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Development of wheat scab symptoms is delayed in transgenic wheat plants that constitutively express a rice thaumatin-like protein gene

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Abstract The possibility of controlling wheat scab (caused by *Fusarium graminearum* Schw.) was explored by engineering wheat plants for constitutive expression of pathogenesis-related (PR) protein genes. A rice thaumatin-like protein (TLP) gene (*tlp*) and a rice chitinase gene (*chi11*) were introduced into the spring wheat cultivar 'Bobwhite' by co-transformation of the plasmids pGL2ubi-tlp (ubiquitin/*tlp*//CaMV 35S/*hpt*) and pAHG11 (CaMV 35S/*chi11*//ubiquitin/*bar*). The transformation was by biolistic bombardment. Bialaphos was used as the selection reagent. The integration and expression of the *tlp*, *bar*, *chi11* and *hpt* genes were analyzed by Southern, Northern and Western blot analyses. The four transgenes co-segregated in the T_1 progeny of the transgenic plant and were localized at the telomeric region of the chromosome 6A long arm by sequential N-banding and fluorescent *in situ* hybridization (FISH) using pAHG11 or pGL2ubi-tlp as the probes. Only the transgenes *tlp* and *bar*, under the control of the ubiquitin promoter-intron, were expressed. No expression of the *chi11* and *hpt* genes, controlled by the CaMV 35S promoter, was detected in T_1 plants. After inoculation with conidia of *F. graminearum*, the symptoms of scab developed significantly slower in transgenic plants of the T_1, T_2 and T_3 generations expressing the *tlp* gene than in non-transformed control plants. This is the first report of enhanced resistance to *F. graminearum* in transgenic wheat plants with constitutive expression of TLP.

Key words *Triticum aestivum* · Genetic transformation · Thaumatin-like protein · Wheat scab · Fluorescent *in situ* hybridization

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Introduction

Scab or *Fusarium* head blight (FHB), caused mainly by *Fusarium graminearum* Schw., is a destructive disease of wheat, barley and other small grains in warm and humid wheat growing areas of the world, especially in China, Japan and increasingly in Southeast Asia and North and South America. In serious epidemic years in China, scab incidence may be 50–100%, and yield is reduced by 10–40%. The harvested grain is contaminated by fungal-secreted mycotoxins that reduce the usefulness of the grain for food or feed consumption. In a recent review, McMullen et al. (1997) documented past and recent epidemics of scab on the world's cereal crops. In the 1993 epidemics, the wheat and barley producers in the tri-state area of Minnesota, North Dakota and South Dakota and the adjacent province of Manitoba in Canada suffered one billion dollars in loss due to reduced yield and quality. This is the greatest loss due to any plant disease in a single year in North America. Local epidemics in these and many other states, especially Illinois, Indiana, Michigan and Ohio, have occurred since then (McMullen et al. 1997).

Control of wheat scab by fungicide application and other practices is neither practical nor sustainable. Christensen et al. (1929) stated that "the only effective method of controlling wheat scab is to grow resistant varieties", and this is still true today. The identification of sources of resistance to scab in wheat and related species and their use in breeding is a top priority in many wheat improvement programs. Here we explore an alternative strategy for scab disease control in wheat through the deployment of pathogenesis-related (PR) protein genes.

The PR-proteins (reviewed by Linthorst 1991; Cutt and Klessig 1992) were divided into 11 subgroups (PR-1 to PR-11) based on serological relations, similarities in molecular masses and amino acid sequence data (Van Loon et al. 1994). PR-proteins have anti-fungal activity in vitro. The genes belonging to 3 PR-protein groups, PR-2 (β-1,3-glucanases), PR-3 (chitinases) and PR-5 (thaumatin-like proteins, TLPs), have been used successfully to enhance plant resistance to fungal pathogens (review: Yun et al. 1997). A combination of PR-proteins is more effective than a single PR-protein (Mauch et al. 1988; Jongedijk et al. 1995).

We recently cloned two TLP cDNAs from a rice cDNA library (Velazhahan et al. 1998). One of these genes, controlled by the CaMV 35S promoter, was transferred into rice. The transgenic rice plants with constitutive expression of TLP had enhanced disease resistance to the pathogen *Rhizoctonia solani* (Datta et al. 1998). Similarly, a rice chitinase gene (Huang et al. 1991), controlled by the CaMV 35S promoter, when introduced into rice plants resulted in over-expression of a class I rice chitinase and enhanced resistance of the transgenic plants to rice sheath blight (Lin et al. 1995). In this study reported here, we introduced the rice TLP gene (*tlp*) and the rice chitinase gene (*chi11*) into wheat (*Triticum aestivum* L.) in an attempt to enhance resistance to fungal pathogens. The integration and expression of the transgenes and the resistance of the transgenic plants to scab were studied. We also report the physical mapping of the transgenes to a specific region of a wheat chromosome by fluorescent *in situ* hybridization (FISH).

Materials and methods

Plant materials and methods of particle bombardment, protein extraction, phosphinothricin acetyltransferase (PAT) assay, SDS-polyacrylamide gel electrophoresis and Western and Southern blot analyses were as described in Chen et al. (1998a). The plasmids pGL2ubi-tlp and pAHG11 were used to transform wheat plants. The plasmid pGL2ubi-tlp contains a rice TLP gene (*tlp*) and the hygromycin phosphotransferase gene (*hpt*). The promoters are ubiquitin-intron (ubi), driving *tlp*, and CaMV 35S, driving *hpt* expression (Fig. 1). The plasmid pAHG11 was described in Chen et al. (1998a) and contains the *chi11* and *bar* genes, driven by CaMV 35S and ubiquitin promoters, respectively.

For Western blot hybridization, a polyclonal anti-TLP-antibody (primary antibody) raised in rabbits against a Pinto bean TLP was induced (Sehgal et al. 1991). The antibody was diluted to 1:1000 (V/V). For Northern blot hybridization, total RNA was extracted from ten florets of control or scab-inoculated plants with TRIZOL® reagent (GIBCO BRL, Grand Island, N.Y.). Aliquots of RNA were analyzed by agarose-formaldehyde gel electrophoresis and the transferred to Hybond-N+ nylon membrane (Amersham, Arlington Heights, Ill.) with 7.5 m*M* NaOH solution (Sambrook et al. 1989). The probe was the coding region of the rice TLP gene. The other procedures for Northern hybridization were similar to those for Southern hybridization (Chen et al. 1998a).

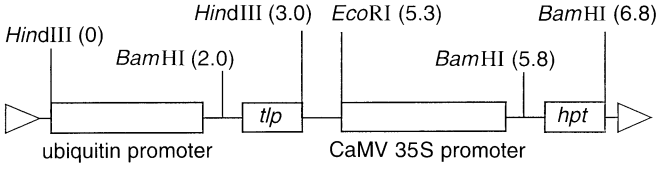

Fig. 1 Schematic representation of pGL2ubi-tlp (7.5 kb). *Numerical values after* restriction sites indicate approximate sizes in kilobases

The procedure for N-banding was similar to that of Gill et al. (1991), except the incubation temperature in the phosphate buffer was 92°C and the slides were not dehydrated in ethanol.

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 Pedersen et al. (1997) and denatured according to Heslop-Harrison et al (1991). Plasmids pAHG11 and pGL2ubi-tlp were labeled by nick-translation in the presence of DIG-11-dUTP. Approximately 12.5 ng of the digoxigenin-labeled probe in hybridization solution (50% formamide, 10% dextran sulphate, 0.1% SDS and 2X SSC) was used per slide. This solution was applied to each slide and incubated at 37°C overnight. After washing to a stringency of 85%, the slides were blocked with 5% IgG-free BSA (Jackson ImmunoResearch 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-anti-sheep antibody. The method of washing slides was according to Leitch et al. (1991).

Resistance to scab was evaluated by a 'single floret injection inoculation method' (Xu and Chen 1993). The first floret of a middle spikelet was injected with 10 µl of a conidial suspension at a density of $5–10\times10^4$ conidia per milliliter. The inoculated plants were grown in a closed inoculation chamber in a greenhouse and sprayed with tap water for 15 s every 10 min for 3 days. The plants were then placed in a greenhouse (26°–30°C, T_1 and T_2 plants) or growth chamber (25°–26°C, T_3 plants). The infected spikelets per inoculated spike were counted on the 7th day after inoculation for greenhouse-grown plants or the 10th day for plants grown in the growth chamber.

Results

DNA hybridization and expression of transgenes

The regenerated plants, obtained from immature embryos co-bombarded with plasmids pAHG11 and pGL2ubi-tlp, were sprayed with 0.2% Liberty in the greenhouse. Only 1 putative transgenic plant (no. 14) survived spraying with this herbicide (Chen et al. 1998b). Seeds from this selfed plant were germinated to obtain T_1 progeny. Southern blot analysis of DNA from a T_1 plant using four probes consisting of coding regions of the *tlp*, *bar*, *chi11* and *hpt* genes indicated that all of the four transgenes were present and integrated into the genome of the transgenic plants (Fig. 2a). However, the expression of the four genes differed when determined by Northern blot analysis (Fig 2b). The presence of mRNA for the *tlp* and *bar* genes, whose expression was driven by the maize ubiquitin promoter, was easily detected in the transgenic plants, but transcripts of the *chi11* and *hpt* genes, driven by the CaMV 35S promoter, were not detected. Western blot and PAT assays of extracts of several Liberty-resistant T_1 plants confirmed that the *tlp* and *bar* genes were expressed at the protein level (Fig. 3a, b). However, no expression of *chi11* was observed in Western blots using a chitinase antiserum (data not shown). These results are consistent with the results of Northern blot analysis.

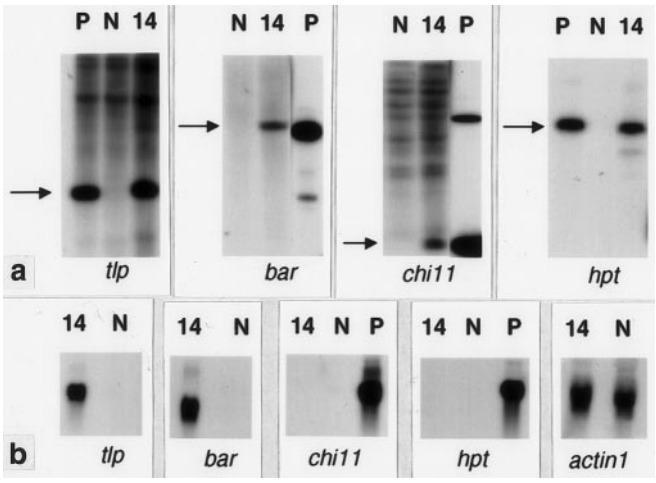

Fig. 2a A T_1 progeny of herbicide-resistant plant no. 14 analyzed by Southern blot hybridization using probes from the coding regions of the *tlp, bar, chi11 and hpt* genes. **b** A T_1 progeny of herbicide-resistant plant no. 14 analyzed by Northern blot hybridization using probes from the coding regions of the *tlp, bar, chi11 and hpt* genes. The RNA blot also was hybridized with a rice *actin 1* cDNA probe to indicate similar amounts of transgenic and control plant RNAs on the blot. The plant and plasmid DNA were digested with *Hin*dIII. *P*, positive control containing a mixture of plant and plasmid pGL2ubi-tlp DNA, or plasmid pAHG11 in Southern blot (*a*), or the RNA of transgenic rice harboring *chi11* and *hpt* (*b*); *N*, DNA or RNA from non-transformed 'Bobwhite' leaves; *14*, DNA or RNA from the transgenic plant leaves. The sizes of the DNA fragments indicated by *arrows* in positive controls are 3.0 kb (*tlp*), 5.8 kb (*bar*), 1.5 kb (*chi11*) and 4.5 kb (*hpt*) in panel a; the sizes of RNA in panel b are 1.2 kb (*tlp*), 0.8 kb (*bar*), 1.3 kb (*chi11*) and 1.2 kb (*hpt*)

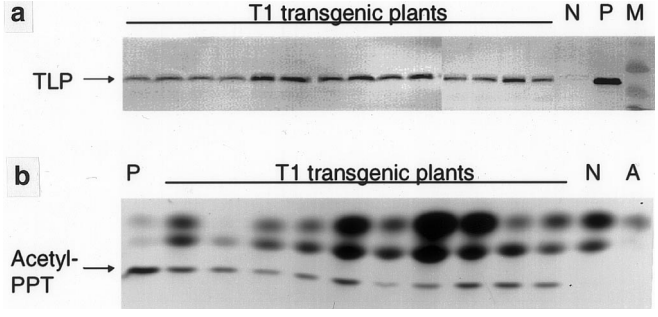

Fig. 3a, b TLP (**a**) and PAT (**b**) expression in Liberty-resistant T_1 transgenic plants by Western blot analysis (**a**) or by thin layer chromatography assay (**b**). *P*, Transgenic tobacco containing the rice *tlp* gene (**a**) or transgenic wheat callus containing the *bar* gene (**b**); *N*, non-transformed 'Bobwhite' extract; *M*, protein molecular-weight marker (the upper band is 30 kDa, and the lower band is 21.5 kDa; *A*, acetyl coenzyme A

To study the segregation of the four transgenes, we analyzed 34 T_1 plants with resistance to 0.2% Liberty and two randomly selected sensitive plants by Southern blotting using the *tlp*, *bar*, *chi11* and *hpt* coding-region fragments as the probes. The results indicated that all of the herbicide-resistant plants contained all four transgenes, while the two sensitive plants lacked all the transgenes. No segregation of the four transgenes was observed among the T_1 progeny (Fig. 4), suggesting that they are linked and presumably integrated at one site. Fluorescent *in situ* hybridization (FISH) was used to obtain additional support for this possibility. The FISH results indicated that the pAHG11 locus, containing the *chi11* and *bar* genes, is located near the telomeric region of the long arm of wheat chromosome 6A. The identity of the chromosome was determined by sequential N-banding and FISH probed with pAHG11 DNA according to Jiang and Gill (1993) (Fig. 5). No additional hybridization loci were detected, indicating that both *chi11* and *bar* transgenes probably were inserted at one locus. When pGL2ubi-tlp DNA was used as the FISH probe, the *tlp* and *hpt* transgenes also were detected at the same region of chromosome 6A (data not shown), indicating that both plasmids were integrated at or near the same locus.

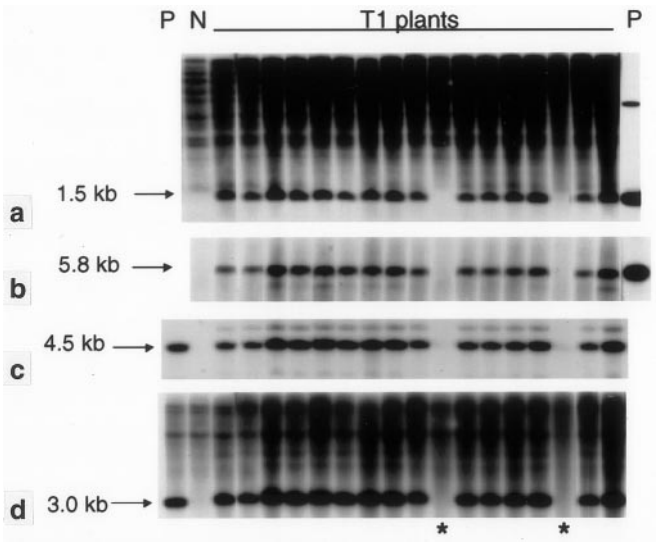

Fig. 4a–d Southern blot analysis of DNA from T_1-14 plants. The DNA blot was hybridized with *chi11* (**a**), *bar* (**b**), *hpt* (**c**) and *tlp* (**d**) coding regions. The T_1 plants were resistant to 0.2% Liberty except for the 2 plants marked with an ∗. Plant and plasmid DNA were digested with *Hin*dIII. *P*, A mixture of pGL2ubi-tlp (40 pg) and non-transformed 'Bobwhite' DNA (**c**, **d**) or pAHG11 (40 pg) (**a**, **b**). *N*, Non-transformed 'Bobwhite' DNA. *Arrows* indicate the sizes of the DNA fragments for the positive control. The results from 17 plants analyzed (15 resistant and 2 sensitive) are shown

Induction of TLP mRNA in scab-infected wheat plants and resistance of transgenic plants to scab disease

In order to study the expression of endogenous TLPs upon infection of scab, we inoculated the florets of non-transformed 'Bobwhite' with conidia of *F. graminearum*. The glumes of the inoculated floret became slightly brown on the second day after inoculation. The floret was dark-brown and became wilted by the third day after inoculation, and the amount of RNA recovered was less than in the uninfected control. Not enough RNA was obtained from ten inoculated florets 3 days after inocula-

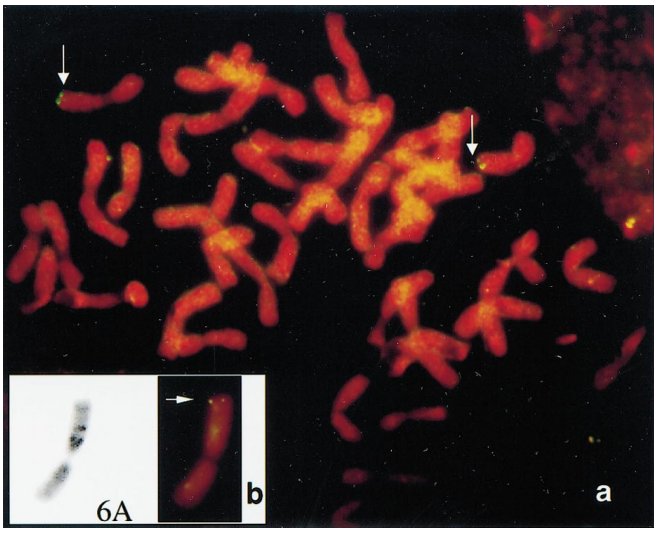

Fig. 5a, b The transgenes were detected and localized by FISH and chromosome N-banding. The signals of the transgenes were seen at the telomeric region of a pair of chromosomes in a homozygous T_3 plant (**a**). For identifying the chromosome carrying the transgenes, metaphase chromosomes were subjected to the N-banding procedure and photographed, and then analyzed by FISH (**b**). The chromosome carrying the transgenes was identified as 6A

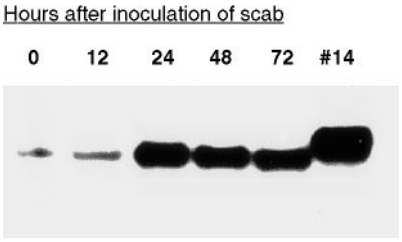

Fig. 6 Endogenous TLP mRNA was expressed in the florets of non-transformed 'Bobwhite' plants upon inoculation with scab. The RNA expression was analyzed by Northern hybridization at the indicated times. Number 14 is the transgenic plant without inoculation with scab. Total RNA per lane is 10 µg

Table 1 Resistance identification of transgenic plants to scab[a]

Materials	Infected spikelets per spike	Number of spikes	*t*-test 0.05
T1-14	2.3	30	A
Bobwhite	3.9	10	B
T2-14	2.2	16	A
Bobwhite	3.3	11	B
T3-14	2.2	42	A
Bobwhite	3.2	93	B

[a] *Fusarium graminearum* isolate is 3639; T_1 and T_2 plants were tested in greenhouse, and T_3 plants were tested in a growth chamber; the results were recorded on the 7th day (T_1 and T_2) or 10th day (T_3) after inoculation. Non-transformed 'Bobwhite' plants were used as controls. The experimental results were analyzed by *t*-test with SAS software

tion. The induction of endogenous *tlp* genes upon scab infection was detected by Northern blot hybridization (Fig. 6). A time-course study indicated that both the uninfected florets and the scab-inoculated florets 12 h after infection had nearly the same levels of TLP mRNA, presumably representing the base level of expression of the gene(s) in this tissue. Between 12 h and 24 h after inoculation of the latter, the TLP mRNA level increased rapidly and remained near this level at 48 h and 72 h. These data indicate that the expression of endogenous *tlp* genes in wheat plants increases rapidly after scab infection. The level of the mRNA in uninfected florets of transgenic plant no. 14 was more than that in the infected control plants. Thus, the constitutive expression levels in transgenic plants may be high enough to be an effective defense.

Hyphae appeared on the spikes of transformed and untransformed plants in the greenhouse on the third day after inoculation with conidia. The number of infected spikelets per spike was scored on the 7th day after inoculation in the greenhouse (T_1 and T_2 plants) or on the 10th day in the growth chamber (T_3 plants). The number of infected spikelets per inoculated spike was significantly lower in transgenic plants from all three generations (T_1, T_2 and T_3) than in non-transformed control plants (Table 1). On day 10 in the greenhouse, 43% (6/14) of spikes in control plants were half-wilted as compared to 16% (12/74) of the spikes in transgenic plants. Fourteen days after inoculation, the frequency of wilted spikes in the control (90%) and transgenic plants (86%) were similar. After 3 weeks, nearly all of the inoculated spikes of both transgenic and control plants were wilted. Therefore, transgenic plants constitutively expressing TLP have a higher level of resistance to scab because the development of disease symptoms was slower than in the control plants.

Discussion

In recent years, there have been several reports of improved resistance of plants to diseases as a result of overexpression of PR-proteins such as chitinases, β-glucanases and TLPs (Broglie et al. 1991; Liu et al. 1994; Zhu et al. 1996; Lin et al. 1995; Datta et al. 1998; Grison et al. 1996; Jongedijk et al. 1995; Tabei et al. 1998; and for review, Yun et al. 1997). Although expression of a single PR-protein has a beneficial effect on slowing disease progression, satisfactory levels of resistance were achieved only when combinations of PR-protein genes were introduced into plants. We attempted to introduce genes encoding 2 PR-proteins with different modes of action, namely a chitinase and a TLP, into wheat plants to enhance resistance to scab disease. Chitinase acts on the cell-wall polysaccharide, chitin (Collinge et al. 1993), whereas TLPs are thought to alter the membrane permeability and/or cellular signal transduction cascades (Vigers et al. 1991; Dudler et al. 1994; Yun et al. 1998).

Because there was detectable expression of chitinase in the T_0 plant (Chen et al. 1998a), it is likely that the

loss of the expression in T_1 plants is due to silencing of the chitinase (and possibly *hpt*) transgene. Only the *tlp* and *bar* genes were expressed in the T_1 generation progeny at the RNA and protein level even though all four transgenes were intact (no rearrangements or deletions were detected), as shown by Southern blot analysis. *In situ* hybridization experiments further revealed that both of the transgenes (*tlp* and *chi11*) tested mapped at the subtelomeric region of chromosome 6A long arm. Therefore, this silencing apparently is not due to the chromosome integration site (position effect) because the closely linked *tlp* and *bar* genes were expressed at the same level as in the parent T_0 plant.

We believe that the differential silencing of the chitinase and *hpt* transgenes, but not of the *tlp* and *bar* genes, is due to differences in the two promoters used to drive their expression. The chitinase and the *hpt* genes are under the control of the CaMV 35S promoter, whereas the *tlp* and the *bar* genes are regulated by the ubiquitin promoter-intron combination. There are examples in the literature in which the expression of CaMV 35S promoter-driven expression has been silenced in T_1 or later generations (Matzke et al. 1994). This appears to be the case especially in wheat. In a previous study of 15 transgenic wheat plants expressing the same rice chitinase gene used in this study, we found that most of the plants expressed the chitinase gene in the T_0 generation, but that there was nearly complete silencing in the T_1 generation (Chen et al. 1998a).

Southern blot analysis of more than 30 T_1 plants indicated co-segregation of all four genes even though they were on separate plasmids during co-bombardment. Integration of one plasmid DNA at a chromosome site is likely to favor subsequent integration of other plasmids at the same site. Alternatively, the combining of two or more plasmids may have occurred prior to chromosome integration. Similarly, co-segregation has been observed in other studies (Kohli et al. 1998).

FISH techniques have been used for localizing repetitive DNA sequences, transgenes and endogenous genes on individual plant chromosomes (Jiang and Gill 1994; Pedersen et al. 1997; Fransz et al. 1996; Jiang et al. 1996; Iwano et al. 1998). The number and location of integration sites on specific chromosomes can be seen clearly and at a high detection frequency. Our FISH results confirmed that there is only one integration site on the chromosome of transgenic line no. 14 for both plasmids used in this study. These results are in agreement with our observations on the presence and segregation of transgenes as revealed by Southern blot analysis of T_1 plants (Chen et al. 1998a, b). Because the transgenes are found on chromosome 6A, this line is particularly valuable as it decreases the possibility of the transfer of the herbicide-resistance gene by an outcross with the closely related weedy species *Aegilops cylindrica* Host. ($C^CC^CD^CD^C$). This species is the only wheat relative that may cross with commercial wheat in the USA.

Increased resistance to fungal infection was observed in transgenic potato and rice plants over-expressing *tlp* genes. Transgenic potato and rice plants had fewer lesions and a delayed onset of disease caused by *Phytophthora infestans* and *Rhizoctonia solani*, respectively (Zhu et al. 1996; Liu et al. 1994; Datta et al. 1998). A similar improvement in resistance was demonstrated for the first time with transgenic wheat plants in this study. Even though we had hoped to pyramid 2 PR-protein genes in an attempt to increase resistance to scab, we could not achieve expression of the transgenic chitinase gene in the T_1 generation, presumably due to transgene silencing. However, the finding that significant improvement in disease resistance can be achieved with elevated levels of TLP alone suggests that further enhancement of resistance can be achieved when stable, high-level expression of additional antifungal genes can be engineered.

Resistance of wheat to scab is inherited as a quantitative trait. Numerous chromosomes have been reported to influence the resistance (Bai and Shaner 1994). Five quantitative trait loci (QTL) were found to be significantly associated with the resistance (Anderson et al. 1998), suggesting the possibility of multiple genes affecting resistance in wheat. TLP and other PR-proteins are possibly among the genes contributing to scab resistance as appears to be true for other QTLs (Faris et al. 1998). The delay in scab symptom development due to TLP over-expression in susceptible cultivar 'Bobwhite' is significant, although the effects were quantitative rather than qualitative. A similar situation was seen in transgenic potato plants expressing transgenic TLPs (Liu et al. 1994; Zhu et al. 1996). We are actively investigating the possibility that we can improve resistance to scab and yield potential of commercial cultivars using *tlp* and other PR-protein genes driven by the ubiquitin promoter.

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References

Anderson JA, Waldron BL, Moreno-Sevilla B, Stack RW, Frohberg RC (1998) Detection of *Fusarium* head blight resistance QTL in wheat using AFLPs and RFLPs. In: Slinkard AE (ed) Proc 9th Int Wheat Genet Symp, vol 2. University Extension Press, Saskatoon, Sask, Canada, pp 135–137

Bai G, Shaner G (1994) Scab of wheat: prospect for control. Plant Dis 78:760–766

Broglie K, Chet I, Holliday M, Cressman R, Biddle P, Knowlton S, Mauvais CJ, Broglie R (1991) Transgenic plants with enhanced resistance to the fungal pathogen *Rhizoctonia solani*. Science 254:1194–1197

Chen WP, Gu X, Liang GH, Muthukrishnan S, Chen PD, Liu DJ, Gill BS (1998a) Introduction and constitutive expression of a rice chitinase gene in bread wheat using biolistic bombardment and the *bar* gene as a selectable marker. Theor Appl Genet 97 (in press)

Chen WP, Chen PD, Liu DJ, Muthukrishnan S, Gill BS (1998b) Constitutive expression of a rice thaumatin-like protein in T_0, T_1 and T_2 transgenic wheat plants. In: Slinkard AE (ed) Proc

9th Int Wheat Genet Symp, vol 3. University Extension Press, Saskatoon, Sask, Canada, pp 169–171
Christensen JJ, Stakman EC, Immer FR (1929) susceptibility of wheat varieties and hybrids to fusarial head blight in Minnesota. Minn Agric Exp Stn Bull 59:1–24
Collinge DB, Kragh KM, Mikkelsen JD, Nielsen KK, Rasmussen U, Vad K (1993) Plant chitinases. Plant J 3:31–40
Cutt JR, Klessing DF (1992) Pathogenesis-related proteins. In: Boller, Meins F (eds) Genes involved in plant defence. Springer, Berlin Heidelberg New York, pp 209–243
Datta K, Velazhahan R, Oliva N, Ona I, Mew T, Khush GS, Muthukrishnan S, Datta SK (1998) Over expression of cloned rice thaumatin-like protein (PR-5) gene in transgenic rice plants enhances environmental friendly resistance to *Rhizoctonia solani* causing sheath blight disease. Theor Appl Genet 97 (in press)
Dudler R, Mauch F, Reimmann C (1994) Thaumatin-like protein. In: Witty M, Higginbotham JD (eds) Thaumatin. CRC Press, Boca Raton, Fla, pp 193–199
Faris JD, Li WL, Liu DJ, Chen PD, Gill BS (1999) Candidate gene analysis of quantitative disease resistance in wheat. Theor Appl Genet 98:219–225
Fransz PF, Stam M, Montijn B, Ten Hoopen R, Wiegant J, Kooter JM, Oud O, Nanninga N (1996) Detection of single-copy genes and chromosome rearrangements in *Petunia hybrida* by fluorescence in situ hybridization. Plant J 9:767–774
Gill BS, Friebe B, Endo TR (1991) Standard karyotype and nomenclature system for description of chromosome bands and structural aberrations in wheat (*Triticum aestivum*). Genome 34:830–839
Grison R, Grezes-Besset B, Schneider M, Lucante N, Olsen L, Leguay J-J, Toppan A (1996) Field tolerance to fungal pathogens of *Brassica napus* constitutively expressing chimeric chitinase gene. Nat Biotechnol 14:643–646
Heslop-Harrison JS, Schwarzacher T, Anamthawat-Jonsson K, Leitch AR, Shi M, Leitch IJ (1991) *In situ* hybridization with automated chromosome denaturation. Techniques 3:1–7
Huang JK, Wen L, Swegle M, Tran HS, Thin TH, Naylor HM, Muthukrishnan S, Reeck GR (1991) Nucleotide sequence of a rice genomic clone that encodes a class I endochitinase. Plant Mol Biol 16:479–480
Iwano M, Sakamoto K, Suzuki G, Watanable M, Takayama S, Fukui K, Hinata K, Isogai A (1998) Visualization of a self-incompatibility gene in *Brassica campestris* L. by multicolor FISH. Theor Appl Genet 96:751–757
Jiang J, Gill BS (1993) Sequential chromosome banding and in situ hybridization analysis. Genome 36:792–795
Jiang J, Gill BS (1994) Nonisotopic *in situ* hybridization and plant genome mapping: the first 10 years. Genome 37:717–725
Jiang J, Hulbert SH, Gill BS, Ward DC (1996) Interphase fluorescence in situ hybridization mapping: a physical mapping strategy for plant species with large complex genomes. Mol Gen Genet 252:497–502
Jongedijk E, Tigelaar H, van Roekel JSC, Bres-Vloemans SA, Dekker I, van den Elzen PJM, Cornelissen BJC, Melchers LS (1995) Synergistic activity of chitinases and β-1,3-glucanases enhances fungal resistance in transgenic tomato plants. Euphytica 85:173–180
Kohli A, Leech M, Vain P, Laurie DA, Christou P (1998) Transgene organization in rice engineered through direct DNA transfer supports a two-phase integration mechanism mediated by the establishment of integration hot spots. Proc Natl Acad Sci USA 95:7203–7208
Leitch IJ, Leitch AR, Heslop-Harrison JS (1991) Physical mapping of plant DNA sequences by simultaneous in situ hybridization of two differently labeled fluorescent probes. Genome 34:329–333
Lin W, Anuratha CS, Datta K, Potrykus I, Muthukrishnan S, Datta SK (1995) Genetic engineering of rice for resistance to sheath blight. Bio/Technology 13:686–691
Linthrost HJM (1991) Pathogenesis-related proteins. Crit Rev Plant Sci 10:123–150
Liu D, Raghothama KG, Hasegawa PM, Bressan RA (1994) Osmotin overexpression in potato delays development of disease symptoms. Proc Natl Acad Sci USA 91:1888–1892
Matzke M, Matzke AJM, Scheid OM (1994) Inactivation of repeated genes – DNA-DNA interaction. In: Paszkowski J (ed) Homologous recombination and gene silencing in plants. Kluwer Academic Publ, Dordrecht, The Netherlands, pp 335–348
Mauch F, Mauch-Mani B, Boller T (1988) Antifungal hydrolases in pea tissue. II. Inhibition of fungal growth by combinations of chitinase and β-1,3-glucanase. Plant Physiol 88:936–942
McMullen M, Jones R, Gallenberg D (1997) Scab of wheat and barley: a re-emerging disease of devastating impact. Plant Dis 81:1340–1348
Pederson C, Zimny J, Becker D, Jahne-Gartner A, Lorz H (1997) Localization of introduced genes on the chromosomes of transgenic barley, wheat and triticale by fluorescence *in situ* hybridization. Theor Appl Genet 94:749–757
Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning – a Laboratory Manuel, 2nd edn. Cold Spring Harbor Laboratory Press. New York, pp 7.43–7.50
Sehgal OP, Rieger R, Mohamed F (1991) Induction of bean PR-4d-type protein in divergent plant species after infection with tobacco ringspot virus and its relationship with tobacco PR-5. Phytopathology 81:215–219
Tabei Y, Kitade S, Nishizawa Y, Kikuchi N, Kayano T, Hibi T, Akutsu K (1998) Transgenic cucumber plants harboring a rice chitinase gene exhibit enhanced resistance to gray mold (*Botrytis cinerea*). Plant Cell Rep 17:159–164
Van Loon LC, Pierpoint WS, Boller T, Conejero V (1994) Recommendations for naming plant pathogenesis-related proteins. Plant Mol Biol Rep 12:245–264
Velazhahan R, Chen-Cole K, Anuratha CS, Muthukrishnan S (1998) Induction of thaumatin-like proteins (TLPs) in *Rhizoctonia solani*-infected rice and characterization of two new cDNA clones. Physiol Plant 102:21–28
Vigers AJ, Roberts WK, Selitrennikoff CP (1991) A new family of plant antifungal proteins. Mol Plant-Microbe Inter 4:315–323
Xu Y, Chen L (1993) Wheat scab: theory and practice of control. (in Chinese). Science and Technology Press of Jiangsu, Jiangsu, pp 201–216
Yun DJ, Bressan RA, Hasegawa PM (1997) Plant antifungal proteins. In: Janick J (ed) Plant breeding review, vol 14. John Wiley & Sons, New York, pp 39–88
Yun DJ, Ibeas JI, Lee H, Coca MA, Narasimhan ML, Uesono Y, Hasegawa PM, Pardo JM, Bressan RA (1998) Osmotin, a plant antifungal protein, subverts signal transduction to enhance fungal cell susceptibility. Mol Cell 1:807–817
Zhu B, Chen THH, Li PH (1996) Analysis of late-blight disease resistance and freezing tolerance in transgenic potato plants expressing sence and antisense genes for an osmotin-like protein. Planta 198:70–77