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Transient expression and insertional mutagenesis of *Puccinia triticina* using biolistics

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Abstract The fungal genus *Puccinia* contains more than 4,000 species. *Puccinia triticina*, causal agent of wheat leaf rust, is an economically significant, biotrophic basidiomycete. Little is known about the molecular biology of this group, and tools for understanding gene function have not yet been established. A set of parameters was established for the transient transformation of urediniospores. The expression of three heterologous promoters (actin, elongation factor 1-α, and *Hss*1, Heat Shock 70 protein), derived from *Puccinia graminis*, was evaluated along with the potential for insertional mutagenesis. The UidA (GUS) gene was used as a marker for transient expression. When transferred into *P. triticina* urediniospores, transient expression was observed across four helium pressures using one size of gold and three sizes of tungsten microprojectiles. Each of the three promoters displayed strong transient expression in germinated urediniospores; however, higher numbers of GUS-positive urediniospores were observed when either the actin or *Hss*1 promoters were used. Possible concomitant insertional mutagenesis of several avirulence genes was selected in wheat cultivars harboring the cognate resistance genes. Using a linearized cloning plasmid, stable integration into the genome was achieved as demonstrated by PCR and sequencing analysis.

Keywords Biolistics · Leaf rust · Stable integration

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Introduction

The genus *Puccinia* is one of the most economically destructive genera of fungal plant pathogens (Hooker 1967). Wheat leaf rust, caused by *Puccinia triticina* (formerly *Puccinia recondita* f. sp. *tritici*), is one of the most widespread and economically important diseases of wheat and occurs wherever wheat is grown. Leaf rust can cause as much as 40% yield loss on susceptible wheat cultivars (Knott 1989) by reducing grain yield and quality. The development of wheat cultivars with stable, long-lasting resistance to leaf rust has been complicated by the highly variable nature of *P. triticina* populations. Over 50 physiological races are typically detected each year in the USA (Long et al. 1998). However, in most wheat-growing areas, the leaf rust fungus reproduces asexually due to the absence of the alternate host *Thalictrum speciosissimum*. Furthermore, many of the isolates lack the ability to produce the sexual stages of the life cycle.

Very little is known about the molecular interaction between rusts and their plant hosts. Many pathogenicity and virulence factors contribute to the host specialization of these strictly biotrophic basidiomycetous fungi. Flor (1971) demonstrated that within a species, an incompatible interaction is mediated by a gene-for-gene system in which host resistance is triggered when plant resistance (*R*) proteins recognize, either directly or indirectly, specific rust-

encoded ligands or elicitors. Detection of these fungal avirulence (*Avr*) or elicitor proteins activates defense signal transduction pathways, culminating in a defense response by the plant and often involves localized hypersensitive cell death (Ellis et al. 2000). Absence or mutation of the plant receptor(s) and/or the pathogen-derived elicitor(s) eliminates recognition and results in disease. Many *R* genes and their cognate pathogen elicitors have been cloned (Bonas and Lahaye 2002; Martin et al. 2003). Dodds et al. (2004) have identified, by map-based cloning, a haustorial secreted protein from *Melampsora lini* that induces the resistance response in flax (*Linum usitatissimum* L.) containing the *L5*, *L6*, and *L7* resistance genes. In wheat, the first two leaf rust *R* genes cloned were *Lr21* (Huang et al. 2003) and *Lr10* (Feuillet et al. 2003).

The genome structure and molecular biology of cereal rusts are largely unknown. Flow cytometry has been used to estimate the size of the *P. triticina* genome to be approximately 90–93 Mb (Eliam et al. 1994). Several avirulence genes have been genetically mapped in the wheat stem rust fungus, *P. graminis* f. sp. *tritici*, a close relative of leaf rust (Zambino et al. 2000), but none of the elicitors or the genes encoding them have been cloned. Recently, studies have begun in *P. triticina* for gene discovery and expression. Thara et al. (2003) identified, *in planta*, several *P. triticina* infection-induced genes. Zhang et al. (2003) employed a cDNA amplified fragment length polymorphism (AFLP) technique to isolate *P. triticina* genes expressed during various stages of compatible interactions between the leaf rust fungus and its wheat host. In other efforts, cDNA libraries have been generated from different life cycle stages of wheat leaf rust on wheat. A large number of clones from these libraries have been sequenced to construct a *P. triticina* expressed sequence tags database that will soon become publicly available (Bakkeren et al., unpublished data).

Using biolistics, transient expression has been demonstrated in a few agronomically important biotrophic fungi including *Erysiphe graminis* (Christiansen et al. 1995; Chaure et al. 2004) and the bean rust fungus *Uromyces appendiculatus* (Bhairi and Staples 1992; Li et al. 1993). More recently, Schillberg et al. (2000) used particle bombardment to achieve transient expression, but not stable integration, in *P. graminis* f. sp. *tritici* urediniospores using a homologous promoter, elongation factor 1α (EF-1α). *E. graminis* f. sp. *hordei*, the causal agent of barley powdery mildew, is the only obligate biotrophic fungus in which stable transformation via biolistics has been achieved (Chaure et al. 2000). Using these other systems as a guide, three objectives were set for this work: (1) to establish parameters for biolistics of wheat leaf rust using a transient expression system; (2) to test the utility of heterologous promoters for use in transient expression assays; and (3) to demonstrate stable integration using insertional mutagenesis of avirulence genes. Here we describe the parameters that were established and describe stable integration within putative mutant *P. triticina* isolates.

Materials and methods

Experimental design

Bombardment conditions reported by Schillberg et al. (2000) were used as a starting point for optimizing leaf rust bombardment parameters. In the present work, the following factors were evaluated: delivery pressures of 400, 900, 1,350, 1,550, and 1,800 psi; microcarrier size/type 1.0 µm gold, M-5 tungsten (~0.4 µm), M-10 tungsten (~0.7 µm), and M-15 tungsten (~1.0 µm); spore concentrations of 0.25 and 1.0 mg; spore hydration of 0 and 2 h; and three plasmid expression vectors pGUS6L20, pCDLActGUS1, and pCDLHssGUS1 (described below in "Plasmids and Constructs").

Delivery pressure, microcarrier type and size, and spore concentration were evaluated in factorial design experiments to identify the combination producing the greatest number of transformed spores. Data were gathered from four replications for each combination of helium pressure, microcarrier type/size, and spore concentration across two separate experiments conducted on different days. Transformation efficiencies between nonhydrated and 2-h hydrated spores were compared in three separate experiments over a range of seven helium pressures and two different tungsten microparticle sizes (0.4 and 0.7 µm). Relative promoter efficiency was tested as a single factor using optimal bombardment parameters. Data were collected from a single experiment using eight replications for each promoter.

Fungal culture

Pure cultures of *P. triticina*, race PBJL (Long and Kolmer 1989), were maintained on highly susceptible, hard winter wheat seedlings, cultivar "TAM107", treated with Cycocel (plant growth regulator (Olympic Horticultural Products, Mainland, PA). Plants were kept in growth chambers under 16-h day/8-h night cycle under cool white fluorescent/incandescent lighting with day and night temperatures of 20 and 16°C, respectively. Urediniospores were harvested with a cyclone harvester and left in dishes on the bench top for 18 h at ambient temperature to enhance spore germination rates (Eversmeyer, personal communication). Spores were weighed in 0.25 or 1.0 mg aliquots and suspended in 40 µl Soltrol (170 isoparaffin solvent (Chevron Phillips Chemical Company, The Woodlands, TX). Aliquots of 0.25 mg were estimated to contain between 6.0×10^4 and 1.0×10^5 urediniospores. The spore suspensions were immediately spotted onto small squares of Hybond-N+ nylon hybridization membrane (Amersham Biosciences, Piscataway, NJ) and placed onto the surface of unamended 1.0% (w/v) water agar in 60×15-mm Petri dishes. Mounted spores were either bombarded immediately, or they were held at 4°C for 2 h prior to bombardment to initiate spore hydration (Schillberg et al. 2000). Culture plates were

placed directly into the particle gun's vacuum chamber for bombardment. Negative controls for each experiment were not bombarded, but otherwise treated the same.

Plasmids and constructs

The expression vector pGUS6L20 was kindly provided by Dr. S. Schillberg (Institut für Biologie, Aachen, Germany). Two additional constructs containing *P. graminis*-derived gene promoters, actin and *Hss*1, were developed. The gene *Hss*1 encoding Heat Shock Protein 70 was cloned by screening a genomic DNA library with a heterologous probe (pUMS2H) from *Ustilago maydis* (Holden et al. 1989). The genomic DNA library was constructed in the lambda EMBL4 vector using DNA isolated from an axenic culture of *P. graminis* f. sp. *tritici* (strain V1B), following standard procedures (Sambrook et al. 1989) with modifications as described by Bhairi et al. (1989). The entire *Hss*1 gene was contained on two *Eco*RI fragments (8.0 and 3.0 kb) subcloned into pGEM-4Z (Promega, Madison, WI) as pCR L18 and pCRL19, respectively (Fig. 1a). The 3.5-kb region, which was sequenced, contained the complete *Hss*1 gene, including 923 bp of 5′ and 444 bp of 3′ noncoding regions (GenBank accession no. U26597). The actin gene had been cloned, and the DNA sequence is available (Gross and Tiburzy, unpublished results; GenBank accession no. X77857).

The promoter region and the 5′ end of the coding region, containing the first introns, were cloned from actin and *Hss*1 genes using PCR primer pairs ActC-F1/ActC-R2 (ActC-F1, GAGCTCGATGATGGTTGATGACTCTGGAAC; ActC-R2, GGATCCCACATACCCGAACCATTGTCG) and HspC-F2/HspC-R2 (HspC-F2, GAGCTCCCGAGGGAG ATGAACTTGAG; HspC-R2, GGATCCTGGAAGTGCTTG ATGTCGGACT), respectively (Fig. 1a,b). The first exon and intron from each gene was included to increase the efficiency of transient expression. Forward primers con-

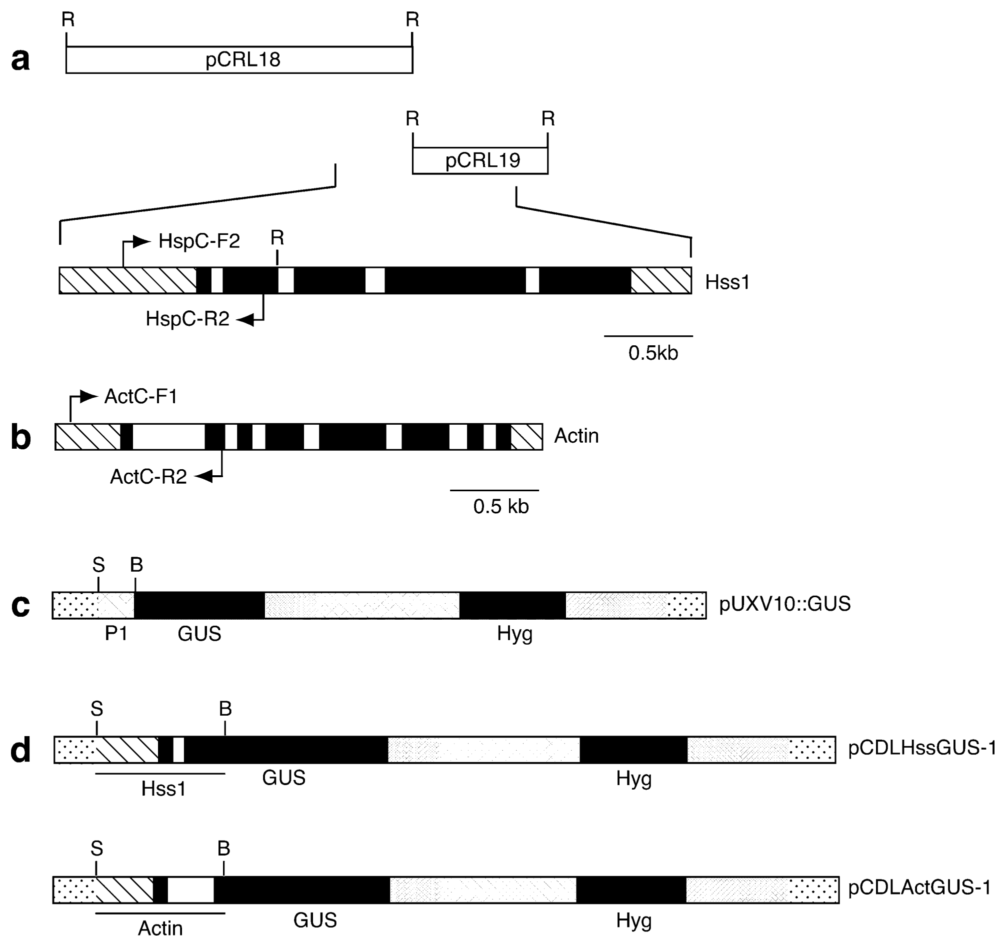

Fig. 1 Schematic representation of plasmid constructs. **a** Clones pCRL18 and pCRL19 containing the complete *Puccinia graminis* Heat Shock Protein 70 gene (*Hss*1). Location of the primers HspC-F2 and HspC-R2 used in PCR amplification and cloning of the *Hss*1 promoter is indicated. **b** Diagram of the *P. graminis* actin gene (X77857) indicating the location of the primers ActC-F1 and ActC-R2 used for the PCR amplification and cloning of the actin promoter. **c** Partial diagram of the *Ustilago maydis* shuttle vector pUXV10::GUS used in the construction of GUS expression vectors. **d** Partial diagram of GUS expression vectors pCDLHssGUS-1 and pCDLActGUS-1 containing *P. graminis*, *Hss*1 and actin promoters, respectively. Exons (*shaded box*), introns (*open box*), *P. graminis* 5′ and 3′ noncoding regions (*striped box*), *U. maydis* promoters (*hatched box*), terminator sequence (*gray box*), and vector sequence (*stippled box*) are indicated. *B*, *Bam*HI, restriction site, *R*, *Eco*RI restriction site, *S*, *Sac*I restriction site, P_1, *U. maydis* GAP promoter; *GUS* β-Glucuronidase coding region, *Hyg* Hygromycin B resistance gene coding region

tained *Sac*I, and the reverse primers contained *Bam*HI restriction sites, which facilitated the construction of these GUS expression vectors. *P. graminis* genomic DNA (isolate CRL 75-36-700-3) and plasmid DNA (pCRL18) were used as templates for the amplification of the actin and *Hss*1 promoters, respectively. PCR-amplified products were purified (QIAquick PCR purification kit, Qiagen, Valencia, CA) and cloned (TOPO TA Cloning Kit for Sequencing, Invitrogen, Carlsbad, CA) as described by the manufacturer. Clones containing the actin (pCDLAct1e) and *Hss*1 (pCDLHss2e) promoters were sequenced for verification.

GUS fusions were constructed by removing the *U. maydis* GAP promoter from the vector pUXV10::GUS (Fig. 1c) and replacing it with the *P. graminis* actin or *Hss*1 promoter. Approximately 2 μg each of pUXV10::GUS and either pCDLAct1e or pCDLHsp2e were mixed and digested with *Sac*I and *Bam*HI restriction enzymes in a total volume of 50-μl buffer (NEBuffer 1, New England BioLabs, Beverly, MA). After the incubation, 2.0 μl of digested DNA was used in ligations containing T4 DNA ligase (New England BioLabs) and transformed by electroporation into Epicurian Coli XL1-Blue MRF′ electroporation competent cells (Stratagene, La Jolla, CA). Transformed colonies were screened by multiplex PCR in which primers for the promoter (ActC-F1/ActC-R2 or HspC-F2/HspC-R2) were mixed with primers specific for the hygromycin resistance gene contained in pUXV10::GUS (HygF1, AACCCT GAACTCACCGGG; HygR1, TTCCTTTGCCCTCGGACG).

Bombardment

Plasmid DNA (7.5 μg) was dried onto tungsten or gold particles (Heiser 1992, Bulletin No. 1688) and delivered under vacuum (25 in. Hg) with a Bio-Rad PDS-1000 He Biolistic Particle Delivery System (Bio-Rad Laboratories, Hercules, CA) at helium pressures listed above. Target distance was 7.0 cm for all experiments. A focusing device described by Torisky et al. (1996) was utilized to narrowly concentrate the impact area for all bombardments. The amount of gold and tungsten microparticles (30 mg) and the concentration of plasmid DNAs (1.0 μg/μl) were held constant across all experiments. Three bombarded controls were included in these experiments: (1) a nonfungal promoter-driven GUS control, pAHC27, which carries a maize ubiquitin promoter driving the UidA (GUS) gene (Christensen and Quail 1996); (2) a plasmid negative control, pBluescript II KS(−) (Stratagene), which lacks a β-glucuronidase gene; and (3) a particle negative control of uncoated tungsten and gold particles.

Histochemical staining

Bombarded spores were germinated on covered water agar plates for 16 h, at ambient temperature, with 100% relative humidity. Spores were assayed for β-glucuronidase activity after transferring to fresh Petri dishes with 3.0 ml of substrate solution [10 mM Na_2EDTA, 0.1% (v/v) Triton-X 100, 100 mM NaH_2PO_4, 0.5 mM $K_3Fe(CN)_6$, 100 mg/ml X-glucuronide] (modified from Sambrook et al. 1989) and incubated at 37°C overnight. Spores were then placed on glass slides and examined at 200× and 630× magnification with a Zeiss Axioplan 2 microscope (Carl Zeiss Micro-imaging, Thornwood, NY). Transformed spores were counted manually, and the data were statistically analyzed using the MEANS and REG procedures of the SAS (System, version 8.2 (SAS Institute, Cary, NC).

Plant lines and selection of avirulence mutants

Three wheat cultivars were used to select for putative transformation mutants of fungal strain PBJL. KS940786-6-7, KS91WGRC11, and KS92WGRC15 carry single rust resistance genes *Lr39*, *Lr42*, and *Lr21*, respectively, and were graciously supplied by Dr. Allan Fritz, Kansas State University, and Dr. Gina Brown-Guedira, USDA-ARS, Manhattan, KS. Screening with single resistance genes prevented confusing epistatic effects from other resistance genes. Bombarded spores were promptly washed from the membranes, resuspended in Soltrol 170, and inoculated onto large trays of wheat seedlings at the three-leaf stage. Seedlings were grown under the influence of Cycocel plant growth regulator to keep the plants short and manageable. Seedlings were maintained in environmental chambers for 8–10 days. Each experiment included the wheat cultivar TAM107 as a susceptible control to insure spore viability and to give an indication of the spore density.

KS940786-6-7, KS91WGRC11, and KS92WGRC15 display a strong resistance reaction “0” or “;” to PBJL using the 0–4 scale described by McIntosh et al. (1995). With this scale, a “0” is an immune host response with no visible uredia, a “;” is very resistant with hypersensitive flecks, and a “4” is susceptible with large uredia without chlorosis. Any pustule identified during seedling screening became an independent candidate transformant. Using a microscalpel, spores from the pustule were transferred onto another seedling plant of the resistant wheat cultivar from which the isolate was originally identified. The pustule was carried through at least four successive single pustule isolations back onto the resistant, selecting cultivar.

Plasmid rescue

Prior to bombardment with pBluescript KS(−), plasmid DNA was linearized by digestion with *Eco*RV and purified using Qiagen Miniprep Spin columns. Bombardment conditions, urediniospore concentration, and inoculation onto plants were as described before. Genomic DNA of putative mutant lines was isolated from spore mats as above. Genomic DNA, 250 ng, was digested with either *Xho*I or *Sac*I (Fig. 2). Digestions were then ligated at 14°C overnight using 1 U of T4 DNA Ligase (Invitrogen) to recircularize the plasmid. Ligation reactions were used as templates for PCR. Primary PCR conditions were 1 μl of

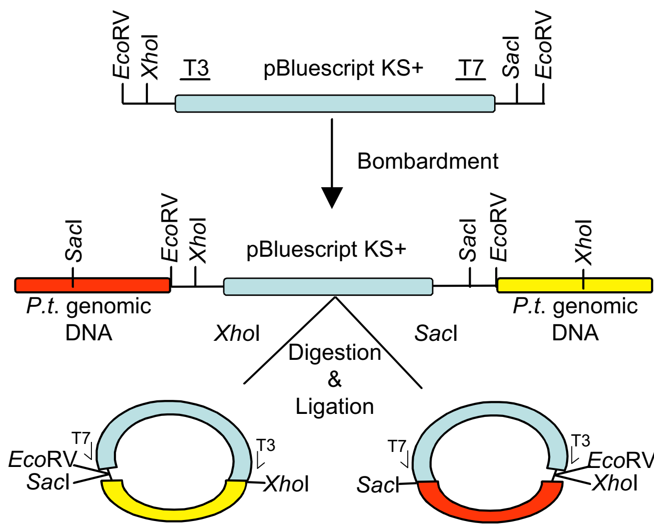

Fig. 2 Flow diagram illustrating plasmid rescue and basis of inverse PCR

the ligation reaction, 10 pmol each of M13F and M13R primers, 1× *Taq* reaction buffer (Sigma), 2.5 mM MgCl, 2.5 mM dNTPs (USB), and 0.25 U of *Taq* (Sigma). Primary PCR reactions were diluted 50-fold, and 1 µl was used for nested PCR. T7 and T3 primers, at 10 pmol each, were used as nested primers. Amplification conditions on the MJ Research PTC-200 consisted of 35 cycles of 94°C for 1 min, 55°C for 2 min, and 72°C for 2 min. PCR fragments were cloned into pCR2.1 (Invitrogen) and transformed into *Escherichia coli*. Purified plasmids were sequenced at the Kansas State University Sequencing Facility.

Sequence data from this article have been deposited with the EMBL/GenBank Data Libraries under accession no. U26597. Mention of trade names or commercial products in this article is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the US Department of Agriculture.

Results

Microprojectile type and size

Initial experiments focused on the three main variables in biolistics: type of particle and size, target, and delivery pressures. Additional experiments evaluated two additional constructs containing *P. graminis*-derived gene promoters, actin and *Hss*1, as alternatives for gene control (Fig. 1). Both tungsten and gold microparticles, when coated with pGUS6L20, resulted in β-glucuronidase gene expression inside bombarded *P. triticina* urediniospores (Fig. 3). The majority of stained spores exhibited a deep-blue color, indicating strong expression of the GUS gene (Fig. 3a). However, light-blue-colored spores were observed occasionally (Fig. 3b). Blue staining was also observed in germinating urediniospores (Fig. 3c). Blue spores that germinated always had blue germ tubes. Variable gene expression levels and stained germination tubes were observed at all tested delivery pressures, except at 1,800 psi. Controls never stained blue (Fig. 3d).

No obvious differences were observed between experiments utilizing gold or tungsten microparticles (data not shown). Therefore, remaining experiments were performed using tungsten projectiles exclusively. Similar urediniospore germination (approximately 90%) was observed between controls using tungsten microparticles with no DNA and nonbombarded spore controls, indicating that the metal produced no detectable toxic effects. When using larger particles (0.7 or 1.0 µm) and lower delivery pressures (450 and 650 psi), the urediniospores appeared to be studded with microparticles, suggesting inefficient penetration through the thick spore wall. In an effort to enhance projectile penetration, smaller (0.4 µm) particles were tested at higher delivery pressures. These projectiles consistently produced greater numbers of histochemically stained spores and germination tubes than did relatively larger particles delivered at equivalent pressures (data not shown).

Spore concentration and hydration

Two different spore densities (0.25 and 1.0 mg, or approximately 8×10^4 and 3×10^5 urediniospores) were tested as bombardment targets. Approximately equal numbers of GUS-positive, nongerminating spores were observed at both spore densities (data not shown); however, urediniospore germination and germ tube elongation were clearly greater in 0.25-mg spore aliquots relative to 1.0-mg concentrations in both bombarded and nonbombarded treatments. Enhanced germination at the lower spore concentration was not totally unexpected since self-inhibition has been demonstrated in several rust species (Staples 2000). The effect of urediniospore prebombardment hydration was also tested. Urediniospores that were hydrated for 2 h consistently produced blue staining in both spores and germ tubes, whereas nonhydrated spores were always GUS-negative.

Helium pressures

Four of the five helium pressures tested produced GUS-positive urediniospores. Considerable variation was observed in the number of blue-staining urediniospores, both within and between experiments (Table 1). Despite wide variability, certain trends were evident. The 900-psi delivery pressure produced the largest mean of GUS-expressing cells per bombardment (7.0±2.2) using 0.4-µm tungsten particles. The data fit a quadratic model with an R^2 value of 0.24. The *t* test of these data supports the hypothesis that the mean number of blue-staining urediniospores is significantly different from zero at a 0.01 confidence level. Transformation efficiency was estimated to be seven transformants per 1.0 µg plasmid DNA using these parameters. Spores transformed at this pressure were also directly inoculated onto detached seedling leaves. Though not

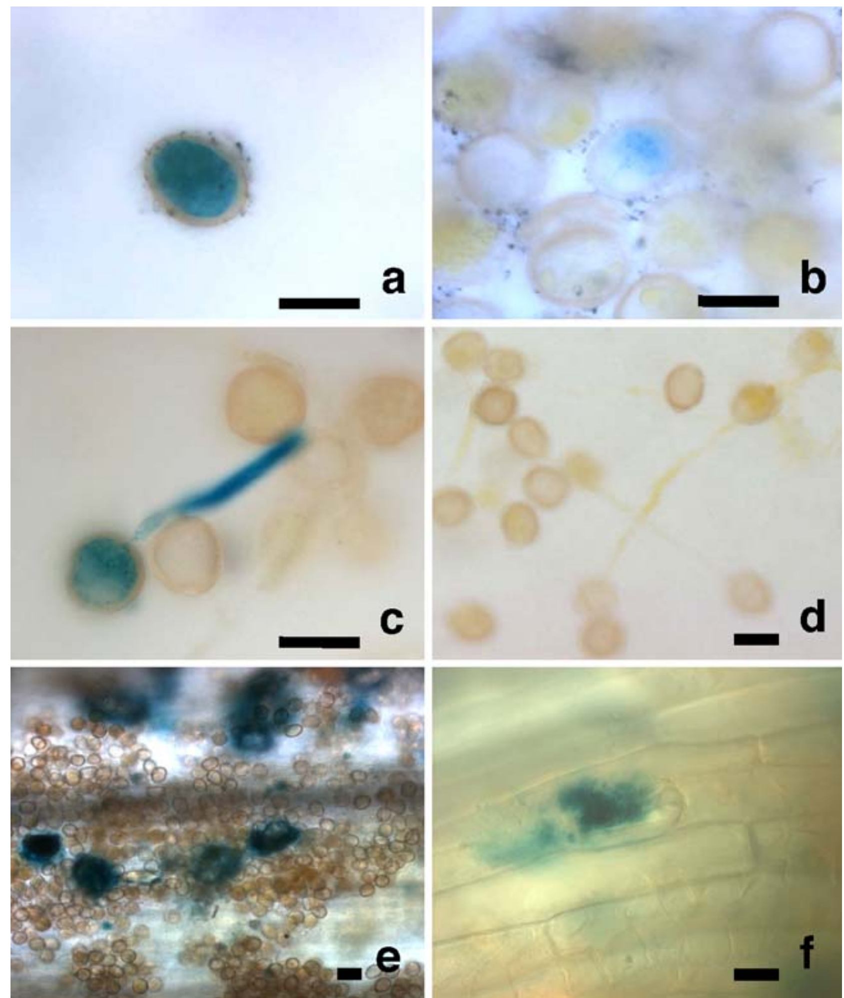

Fig. 3 β-Glucuronidase expression in *Puccinia triticina* urediniospores. **a** Bombardment at 1,550 psi; **b** weak transient GUS expression from a *P. triticina* urediniospore bombarded at 1,350 psi; **c** bombarded at 1,350 psi, GUS-stained spore, and germination tube; **d** non-bombardment control; **e** GUS expression of bombarded spores germinated on a detached leaf. **f** GUS expression in a germ tube associated with a stomate. Magnification bars indicate 20.0 μm

quantified, higher number of transformed spores could be seen on leaves vs agar (Fig. 3e). GUS-expressing and germinating spores were also associated with leaf stomates (Fig. 3f).

The lowest delivery pressure of 450 psi resulted in a mean of 1.3±0.6 blue-staining urediniospores, whereas higher pressures of 1,350 and 1,550 psi produced means of 0.8±0.4 and 1.4±1.0 GUS-positive urediniospores per bombardment, respectively (Table 2). Only the 1,800-psi treatments failed to produce blue-staining urediniospores. Spore displacement and damage levels appeared correlated with an increase in helium delivery pressures (data not shown).

Actin and *Hss*1 promoter constructs

Transient expression assays were also carried out with the plasmid constructs pCDLActGUS1 and pCDLHssGUS1, containing *P. graminis* actin and *Hss*1 promoters, respectively. Optimal bombardment parameters of 0.4-μm tungsten microparticles, 900-psi delivery pressure, and 0.25 mg of target urediniospores were used. Bombardment of either vector produced GUS-positive urediniospores. Furthermore, the pCDLActGUS1 and pCDLHssGUS1 constructs gave a greater mean of GUS-positive urediniospores per bombardment (3.0±1.5 and 2.9±1.8, respectively) than did the pGUS6L20 construct (0.6±0.2; Table 2).

Table 1 Bombardment data comparing five helium delivery pressures

	Helium delivery pressure (psi)				
	450	900	1,350	1,550	1,800
Expt. 1[a]	0, 0, 3, 5	0, 2, 2, 12	0, 0, 2, 2	0, 0, 0, 1	0, 0, 0, 0
Expt. 2	0, 0, 1, 1	4, 8, 10, 18	0, 0, 0, 2	0, 0, 2, 8	0, 0, 0, 0
Mean[b]	1.3±0.6	7.0±2.2	0.8±0.4	1.4±1.0	0.0±0.0

[a]Values represent the number of GUS-positive spores per replication for each experiment. Experiments one and two were conducted on two different days

[b]Mean number of GUS-positive spores and standard error of the mean, calculated from eight total replications per pressure treatment

Table 2 *Puccinia graminis* promoter activity in wheat leaf rust

	Relative promoter expression		
	Actin	*Hss*1	EF-1α
Expt. 1[a]	0, 0, 0, 1	0, 0, 0, 0	0, 0, 0, 1
Expt. 2	1, 3, 8, 11	1, 3, 4, 15	1, 1, 1, 1
Mean[b]	3.0±1.5	2.9±1.8	0.6±0.2

[a]Values represent the number of GUS-positive spores per replication from a single experiment

[b]Mean number of GUS-positive spores and standard error of the mean, calculated from eight total replications per promoter treatment

Isolation of stable integration events

At present, none of the selective markers used for fungal transformation work with rust fungi because obligate biotrophs can only be selected for on their host. Preliminary screens using detached leaves infected with the wheat stem rust fungus (*P. graminis*) indicated that the reduction of fungal growth occurred only as a result of host death (data not shown). As an alternative to the insertion of a positive selective marker, insertional mutagenesis of avirulence genes was used for the selection of stable insertion into rust fungi. Near immunity of the wheat host to leaf rust harboring certain avirulence genes provides ideal tight selection control. It was envisaged that insertion of a linearized plasmid in a certain avirulence gene during transformation would allow the resulting mutant to no longer be recognized by the cognate resistance gene resulting in uredia.

pGUS6L20 Two wheat cultivars were used to select for putative transformation mutants of fungal strain PBJL. KS940786-6-7 and KS91WGRC11 carry single rust resistance genes *Lr39* and *Lr42*, respectively. Screening with single resistance genes prevented confusing epistatic effects from other resistance genes. KS940786-6-7 and KS91WGRC11 each displayed a strong resistance reaction “0” or “;” to PBJL (McIntosh et al. 1995). Using the parameters of 900 psi, 0.4-μm tungsten particles, and the pGUS6L20 plasmid, ten nylon disks each containing 0.25 ng of urediniospores were bombarded, combined, and inoculated onto KS940786-6-7, KS91WGRC11, and TAM107 seedlings. After inoculation of leaves with bombarded urediniospores, two to five pustules per experiment were identified with altered phenotypes ranging from “1 to 4” (Fig. 4a). Tungsten-only bombarded urediniospores produced no pustules. Urediniospores collected from individual pustules were then used to inoculate fresh single plants. Urediniospore increases for each mutant (integration event) isolate were made by four successive rounds of selection on wheat lines containing the appropriate *R* gene.

Sixteen putative insertional mutants were identified using the pGUS6L20 plasmid: 12 PtAvrLr39 and four PtAvrLr42. Mutant isolates exhibited altered phenotypes ranging from intermediate (1-2C) to a highly virulent (3+) phenotype (Fig. 4b) compared to the wild-type highly resistant phenotypes of “0” or “;”. Six of the PtAvrLr39 mutants were difficult to grow due to mild alterations of phenotype and low pustule formation. The remaining six transformants were tested by polymerase chain reaction (PCR) and Southern blotting. PCR primer pairs were designed to detect the ampicillin resistance gene present in pGUS6L20. Agarose gels containing these fragments were blotted and probed with pBluescript II KS(−) plasmid. Under high stringency conditions, PCR amplicons hybridized strongly with the pBluescript probe (Fig. 5) and verified accurate amplification. DNA from the untransformed wild-type rust strain PBJL did not reveal this diagnostic fragment (Fig. 5, lane WT). This strongly suggests that we have successfully integrated a plasmid vector sequence into the leaf rust genome, and that the integration remains stable throughout multiple rounds of selective purification. What is most important is that this shows that the procedure is repeatable for stable integration of two different plasmids and selected for different *Lr* genes.

pBluescript II KS(−) The experiments with pGUS6L20 verified that transient transformation and stable integration could be achieved using biolistics. However, the orientation of the plasmid integration could not be determined without whole genome library construction. pBluescript II KS(−) provides numerous restriction and primer sites and

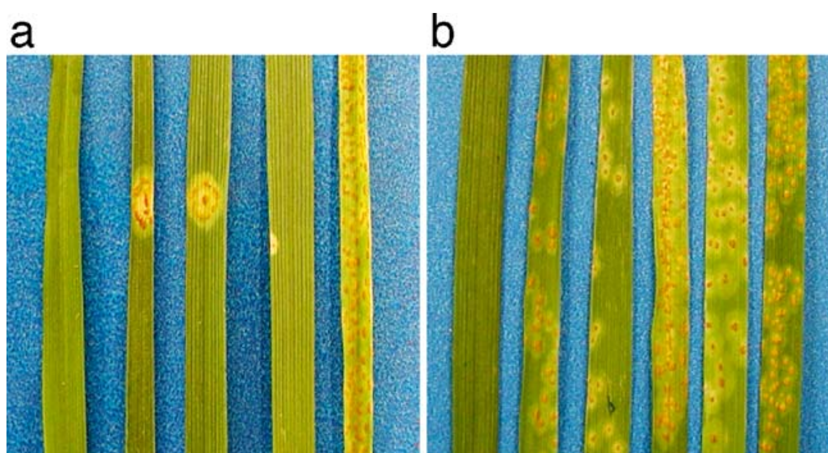

Fig. 4 Phenotypes of putative mutant pustules on seedling leaves of KS940786-6-7. **a** *Left to right*: KS940786-6-7 inoculated with wild type; three individual pustules on KS940786-6-7 selected for changes in virulence to *Lr39*; TAM107 susceptible control. **b** Individual putative mutant lines after four rounds of selection on *Lr39*. *Left to right*: KS940786-6-7 inoculated with wild type; PtAvrLr39-1 infection type (IT) “3+”; PtAvrLr39-2 IT “1c-2c”; PtAvrLr39-3 IT “3+”; PtAvr39-4 IT “2c-3c”; TAM107 susceptible control

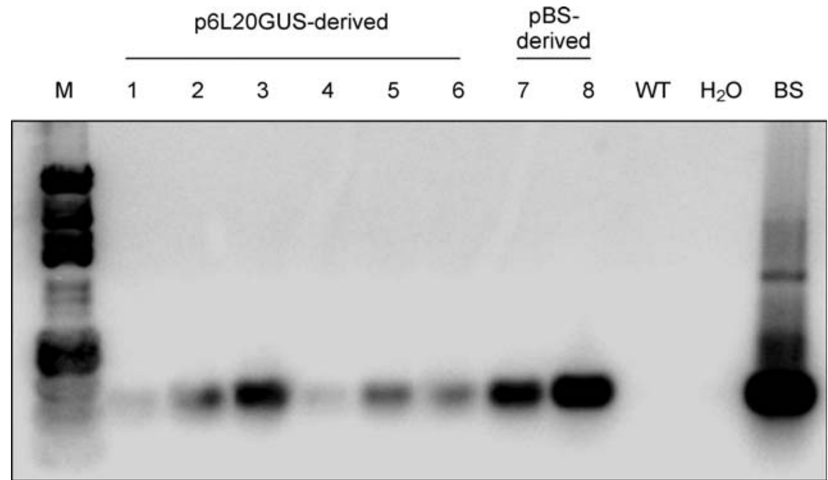

Fig. 5 Verification of the presence of the bombardment plasmid in putative leaf rust mutants by Southern hybridization of the pBluescript plasmid to PCR amplicons from mutant rust genomic DNA. *M*, Hyperladder I size standard; *1–6*, putative avirulence mutants PtAvrLr39-1 through 6; *7–8*, putative mutants PtAvrLr42-1-2); *WT*, wild-type PBJL; *H_2O* water-only PCR control, *BS* pBluescript II KS plasmid PCR control

thus can be rescued from the genome, without library construction as illustrated in Fig. 2. Two rust variants were identified from seedlings containing *Lr42* and two from *Lr21*. Vector-specific PCR primers amplified fragments from the genomes of the two PtAvrLr42 variants and hybridized strongly to the pBluescript probe (Fig. 5). From

Fig. 6 Sequence from plasmid rescue clones from two individual mutant rust lines **a** PtavrLr21-1 and **b** PtavrLr21-2 and the top database alignments. pCR2.1 is a PCR cloning vector and pBluescript is the bombardment vector

a

PtavrLr21-1

```
-------------------pCR2.1-----------⇐][----------pBlue-
GGCACGCACAGTNTGATGGATATCTGCAGAATTCGGCTTAATACGACTCACTACAG
scriptII KS(-)----------] XhoI
GGTCGAATTGGGTACCGGGCCCCCCCTCGAGGTCGACGGTATCGATAAGCTTGATAT
CGAATTCCTGCAGGTTCGAGTACGCAATCGCCCATTGGTTCCAAAAGGCTGCTGAGC
ATGTGTTCGGCTGTGTTTTGTGTGCCCCCGGTAGCTTCTCTCTGTTTCGTGCTTCTG
CTCTCATGGATGACAATGTGATGCACAAATACACCAAAACTGCCTCCGAACCACGAC
ATTGGATCCACTAGTTCTAGAGCGGCCGCCACCGCGGTGGAGCTCCAGCTTT
                                        SacI [------
TGTTCCCTTTAGTGAGGGTTAATAAGCCGAATTCCA
--pBluescriptII–KS(-)--][⇒pCR2.1---
```

E

Sequences producing significant alignments:		E Value
gil17506291lrefINP_492113.1l	chitin synthase (chi-1Co).	1e-25
gil39582483lemblCAE66574.1l	Hypothetical protein CB	1e-25
gil12231691lgblAAG49219.1l	chitin synthase [Brugia m	2e-24
gil11640954lgblAAG39382.1l	chitin synthase [Dirofilaria	2e-22

b

PtavrLr21-2

```
-------------------pCR2.1-----------⇐][----------pBlue-
GGCACGCACAGTNTGATGGATATCTGCAGAATTCGGCTTAATACGACTCACTACAG
scriptII KS(-)----------] XhoI
GGTCGAATTGGGTACCGGGCCCCCCCTCGAGGTCGACGGTATCGATAAGCTTGATAT
CGAATTCCTGCAGCCCACCAACGAGGACATGTAGAAGCAGCACTTCGTGTGTTTGAT
GGGATAGACATATCTTCGTTAGTTCCTAAGATGAAAATCTCAATTGCTAGAAAAGCA
CATCGCCGGAAGACTCGTTCACAATGGGATGCTCCACCAATGCCCTTGCATGCTGTG
AGCCTACTTATGGAGGCCATATATCTCAAAGCAAGAGCACTTCATGATCTCGGAAAG
TTCAAAGATGCTGCACAAGAGTGCAGAATGATATTGGATATCGTGGAAGCAGCAGTG
GGGGATCCACTAGTTCTAGAGCGGCCGCCACCGCGGTGGAGCTCCAGCTT
                                       SacI [-----
TTGTTCCCTTTAGTGAGGGTTAATAAGCCGAATTCCA
--pBluescriptII–KS(-)---][⇒pCR2.1---
```

Sequences producing significant alignments:		E Value
gil7487684lpirllT10654	hypothetical protein T5F17.50	5e-19
gil22329002lrefINP_194589.2l	calmodulin-binding protein.	5e-19
gil18396347lrefINP_564285.1l	calmodulin-binding protein.	1e-16

the plasmid rescue, primary PCR amplified the insert, whereas the nested primers insured that the amplicons were accurate. No amplicons were found in the wild-type control DNA. Fig. 6 illustrates that fungal sequence was obtained from both PtavrLr21-1 and PtavrLr21-2 using plasmid rescue, and that the sequence has strong BLASTN alignments to known open-reading frames of a chitin synthase and a calmodulin binding protein.

Discussion

At present, little is known regarding the optimal conditions for transformation of obligate fungal parasites using particle bombardment methods. Our results indicate that either tungsten or gold are suitable microprojectile carriers for biolistic experiments involving *P. triticina*. Both materials have been utilized in other rust urediniospore transformations with equal success (Bhairi and Staples 1992; Li et al. 1993; Schillberg et al. 2000).

In our urediniospore-targeting experiments, delivery of smaller particles produced greater numbers of expressing cells per bombardment than did larger-sized particles. It is possible that the delivery of the smaller particles results in less spore-wall damage, thus allowing for expression in more cells. With the small target of urediniospores, large-sized particles may not have the ability to penetrate consistently without lethal consequences. Bhairi and Staples (1992) reported that M-5 tungsten particles appeared less efficient than larger M-10 particles in their *U. appendiculatus* transient expression studies, but acknowledge that their study did not focus on the effect of size. Li et al. (1993) used 1.0-μm gold particles to achieve transient expression of the β-glucuronidase gene in *U. appendiculatus* urediniospores.

In plant transformation systems, microprojectile size has been shown to have an effect on transformation efficiencies. The use of large particle sizes (1.5–3.0 μm) resulted in increased transient expression in microspores of rapeseed (Nehlin et al. 2000), maize (Jardinaud et al. 1993), and white spruce (Li et al. 1994) relative to smaller particles. In contrast, bombardment studies in wheat microspores (Folling and Olesen 2001) and scutellum and inflorescence tissue (Rasco-Gaunt et al. 1999) demonstrated that particle size did not significantly affect transient expression. In maize callus transformation experiments, Randolph-Anderson et al. (1997) demonstrated that smaller particle sizes resulted in greater transformation efficiency relative to larger particle sizes.

A delivery pressure of 900 psi was determined to be optimal for our *P. triticina* urediniospore bombardment experiments using M-5 tungsten microprojectiles. Quadratic modeling of our data suggests that either too little or too much delivery pressure can affect transformation efficiency. Schillberg et al. (2000) delivered 1.0-μm gold particles at bombardment pressures of 400 and 1,500 psi to achieve GUS expression in *P. graminis* urediniospores, but made no quantification of transformation rates. At 400 psi, they observed blue spores and spores with germ tubes that expressed GUS along their entire length. When their construct was delivered into *P. graminis* spores at 1,500 psi, they observed GUS expression, but the spores aggregated and failed to germinate. With our methods, pressure-dependent spore aggregation was not observed. Intermediate delivery pressures between 400 and 1,500 psi were apparently not examined in their studies. Bhairi and Staples (1992) and Li et al. (1993) achieved transient expression from *U. appendiculatus* urediniospores by delivering plasmid-coated particles at 450 and 900, and 1,550 psi, respectively. Similar to our observations, Bhairi and Staples (1992) reported variable GUS-staining intensities from bombarded urediniospores. They suggested that gene copy number might influence this staining phenomenon.

Using three different GUS constructs containing EF-1α, *Hss*1, and actin promoters from *P. graminis* f. sp. *tritici*, we have achieved transient expression in the wheat leaf rust fungus *P. triticina*. The results reported here clearly demonstrate that the heterologous *P. graminis* f. sp. *tritici* actin, EF-1α, and Hsp70 promoters are active when transferred to *P. triticina* urediniospores. Furthermore, our results confirm the work of Schillberg et al. (2000) regarding the expression of EF-1α in rust urediniospores, although no comparisons in efficiency can be made because they did not quantify expression nor show stable integration. Successful use of actin and Hsp70 promoters for rust transformation has not been previously reported in the literature. Other potentially useful gene promoters have been tested in rust fungi expression work. Bhairi and Staples (1992) utilized the homologous infection structure INF24 promoter to drive the GUS gene in bean rust urediniospores, whereas Li et al. (1993) used the heterologous cauliflower mosaic virus (CaMV) 35S promoter fragment to drive the expression of the GUS gene in their bean rust transformation experiments. The transfer of heterologous promoters between basidiomycete fungi has been shown to be effective (Ogawa et al. 1998; Schuren and Wessels 1994).

While we were successful in achieving transient expression with the methods detailed above, variation between and within experiments was high, and transformation efficiencies remained at unsatisfactorily low levels. It is unclear as to whether homologous *P. triticina*-derived elongation factor, actin, or HSP70 promoters would markedly increase efficiencies in this type of assay. The detached leaf assays did show that more GUS expression was observed *in planta*, suggesting that using agar germination may not be an accurate method of quantification, and that transformation is, in fact, higher.

To obtain stable transformants, a selection scheme had to be devised because leaf rust is not amendable to manipulation in vitro, and transformable wheat lines do not have the desired leaf rust resistance genes. Resistance genes provide a strong selection for mutants in the population that can overcome host resistance. *P. triticina* is dikaryotic, and there is concern that both avirulence genes need to be knocked out before a change in phenotype can be observed. However, heterozygous AVR genes of *P. triticina* have an intermediate phenotype, indicating that mutation of one of

the homozygous genes could be phenotypically visualized (Kolmer and Dyck 1994). Wheat lines carrying single rust resistance genes were chosen to provide a strong selection. We recovered several rust lines that had regained virulence, and we suspected that this was due to insertion of the transforming plasmid into *AvrLr39*, *AvrLr42*, and *AvrLr21*. The insertion of the plasmids pGUS6L20 and pBluescript II KS(−) in the genome was stable over four successive passages on selective plants. Stable transformation was also shown in *E. graminis* after 24 asexual cycles with and without selection (Chaure et al. 2000).

Mutants were developed by bombardment and were selected due to a change in avr/vir phenotype. Using PCR, we determined pBluescript II KS(−) inserted within the open-reading frames of two genes (Fig. 6). Nested PCR further verified the presence of pBluescript and the presence of the *Xho*I and *Sac*I cloning sites within pBluescript II KS(−). The sequence alignments are encouraging because chitin synthase has been found in differential cDNA-AFLP analysis of leaf-rust-infected tissue (Zhang et al. 2003), and calmodulin-binding proteins have been associated with MLO regulation of defense against mildew in barley (Kim et al. 2002). It is understood that these may be genes in a pathway and not the avirulence genes themselves; however, it does verify that the selection scheme is successful in identifying mutants and has the potential for insertional mutagenesis.

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References

Bhairi SM, Staples RC (1992) Transient expression of the β-glucuronidase gene introduced into *Uromyces appendiculatus* uredospores by particle bombardment. Phytopathology 82:986–989

Bhairi SM, Staples RC, Freve P, Yoder OC (1989) Characterization of an infection structure-specific gene from the rust fungus *Uromyces appendiculatus*. Gene 81:237–243

Bonas U, Lahaye T (2002) Plant disease resistance is triggered by pathogen-derived molecules: refined models of specific recognition. Curr Opin Microbiol 5:44–50

Chaure P, Gurr SJ, Spanu P (2000) Stable transformation of *Erysiphe graminis*, an obligate biotrophic pathogen of barley. Nat Biotechnol 18:205–207

Christensen AH, Quail PH (1996) Ubiquitin promoter-based vectors for high-level expression of selectable and/or screenable marker genes in monocotyledonous plants. Transgenic Res 5:213–218

Christiansen SK, Knudsen S, Giese H (1995) Biolistic transformation of the obligate plant pathogenic fungus, *Erysiphe graminis* fsp *hordei.* Curr Genet 29:100–102

Dodds PN, Lawrence GJ, Catanzariti A, Ayliffe MA, Ellis JG (2004) The *Melampsora lini AvrL567* avirulence genes are expressed in haustoria and their products are recognized inside plant cells. Plant Cell 16:755–768

Eliam T, Bushnell WR, Anikster Y (1994) Relative nuclear DNA content of rust fungi estimated by flow cytometry of propidium iodide-stained pynciospores. Phytopathology 84:728–735

Ellis J, Dodds P, Pryor T (2000) Structure, function and evolution of plant disease resistance genes. Curr Opin Plant Biol 3:278–284

Feuillet C, Travella S, Stein N, Albar L, Nublat A, Keller B (2003) Map-based isolation of the leaf rust disease resistance gene *Lr10* from the hexaploid wheat (*Triticum aestivum* L.) genome. Proc Natl Acad Sci U S A 100:15253–15258

Flor HH (1971) Current status of the gene-for-gene concept. Annu Rev Phytopathol 9:275–296

Folling L, Olesen A (2001) Transformation of wheat (*Triticum aestivum* L.) microspore-derived callus and microspores by particle bombardment. Plant Cell Rep 20:629–636

Heiser W (1992) Optimization of Biolistic™ transformation using the helium driven PDS-1000/He system Bio-Rad Laboratories, US/EG Bulletin No. 1688

Holden DW, Kronstad JW, Leong SA (1989) Mutation in a heat-regulated hsp70 gene of *Ustilago maydis*. EMBO J 8:1927–1934

Hooker AL (1967) The genetics and expression of resistance in plants to rusts of the genus *Puccinia*. Annu Rev Phytopathol 5:163–182

Huang L, Brooks SA, Li W, Fellers JP, Trick HN, Gill BS (2003) Map-based cloning of leaf rust resistance gene *Lr21* from the large and polyploid genome of bread wheat. Genetics 164:655–664

Jardinaud M-F, Souvré A, Alibert G (1993) Transient GUS gene expression in *Brassica napus* electroporated microspores. Plant Sci 93:177–184

Kim MC, Panstruga R, Elliott C, Muller J, Devoto A, Yoon HW, Cho MJ, Schulze-Lefert P (2002) Calmodulin interacts with MLO protein to regulate defense against mildew in barley. Nature 416:447–450

Knott DR (1989) The wheat rusts-breeding for resistance. Springer-Verlag, Berlin Heidelberg New York

Kolmer JA, Dyck PL (1994) Gene expression in the *Triticum aestivum–Puccinia recondita* fsp *tritici* gene-for-gene system. Phytopathology 84:437–440

Li A, Altosaar I, Heath MC, Horgen PA (1993) Transient expression of the beta-glucuronidase gene delivered into urediniospores of *Uromyces appendiculatus* by particle bombardment. Can J Plant Pathol 15:1–6

Li Y-H, Tremblay FM, Séguin A (1994) Transient transformation of pollen and embryogenic tissues of white spruce (*Picea glauca* (Moench) Voss) resulting from microprojectile bombardment. Plant Cell Rep 13:661–665

Long DL, Kolmer JA (1989) A North American system of nomenclature for *Puccinia recondita* f sp *tritici.* Phytopathology 79:525–529

Long DL, Leonard KJ, Roberts JJ (1998) Virulence and diversity of wheat leaf rust in the United States in 1993 to 1995. Plant Dis 82:1391–1400

Martin GB, Bogdanove AJ, Sessa G (2003) Understanding the functions of plant disease resistance proteins. Annu Rev Plant Biol 54:23–61

McIntosh RA, Wellings CR, Park RF (1995) Wheat rusts: an atlas of resistance genes, CSIRO Australia. Kluwer Academic Publishers, Dordrecht

Nehlin L, Möller C, Bergman P, Glimelius K (2000) Transient β-*gus* and *gfp* gene expression and viability analysis of microprojectile bombarded microspores of *Brassica napus* L. Plant Physiol 156:175–183

Ogawa K, Yamazaki T, Hasebe T, Kajiwara S, Watanabe A, Asada Y, Shishido K (1998) Molecular breeding of the basidiomycete *Coprinus cinereus* strains with high lignin-decolorization and degradation activities using novel heterologous protein expression vectors. Appl Microbiol Biotechnol 49:285–289

Randolph-Anderson B, Boynton JE, Dawson J, Dunder E, Eskes R, Gillham NW, Johnson A, Perlman PS, Suttie J, Heiser WC (1997) Sub-micron gold particles are superior to larger particles for efficient Biolistic® transformation of organelles and some cell types. Bio-Rad, US/EG Bull 2015

Rasco-Gaunt S, Riley A, Barcelo P, Lazzeri PA (1999) Analysis of particle bombardment parameters to optimize DNA delivery into wheat tissues. Plant Cell Rep 19:118–127

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: a laboratory manual, 2nd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY

Schillberg S, Tiburzy R, Fischer R (2000) Transient transformation of the rust fungus *Puccinia graminis* f sp *tritici*. Mol Gen Genet 262:911–915

Schuren FH, Wessels JG (1994) Highly efficient transformation of the homobasidiomycete *Schizophyllum commune* to phleomycin resistance. Curr Genet 26:179–183

Staples RC (2000) Research on the rust fungi during the twentieth century. Annu Rev Phytopathol 38:49–69

Thara VK, Fellers JP, Zhou J-M (2003) *In planta* induced genes of *Puccinia triticina*. Mol Plant Pathol 4:51–56

Torisky R, Fellers JP, Collins GB (1996) A focusing device for tissue transformation with the DuPont/BioRad PDS1000 helium microprojectile system. Plant Mol Biol Rep 14:124–133

Zambino PJ, Kubelik AR, Szabo LJ (2000) Gene action and linkage of avirulence genes to DNA markers in the rust fungus, *Puccinia graminis*. Phytopathology 90:819–826

Zhang L, Meakin H, Dickinson M (2003) Isolation of genes expressed during compatible interactions between leaf rust (*Puccinia triticina*) and wheat using cDNA-AFLP. Mol Plant Pathol 4:469–477