2 and #32C3, when probed with the DIG-11-dUTP-labelled pAHCUbi383 plasmid DNA. The identical location of signals on homologous chromosomes served as further proof that these two lines came from the same transgenic events and are identical clones. Arrows indicate the transgene insertion sites.

Transgene silencing is correlated with the methylation of transgene regions

The genomic DNA from the homozygous T3 plants of lines 32A2#3 and 32C3#4 were digested with four different restriction enzymes (*Eco*RV, *Bam*HI, *Kpn*1 and *Eco*RI) and analysed by Southern blotting using radiolabelled DNA fragments from the *bar* gene, *Ubi1* promoter, 383 chitinase and 638 glucanase genes. Autoradiography revealed identical banding patterns in the DNA from both lines digested with *Eco*RV, *Bam*HI and *Kpn*1 (Figure 7). However, the banding patterns for the *Eco*RI-digested DNAs of these two lines were different. Line 32C3#4 had a strong 5.4 kb band which was

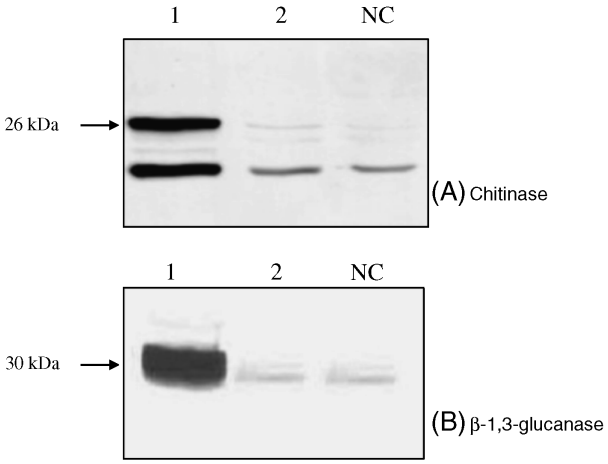

Figure 6 Western blot analyses for detecting the expression of the transgene-encoded proteins in the homozygous T_3 plants. Total leaf protein was extracted at 12 000 ***g*** and fractionated by SDS-PAGE, transferred to a nitrocellulose membrane, and probed with polyclonal antisera for (A) chitinase and (B) β-1,3-glucanase. Lanes: 1, 32A2#3; 2, 32C3#4 and NC,-non-transformed 'Bobwhite' negative control. Arrows indicate the expected bands.

completely missing in line 32A2#3. Instead, line 32A2#3 showed the presence of multiple bands in the 1.5–1.8 kb size range (representing the *Ubichi/Ubiglu* and *CaMVUbi* fragments, see Figure 1) along with a distinct band of 4.2 kb when probed with the *Ubi1* promoter fragment. Based upon a comparison with the expected bands from the *Eco*RI sites in the transforming plasmids (see Figure 1), we predict that in both lines several *Eco*RI sites in the transgenes were subjected to methylation.

The *Eco*RI sites in the transgene locus in 32C3#4 DNA were heavily methylated when compared with DNA from the 32A2#3 line. Based on the plasmid map, we deduced that the 5.4 kb band detected by the *Ubi1* probe in the silenced line 32C3#4 resulted from methylation of the *Eco*RI site at the 3′-end of the NOS terminator and included the DNA sequences from the vector backbone (see Figure 1). The banding patterns of the *Eco*RI digests of these two DNAs were also different when hybridized with the *bar* probe. The expected 1.3 kb band was fainter in line 32C3#4, compared with line 32A2#3. On the other hand, a larger 5.4 kb band was detected with the *bar* probe in 32C3#4, which was absent in 32A2#3 DNA. Line 32C3#4 lacked the 2.8 kb band that was detected in 32A2#3 plants, providing further evidence for additional methylation sites in the transgenes in this line (Figure 7). Following hybridization with the coding region fragment of chitinase DNA in line 32C3#4, the expected 1.3 kb *Eco*RI fragment was mostly replaced by bands in the region of 3.4 kb and 5.4 kb that were absent in the expressing line, 32A2#3 (data not shown). These observations with Southern blots of the *Eco*RI digested DNA clearly indicated the difference in the extent of methylation of transgenes between these two lines, even though they were apparently genetically identical, as revealed by the identical banding patterns for several other restriction enzyme digestions (*Bam*HI, *EcoR*V, *Kpn*1 and *Hin*dIII; see Anand *et al*., 2003 for the *Hin*dIII digestion pattern).

Further evidence for methylation of the ubiquitin promoter-intron in line 32C3#4 was obtained by digesting the genomic DNA with a pair of enzymes (*Hpa*II and *Msp*1), both of which have the common recognition sequence CCGG, but differ in their ability to digest methylated recognition sites. *Msp*1 digestion of DNA from both lines yielded identical banding patterns, with the *Ubi1* promoter probe resulting in bands of 0.9 kb, 0.75 kb and 0.25 kb, as predicted from the sequence data for the ubiquitin promoter-intron (Christensen *et al*., 1992; Figure 8). With the 383 chitinase and 638 glucanase, a strong smear in the region of 20 kb was detected, indicating the highly methylated state of these genes (data not shown). The *Hpa*II digestion pattern in the 32A2#3 and

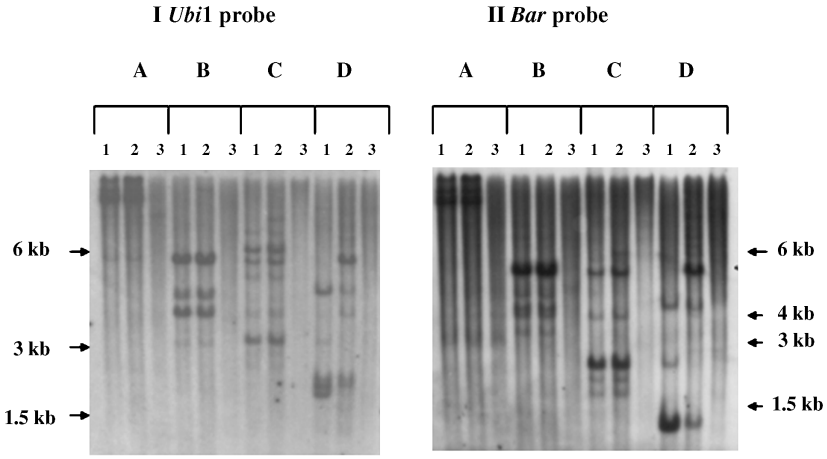

Figure 7 Southern blot analyses of genomic DNA of the homozygous T_3 plants. Genomic DNA was digested with (A) *EcoR*V, (B) *Bam*HI, (C) *Kpn*1, or (D) *Eco*RI. The *Ubi1* probe was used for hybridization in blot I, and the *bar* gene probe was used for hybridization in blot II. Following autoradiography, blot I was stripped and rehybridized with the probe for the *bar* gene. Lanes: 1, 32A2#3 (expressing line); 2, 32C3#4 (silenced line) and 3, DNA from untransformed plant. Arrows indicate the migration positions of DNA size markers.

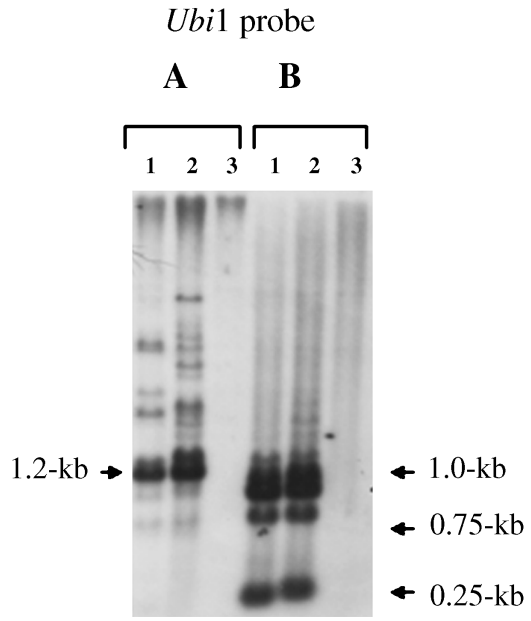

Figure 8 Methylation status of CCGG sites in the ubiquitin promoter sequences. Genomic DNA (12 μg) from lines 32A2#3 and 32C3#4 were digested with 80 units of methylation-sensitive enzymes (A) *Hpa*II or (B) *Msp*I. Southern blotting was carried out with a ^{32}P-labelled 2.0 kb fragment corresponding to the *Ubi1* promoter followed by autoradiography. Lanes: 1, 32A2#3; 2, 32C3#4 and NC, DNA from untransformed plant. Arrows indicate the expected bands in the *Msp*1 digest based on the distribution of CCGG sequences in the ubiquitin promoter.

32C3#4 lines with the *Ubi1* promoter probe resulted in a nearly complete elimination of all three major bands seen in the *Msp*1 digest (a faint 0.75 kb band was barely visible), showing all CCGG sites in the *Ubi* promoter region were completely methylated in both lines. A major 1.2 kb band was detected, also suggesting the methylation of the CCGG sequences in this region (Figure 8). The *Hpa*II digests of DNA from the silenced line 32C3#4 had several additional larger bands when compared with the DNA from expressing line 32A2#3, suggesting the additional methylation of several CCGG sites in the *Ubi* promoter regions of line 32C3#4 (Figure 8).

Transgene activity is not reversed in the presence of azaC

Germinating seeds of line 32C3#4 and a few other silenced wheat transgenic plants (data not presented) in presence of 100 mg/l 5-azacytidine did not lead to a reversion of the silenced state of the transgenes, as revealed by RT-PCR and Western blot analysis. DNA was extracted from untreated and azaC-treated 32C3#4 plants and digested with the methylation-sensitive enzymes *Hpa*II and *Msp*I. There was no evidence of demethylation of CCGG sequences in the *Ubi*1 promoter sequences, since the *Hpa*II digestion pattern of DNA from azaC-treated plants did not contain smaller bands as compared to untreated plants (data not shown). It therefore appears that azaC treatment did not reverse the methylation of the promoter sequences and the associated transgene silencing. Western blot analyses also confirmed that there was no reversal of transgene silencing in these plants (data not shown).

Discussion

The use of particle bombardment as an effective means for the introduction of genes controlling important traits into wheat has revolutionized wheat research over the last decade. In recent years, this approach has emerged as the most reproducible and viable means for the introduction of agronomically important genes for quality improvement (Altpeter *et al*., 1996b; Blechl and Anderson, 1996; Drakakaki *et al*., 2000), drought stress (Sivamani *et al*., 2000), and disease resistance (Bieri *et al*., 2000; Bliffeld *et al*., 1999; Chen *et al*., 1999; Clausen *et al*., 2000; Oldach *et al*., 2001; Schweizer *et al*., 1999; Stoger *et al*., 1999; Zhang *et al*., 2001). However, gene transfer technology is limited by the low frequency of generation of transgenic plants with high level expression of the transgenes. The problem is exacerbated by reports of transgene silencing in wheat over several generations (Alvarez *et al*., 2000; Demeke *et al*., 1999; Muller *et al*., 1996).

Transgenic wheat expressing defence genes has been described by several groups (Bieri *et al*., 2000; Bliffeld *et al*., 1999; Chen *et al*., 1999; Oldach *et al*., 2001; Schweizer *et al*., 1999) but the stability of transgene expression in these reports has not been extensively investigated. Only one report by Chen *et al*. (1999) has dealt with silencing of PR-protein genes whose expression was followed for several generations. These authors reported that transgene silencing was a function of the promoter used to drive its expression. All transgenic wheat plants with the rice chitinase gene (*chi11*) or the *hpt* gene under the control of cauliflower mosaic virus (CaMV-35S) promoter were completely silenced in T_2 generation. A single transgenic wheat plant expressing the *bar* gene and a rice thaumatin-like protein (*tlp*) gene under the control of maize ubiquitin promoter-intron (*Ubi1*) showed stable expression and inheritance of both transgenes for up to four generations. These results prompted us to utilize the *Ubi1* promoter-intron to drive the expression of all transgenes, including the selectable marker. In the present investigation, we found that 20 of the 24 primary transgenic

lines utilizing the *Ubi1* promoter were completely silenced for the expression of all the transgenes in either the T_1 or T_2 generation plants. Even though all 24 primary transgenic plants showed expression for the transgenes at maturity, as evidenced from the Liberty-painting assay and Western blot analyses, T_1 progeny from 18 of these plants had no detectable expression of PR-protein genes and were susceptible to Liberty. Presumably gene silencing either occurred during seed formation in T_0 plants or at the early stages of development in the T_1 generation plants. In the next generation, two more lines exhibited the same phenomenon of transgene silencing. Our experiments indicate that the *Ubi1* promoter is not immune to gene silencing in transgenic wheat. Therefore, this report contradicts our earlier finding that the transgenes under control of the CaMV-35S promoter were silenced in T_1 or subsequent generations, whereas maize ubiquitin promoter-intron driven genes were stably expressed in the T_2 and later generations (Chen *et al*., 1998, 1999). This apparent contradiction may be attributed to the fact that our conclusion was based upon the behaviour of two ubiquitin promoter-intron driven genes in a single transgenic plant, which fortuitously stably expressed the transgenes for several generations. The present study, which has involved a larger number of transgenic plants, clearly indicates that the *Ubi1* promoter is also susceptible to gene silencing in transgenic wheat plants although at a lower frequency than the CaMV-35S promoter. Chen *et al*. (1998) reported the silencing of transgenes in a vast majority of the 18 transgenic wheat plants with CaMV-35S promoter which they studied in the T_1 generation and a complete loss of transgene-expression by the T_2 generation, demonstrating 100% frequency of gene silencing. The fact that four of the 24 lines reported in this study had stable transgene expression up to the T_4 generation offers hope that some integration events may result in the stable expression of the trangenes. The reason why these four plants escaped silencing is not obvious and may be due to a 'position effect' or a 'transgene copy number' effect. Alternatively, it may reflect the random nature of transgene silencing.

The high frequency of gene silencing might have involved homology-dependent silencing (Meyer and Saedler, 1996), even though we were able to detect the expression of endogenous PR-protein genes upon infection in the silenced transgenic plants bioassayed against *Fusarium* head blight under greenhouse conditions (data not shown). Gene silencing could also have been due to the presence of vector backbone elements in high-copy-number integration events that might have led to homologous recombination. A majority of the transgenic plants that we have studied had transgene copy-numbers varying anywhere between 3 and 15 (data not presented). Southern blot analysis of DNAs digested with *Hin*dIII from almost all the transgenic plants detected a strong band at 5.7 kb (the expected size for the fragment containing the *bar* gene) or a band in the region of 3.2 kb (expected for the genes of interest), consistent with tandem insertion of the transformation plasmid(s) as well as bands of other sizes (representing end fragments or rearranged fragments). It is also possible that in some tandem insertions, genomic DNA could be interspersed between transgenic sequences (Jackson *et al*., 2001; Kohli *et al*., 1998; Salomon and Puchta, 1998). The simultaneous integration of a large number of copies of the transgene in a damaged chromosome being repaired by an active DNA repair complex could lead to the interspersed insertion of the transgene that might result in the inactivation of the transgenes. Kohli *et al*. (1998) demonstrated that even a single copy of the integrated transgene could undergo recombination with endogenous plant DNA, inactivating the transgene. The percentage of transgenic wheat plants that underwent transgene silencing (Chen *et al*., 1998; this study) was much higher than the levels reported in other cereals such as rice or sorghum (Chareonpornwattana *et al*., 1999; Krishnaveni *et al*., 2001; Kumpatla *et al*., 1997). This may be attributed to the larger genome size of wheat and/or its polyploid nature. The explanation that the high percentage of transgene silencing reported in previous studies of transgenic wheat was due to the use of a viral promoter or to the absence of an intron, is ruled out by the present investigation wherein the maize ubiquitin-promoter-exon1-intron1 was used to drive the expression of the transgenes.

DNA methylation has been implicated in establishing and maintaining the inactive state of the gene by rendering the chromatin structure inaccessible to the transcriptional machinery (Razin, 1998), either by prevention of the binding of transcriptional factors to the regulatory regions or by the recognition of the methylated residues by specific repressor molecules (Kass *et al*., 1997). This mechanism suggests that methylation results in reduced gene expression. Our data on the behaviour of genetically identical lines arising from the same transgenic event supports this model. The digestion of total genomic DNA from these lines with *Hpa*II/*Msp*I enzymes, followed by hybridization with the DNA probe corresponding to the *Ubi1* promoter fragment, provided evidence for additional methylation of this promoter in the silenced line. The detection of larger size bands in the silenced line (32C3#4), when compared with the expressing line (32A2#3) demonstrated a more extensive methylation of CCGG sites in the promoter region of the transgenes in the

silenced line (Figure 7). Measurements of RNA by RT-PCR indicate that gene silencing is most likely at the transcriptional level.

The transcriptional silencing associated with the enhanced methylation of transgene sequences could not be reversed by the application of azaC, as was reported in rice (Kumpatla and Hall, 1998a,b; Kumpatla *et al*., 1997) and wheat (Muller *et al*., 1996). The treatment of a few silenced lines including 32C3#4 (data for other silenced lines is not shown) with azaC did not reverse the methylation of the CCGG sites of ubiquitin promoter. Furthermore, the transgene silencing appears to be irreversible because the azaC-treated plants did not show any detectable transgene expression (data not shown). Co-suppression of high molecular weight glutenin genes has been reported in transgenic wheat (Alvarez *et al*., 2000). However, we did not observe the phenomenon of co-suppression of the endogenous chitinase and glucanase genes when silenced plants were inoculated with wheat head scab pathogen (data not presented).

In conclusion, gene silencing in wheat appears to be a random phenomenon that is progressive. It does not appear to be restricted to viral promoters, even though the ubiquitin promoter is somewhat less prone to silencing. It is not clear what features of the four lines with stable expression of transgenes allowed them to escape silencing. There is a likely possibility that the biolistic transformation results in numerous chromosomal rearrangements at the integration sites, leading to transgene inactivation. This problem can be expected to be exacerbated in plants with several copies of the transforming plasmid with large vector sequences. The recurrent onset of silencing associated with mutli-copy transgene insertion has already been reported in rice (Kumpatla and Hall, 1998a,b). Some of these complications could be avoided by the development of an efficient transformation protocol using *Agrobacterium* transformation or by optimizing biolistic protocols to obtain a single 'clean' transgene copy or by the use of minimal transformation cassettes with no vector sequences (Fu *et al*., 2000). Alternatively, the application of the *cre-lox* system for generating single-copy transgenic wheat plants from primary transgenics with complex integration events may be utilized to obtain transgenic plants with stable transgene expression (Srivastava *et al*., 1999).

Experimental procedures

Plasmids

The plasmid constructs, pAHCUbi194 and pAHCUbi383 (encoding wheat chitinases) and pAHCUbi289 and pAHCUbi638 (encoding wheat β-1,3-glucanases) described by Anand *et al*. (2003), were used to transform the wheat calli. The clones encoding the chitinases and β-1,3-glucanases used in this study were isolated from a cDNA library constructed from scab-infected Sumai-3 wheat (Li *et al*., 2001). These cDNAs were ligated to a 2.0 kb fragment containing the maize ubiquitin promoter-intron and another 0.25 kb fragment containing the cauliflower mosaic virus transcription terminator (CaMV polyA) fragment in pBluescript vector. The *Hin*dIII fragments carrying the promoter-cDNA-CaMV polyA cassette were recovered and inserted into the unique *Hin*dIII site of the plasmid, pAHC20 (Christensen *et al*., 1992). The plasmid pAHC20 contains the *bar* gene under control of the maize ubiquitin promoter-intron. The *bar* gene confers resistance to the herbicide, glufosinate (Liberty®, Aventis, Research Triangle Park, NC). The pAHCUbi383 plasmid contains the opening reading frame (ORF) from the wheat cDNA clone 383 encoding a class IV chitinase, while the pAHCUbi638 plasmid contains the ORF for the wheat cDNA clone 638, which encodes an acidic glucanase (Figure 1A, B; Li *et al*., 2001). Transgenic wheat plants arising from a biolistic transformation with these plasmids in combinations are described here. One line derived from a particular transformation event (#32) with a combination of 383 chitinase and 638 glucanase transgenes, was analysed extensively in this paper.

Plant materials and transformation

Wheat plants (*Triticum aestivum* L. cv 'Bobwhite') were grown in growth chambers with a 16 h:8 h photoperiod at a light intensity of 800 μE/m^2/s and a temperature regime of 20 °C:18 °C (light:dark). Approximately 14 days post-anthesis, spikes were collected and immature caryopses were sterilized in a solution containing 20% commercial bleach and 0.1% Tween-20 for 20 min and then rinsed three times with sterile, distilled water. Immature embryos were excised and placed on CM4 medium (Zhou *et al*., 1995), with the embryo-side in contact with the medium, for 5–7 days in the dark at 25 °C to initiate callus formation. Induced embryogenic calli were transferred to CM4 + osmoticum (0.2 M mannitol and 0.2 M sorbitol) 4–16 h prior to genetic transformation. Genes were introduced into embryogenic calli with the particle inflow gun (Finer *et al*., 1992).

Methods for the selection and recovery of transgenic plants were similar to those of Altpeter *et al*. (1996a), with slight modifications. Sixteen hours after particle bombardment, wheat calli were placed on CM4 medium containing 5 mg/l glufosinate and cultured in the dark at 25 °C for

8–10 days. The cultures were then transferred to shoot production medium (MSP), containing MS (Murashige and Skoog, 1962) basal salt mixture, B5 vitamins (Gamborg *et al*., 1968), 0.2 mg/L 2,4-D, 40 g/L maltose, 100 mg/L ascorbic acid, 10 mM MES, 5 mg/L glufosinate, and 2 g/L Gelrite, pH 5.7) and cultured in a 16 h:8 h (light:dark) cycle at 25 °C for 2 weeks. Following induction of green shoots, the cultures were transferred to elongation and rooting medium (MSE) containing 5 mg/L glufosinate and cultured for 2–3 weeks in the light (MSE medium is the same as MSP except for the omission of 2,4-D). The plantlets were transferred to soil and grown in environmentally controlled growth rooms (16 h:8 h light:dark and light intensity of 800 μE/m^2/s).

Leaf painting assay

To examine the expression of the *bar* gene in the transgenic plants, a freshly prepared aqueous solution of the herbicide, Liberty (0.2% v/v) was applied on the mid-lamina portion (about 2.5 cm long) of the second/third youngest leaf using a cotton plug. The painted area was marked with a marker pen and visual observations of damage were recorded for 3–4 days after painting.

Polymerase chain reaction (PCR)

For PCR, DNA was isolated from 20–30 mg of fresh leaves according to the methods of Riede and Anderson (1996). Detection of the *bar* gene and genes of interest by PCR was performed in 0.2 mL microcentrifuge tubes in a 50 μL reaction mixture containing 200 ng of DNA as template, 0.4 mM dNTP, and 0.2 μM of each oligonucleotide primer. PCR amplification was carried out using BIOLASE™ DNA polymerase and reaction buffer (Bioline, Springfield, NJ), following the manufacturer's instructions. The following primer combinations were used: Bar F, 5′-CCTGCCTTCATACGCTATTTATTTGCC-3′ (forward primer) and Bar R, 5′-CTTCAGCAGGTGGGTGTAGAGCGTG-3′ (reverse primer); and Ubi A, 5′-GCCCTGCCTTCATACGCTAT-3′ (forward primer in the intron of ubiquitin promoter) and PolyA-R, 5′-GGAATTCAAGCTTCATCGAGCTCGGTA-3′ (reverse primer in the CaMV polyA). DNA amplifications were performed in a thermal cycler using initial denaturation at 94 °C for 5 min, followed by 30 cycles of 1.0 min at 94 °C, 1.5 min at 62.1 °C (for *bar* gene)/62.4 °C (for chitinase and glucanase genes) and 2.0 min at 72 °C. One additional extension cycle was performed for 10 min at 72 °C. The amplification products for the *bar* gene were detected in a 0.8% w/v agarose gel, while the amplification products for the chitinase and glucanase transgenes were resolved on 1.4% w/v agarose gels and visualized by ethidium bromide staining.

Restriction enzyme digestion and Southern blot analysis of genomic DNA

The methods used for digestion of genomic DNA with different restriction enzymes and Southern blot analysis have been described previously (Chen *et al*., 1998). DNA methylation analysis was performed by digesting 12 μg of the genomic DNA with the methylation-sensitive restriction enzyme, *Hpa*II, and *Msp*I (isoschizomer which recognizes CCGG and its methylated forms) followed by hybridization to a ^{32}P-radiolabelled coding fragment of *Ubi1* promoter, 383 chitinase, 638 glucanase and *bar* gene. To confirm the genetic identity of the two lines derived from the same callus, similar amounts of genomic DNA were also digested with four different enzymes, *Bam*HI, *Eco*RV, *Kpn*1 and *Eco*RI, and Southern blots were probed with ^{32}P-labelled coding region fragments of the *bar*, *Ubi1* promoter, 383 chitinase and 638 glucanase genes.

Northern blot analysis

For the Northern blot hybridization, total RNA was extracted from 150 mg leaves with Trizol® reagent (Gibco BRL, Grand Island, NY) according to the manufacturer's instructions. Aliquots of RNA (10 μg) were analysed by formaldehyde–agarose gel electrophoresis and blotted on to Hybond-N^+ nylon membranes (Amersham, Arlington Heights, IL). Membranes were probed with ^{32}P-labelled coding region fragments of the wheat chitinase or glucanase genes. The other steps for Northern hybridization were similar to those for Southern hybridization (Chen *et al*., 1998).

Reverse-transcriptase (RT-)PCR

RT-PCR reactions were performed with 0.5 μg of total RNA using the Advantage One-Step RT-PCR kit (Clontech, Palo Alto, CA) as per the manufacturer's instructions. In order to distinguish the transgene-specific transcripts, a forward primer in the first exon of the ubiquitin promoter, 5′-CGTGTTGTTCGCAGCGCACAC-3′, and a reverse primer in the CaMV polyA fragment (see Figure 1), 5′-GCTCAACACATGAGCGAAACCC-3′, were used in the RT-PCR reaction. The RT-reaction was performed in a thermal cycler at 50 °C for 1 h, followed by PCR amplification of 15–25 cycles at 94 °C for 0.5 min, 64.7 °C for 0.5 min and 1 min at 72 °C. A final extension for the PCR products was performed at 72 °C for 10 min. The transgene products were resolved on a 1.6% w/v agarose gel.

Protein analyses

Plant materials and methods of protein extraction, SDS-polyacrylamide gel electrophoresis and Western blot analyses were all carried out as described by Chen *et al*. (1998). For the Western blot hybridization, polyclonal antibodies raised in rabbits against a barley chitinase (Swegle *et al*., 1992) and a barley β-1,3-glucanase (a gift from Dr Murray Ballance, University of Manitoba, Winnipeg), respectively, were used. The antibodies were used at 1 : 1000 (v/v) dilutions.

Fluorescent *in situ* hybridization (FISH)

For FISH analysis, chromosome preparations were made by squashing root-tip meristems in a drop of 45% acetic acid on a glass slide. The slides were treated as outlined by Pederson *et al*. (1997) and denatured according to Heslop-Harrison *et al*. (1991). Plasmids pAHCUbi383 and pAHCUbi638 were labelled by nick-translation in the presence of DIG-11-dUTP (Jackson *et al*., 2001). Approximately 12.5 ng of the digoxigenin-labelled probe in hybridization solution (50% formamide, 10% dextran sulphate, 0.1% SDS and 2× SSC) was used per slide. This solution was applied to each slide and incubated overnight at 37 °C. After washing to a stringency of 85%, the slides were blocked with 5% IgG-free BSA (Jackson Immuno Research Laboratories, West Grove, PA). Hybridization signals were detected using fluorescein isothiocyanate (FITC)-conjugated sheep-antidigoxigenin antibody. The fluorescence signal was enhanced with FITC-conjugated rabbit-antisheep antibody. The method of washing slides was as according to Leitch *et al*. (1991).

5-Azacytidine (azaC) treatment

Seeds from the silenced homozygous T3 plants were germinated in the presence of 100 mg/L azaC or in its absence (controls). The azaC-treated seeds and controls were germinated in the dark until the seedlings were about 10–15 cm in length and subsequently transferred to light. Once the seedlings turned green, they were moved into one gallon (≈ 4.5 L) pots and grown to maturity in growth chambers (16 h:8 h light/dark, 20 °C:18 °C day/night temperature, 600 $\mu E/m^2/s$). Leaves were harvested 4–5 weeks after transplantation for protein, RNA and DNA analyses.

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