Technical Advance

A Transient Assay System for the Functional Assessment of Defense-Related Genes in Wheat

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The generation and characterization of transgenic wheat plants is a tedious and time-consuming process that limits the number of putatively important transgenes that can be tested. We therefore established a transient assay system based on wheat leaves to study the effect of transiently expressed genes on the interaction with the wheat powdery mildew fungus *Erysiphe* (syn. *Blumeria*) *graminis* f. sp. *tritici*. Young wheat leaves were bombarded with tungsten particles coated with a mixture of plasmids carrying the β -glucuronidase (*GUS*) reporter gene and a test gene. Leaves were subsequently challenge inoculated with *E. graminis* and the fungus was allowed to develop for 40 h. After being stained for *GUS* enzymatic activity as well as for epiphytic fungal structures, the phenotype of transformed epidermal cells was evaluated by bright-field microscopy. The fungus was routinely found to penetrate cells transiently expressing *GUS* with an efficiency of approximately 35%, which should suffice to detect putative transgene effects. Transgenes encoding a low-molecular-weight cell-wall protein of wheat (*WIR1*), a thaumatin-like protein, and a glucanase had no effect on fungal penetration of transformed epidermal cells. On the other hand, transgenes encoding a pathogen-induced wheat protein of unknown function (*WC15*), a chitinase, a glucose oxidase, and a putative peroxidase significantly reduced fungal penetration.

Wheat is included in many breeding programs worldwide (see, e.g., Bushuk 1998; McIntosh 1998), one important breeding criterion being pathogen resistance. In recent years, transgenic plants have become more and more important in plant breeding efforts and, since the breakthrough in stable genetic transformation of wheat (Vasil et al. 1992), the transgenic approach is also in principle applicable for wheat. However, the generation and assessment of transgenic wheat plants is still a tedious and time-consuming process that limits the number of putatively antimicrobial transgenes that can be tested (Altpeter et al. 1996; Nehra et al. 1994). It would therefore be helpful to first generate functional data concerning the putatively antimicrobial transgene in a transient expression system based on differentiated tissue that is challenge inocu-

lated with a suitable pathogen. A widespread method for transient expression of foreign genes is bombardment with gold or tungsten particles coated with plasmid DNA carrying the gene(s) of interest (see, e.g., Gopalan et al. 1996; Hensgens et al. 1993; Leister et al. 1996). The pathosystem of cereals with the powdery mildew fungus *Erysiphe* (syn. *Blumeria*) *graminis* is particularly amenable to such a functional transient approach, since the fungus attacks only the outermost, epidermal cell layer that is the primary target for transformation by particle bombardment. Indeed, transient assay systems for assessment of defense-related genes in barley were recently described. The first system is based on barley coleoptile cells co-expressing the *B-peru* gene and defense-related genes (Nelson and Bushnell 1997). The second system is based on barley leaves co-expressing the green fluorescent protein (*GFP*) gene and test genes like *Mlo* or *mlo* alleles (Shirasu et al. 1999). Here, we describe a functional, transient test system for defense-related genes in wheat leaves.

RESULTS AND DISCUSSION

Transient gene expression in leaves.

Tungsten particles coated with plasmid DNA encoding four different transgenes under the control of the cauliflower mosaic virus (CaMV) 35S promoter (Fig. 1) were shot into wheat leaf segments by a particle inflow gun (Finer et al. 1992). The *uidA* β -glucuronidase (*GUS*) gene of *Escherichia coli* and the *GFP* gene of *Victorea aquorea* are commonly used reporter genes (Cubitt et al. 1995; Jefferson et al. 1987), whereas the wheat *germin gf-2.8* gene (Lane et al. 1991), which encodes a protein with oxalate oxidase (OXOX) activity, and the glucose oxidase (*GOX*) gene of *Aspergillus niger* (Frederik et al. 1990) represent novel, putatively useful reporter genes for transient expression. *germin* and *GOX* may be suitable reporter genes since no equivalent, endogenous genes are known to be expressed in the epidermis of healthy or pathogen-attacked wheat leaves (P. Schweizer, A. Christoffel, and R. Dudler, *unpublished*; Zhou et al. 1998), and since the sensitivity of detection was comparable to that of *GUS* or *GFP*. In a typical experiment, we detected 7.3 ± 2.9 *GUS*-expressing cells per leaf bombarded with pNcoGUS. The respective numbers were 11.9 ± 1.5 positive cells for the *GFP* gene, 9.7 ± 0.5 positive cells for the *OXOX* gene, and 10.4 ± 1.7 positive cells for the *GOX* gene. Figure 2 shows expression of each of the four reporter genes in epidermal cells, detected

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either by direct autofluorescence (*GFP*) or by in situ enzymatic activity (*GUS*, *germin*, *GOX*) of the respective gene products. The in situ activity of *germin* (OXOX) and *GOX* in bombarded leaves, as revealed by the oxidation of 4-chloro-1-naphthol in a coupled peroxidase reaction, was dependent on the corresponding reporter gene as well as on exogenously added oxalic acid and glucose, respectively (data not shown). This demonstrates that oxidation of 4-chloro-1-naphthol was not due to spontaneous H_2O_2 production of bombarded leaves. The white dot inside the *GFP*-expressing cell represents the extremely brightly fluorescing cell nucleus, where the *GFP* protein appeared to be enriched for unknown reasons (Fig. 2B). The dark border of the *germin*-expressing cell may reflect apoplastic localization of the *germin*-transgene product (Fig. 2C). The halo of 4-chloro-1-naphthol oxidation around the *GOX*-expressing cell might be due to some diffusion of the apoplastic *GOX*, which is not known to be cell wall associated, in contrast to *germin* (Berna and Bernier 1997) (Fig. 2D). Different parameters of the bombardment protocol were varied in order to find an optimal compromise between transformation efficiency and tissue damage (Table 1). Reducing the pressure from 1.4 to 1.0 MPa and the particle density from 50 mg ml⁻¹ to 25 mg ml⁻¹ reduced the damaging impact of the bombardment and enhanced fungal penetration rate (data not shown). A pressure below 1 MPa drastically reduced the transformation efficiency (data not shown). A comparison of the CaMV 35S and polyubiquitin1 promoters (Christensen et al. 1992) revealed a similar number of *GUS*-expressing cells, although the polyubiquitin1 promoter caused a slightly higher intensity of *GUS* staining per cell than did the CaMV 35S promoter (Table 1; and data not shown). Thus, both promoters appeared suitable for transient expression in wheat leaves. All subsequent experiments were conducted at 1 MPa He₂ pressure with 1.1- μ m-diameter tungsten particles. Per shot, 125 μ g of tungsten particles, coated with 1 μ g of supercoiled plasmid DNA, was delivered.

Interaction of transformed cells with *E. graminis* f. sp. *tritici*.

For functional testing of defense-related genes, a co-expression strategy with the *GUS* reporter gene was chosen, as outlined in Figure 3. Leaves were bombarded with tungsten particles coated with a 1:2 (wt/wt) mixture of plasmids carrying the test gene under the control of the CaMV 35S promoter and the *GUS* gene under the control of the polyubiquitin1 promoter, respectively. Four hours later, leaves were challenge inoculated with *E. graminis* f. sp. *tritici*. The fidelity of co-expression in inoculated leaves was determined in double-staining experiments with leaves that had been bombarded with particles coated with a 1:1 (wt/wt) mixture of pUbiGUS and pGermin (Fig. 1). This combination of plasmids resulted in 22 $\pm$ 5% of transformed cells expressing only *GUS*, 5 $\pm$ 1% expressing only *germin*, and 73 $\pm$ 5% with detectable expression of both reporter genes. This co-expression rate should be sufficiently high for detecting eventual antifungal effects of transgenes whose expression cannot be directly visualized. Figure 2E shows an example of a double-stained cell in a non-inoculated leaf (see also Materials and Methods). While expression of *GUS* alone resulted in a more or less uniform blue staining of the whole cell, co-expression of *GUS* and *germin* resulted in cells with a blue staining at the center due to *GUS*

expression and a dark, brownish staining at the periphery, which was due to *germin* expression (compare Figure 2E with 2A and C). Figure 2F shows that the fungus was able to establish an apparently susceptible interaction with a transformed cell expressing *GUS*, as reflected by the formation of a mature haustorium and elongating secondary hyphae. For scoring of the interaction phenotype with transformed cells, only epidermal cells of type A, B, and C were considered, according to Koga et al. (1990) (Fig. 2G). Trichomes and stomata, including companion cells, were disregarded because haustoria were hardly visible in these *GUS*-stained cells. Slim cells between trichomes were disregarded because this cell type showed an enhanced resistance to fungal penetration (data not shown). The *GUS* reporter gene was used to mark transformed cells for the following practical reasons: (i) microscopic slides with stained leaves can be stored for several weeks without

Fig. 1. Plasmid constructs used for transient expression. Constructs are not drawn to scale. Abbreviations used: 35S, 540-bp fragment of the cauliflower mosaic virus (CaMV) 35S promoter; *Polyubiquitin1*, 1,850-bp fragment of the maize polyubiquitin1 promoter followed by the polyubiquitin1 intron in the 5' nontranslated leader; *GUS*, β -glucuronidase (*uidA*) gene; *germin*, wheat *germin* *gf-2.8* gene (syn. oxalate oxidase [*OXOX*]); *GOX*, glucose oxidase gene from *Aspergillus niger*; *GFP*, green fluorescent protein gene from *Aequorea victoria*; *WIR1-3* and *WCI5*, pathogen-induced genes from wheat; *Gluc*, pathogen-induced β -1,3 glucanase-encoding gene from wheat; *CHI26*, chitinase-encoding gene active in barley grain. Hatched box indicates the CaMV 35S transcriptional terminator.

loss of information; (ii) epiphytic fungal structures can be stained with Coomassie blue without disturbing the detection of transgene expression; (iii) the fungal haustorium or the surrounding extrahaustorial matrix is strongly stained by the GUS product in *GUS*-expressing cells, which facilitates microscopic evaluation of interaction types; (iv) *GUS* is an intracellular protein and does not come into contact with the

haustorium that is invaginating the host plasmalemma (Thordal-Christensen et al., *in press*), thereby reducing the risk of some direct effect of *GUS* on *E. graminis* f. sp. *tritici*. In general, in our hands, the use of the *GUS* reporter gene was more convenient, especially for analysis of thousands of transformed leaves, than the *GFP* reporter gene that was used in a transient assay system of barley (Shirasu et al.

Fig. 2. Transient reporter gene expression and interaction of transformed cells with *Erysiphe graminis* f. sp. *tritici*. **A**, β -Gucuronidase gene (*GUS*)-expressing epidermal cell of a wheat leaf bombarded with tungsten particles coated with pNcoGUS carrying the *uidA* (*GUS*) gene. **B**, Green fluorescent protein gene (*GFP*)-expressing cell after bombardment with pGFP carrying the *GFP* gene. **C**, *germin* (wheat *germin gf-2.8* gene)-expressing cell after bombardment with pGermin carrying the *germin gf-2.8* (syn. *OXOX*) gene. **D**, Glucose oxidase gene (*GOX*)-expressing cell after bombardment with pGOX carrying the *GOX* gene. **E**, Co-expression of *GUS* and *germin* (*OXOX*) genes in a single epidermal cell that had been bombarded with a 1:1 (wt/wt) mixture of pUbiGUS and pGermin (compare also with **A** and **C**). **F**, Leaves were bombarded with tungsten particles coated with a 1:2 mixture of pUbiGUS and pGY1 empty vector and subsequently challenge inoculated with *E. graminis* f. sp. *tritici*. A conidium (CON) of *E. graminis* formed an appressorium (APP) that successfully penetrated a cell transiently expressing *GUS* and formed a haustorium (HAU) and elongating secondary hyphae (ESH). Epiphytic fungal structures were stained with Coomassie blue. Picture was taken 40 h post inoculation. **G**, Scanning electron microscopic picture of adaxial leaf surface of wheat showing different cell types. Cells marked in red, including trichomes, extra slim cells between trichomes lying overneath vascular bundles, as well as stomata, were not used for evaluation of interaction phenotypes. **A**, **B**, and **C**, Cells marked in blue, green, and yellow, respectively, were used for evaluation of interaction phenotypes. **A–G**, For plasmid constructions see Figure 1. Twenty-four hours (in **F**: 44 h) after bombardment, leaves were infiltrated with the respective staining solutions for in situ detection of *GUS*, *OXOX*, or *GOX* activity. For *GFP* expression, leaves were observed by incident blue light fluorescence microscopy 24 h after bombardment. Top view of whole leaf segments. Scale bar = 50 μ m.

1999). A measure for resistance against *E. graminis* is the penetration efficiency (PE), which is defined as the percentage of mature appressoria that are associated with a haustorium. Table 2 shows that bombardment resulted in a drop of PE on both *GUS*-expressing cells as well as on neighboring, nontransformed cells, compared with intact plants or non-bombarded leaf segments placed on phytoagar. The data also show that the senescence inhibitor benzimidazole that was included in the phytoagar had no impact on early fungal development (Leath and Heun 1990). The fact that *GUS*-expressing cells appeared more susceptible than nontransformed neighboring cells probably reflects the presence of damaged or dead neighboring cells that no longer support normal fungal development. Although Nelson and Bushnell (1997) reported no significant reduction of PE on transformed coleoptile cells expressing the B-peru gene, compared with nonbombarded coleoptiles, the absolute PE values in that system were still lower than PE on *GUS*-expressing wheat cells in our system (20 vs 35%). The routinely observed PE of *GUS*-expressing cells should be sufficiently high to detect putative antifungal transgene effects upon co-expression of *GUS* and a test gene (see below).

A critical factor for the transient assay system is timely transgene expression in relation to fungal development. Figure 4 shows that OXOX accumulation of transformed cells preceded appressorial germ tube formation of the fungus if the time interval between bombardment and challenge was 4 h. Thus, putatively antifungal transgene products may have accumulated in high enough amounts prior to attempted fungal penetration, which occurs 10 to 12 h post inoculation, to interfere with this important step of fungal development. Even upon challenge inoculation immediately after the bombardment, the accumulation of the transgene product was near its maximum at the time of attempted fungal penetration.

Control of test gene expression.

Unexpectedly, we occasionally observed a drop in *germin* expression when leaves bombarded with pGermin had been inoculated with *E. graminis*. The same phenomenon was observed with two other members of the *germin*-like gene family (P. Schweizer, A. Christoffel, and R. Dudler, *to be published elsewhere*). These unpredictable expression levels

Fig. 3. Experimental scheme for the transient assay system in wheat. Egt, *Erysiphe graminis* f. sp. tritici.

Table 1. Parameter optimization for transient expression in wheat leaves^a

Experiment	Pressure (MPa)	Particle material	Particle diameter (μm)	Particle density (mg ml ⁻¹)	DNA per shot (μg)	Promoter	GUS ⁺ cells per leaf
A	1.0	Tungsten	1.5	50	1.0	CaMV 35S	9.8 ± 0.2
A	1.0	Gold	1.5	50	1.0	CaMV 35S	3.3 ± 1.0
B	1.0	Tungsten	1.5	50	1.0	CaMV 35S	16.7 ± 2.0
B	1.0	Tungsten	1.5	50	2.5	CaMV 35S	12.1 ± 2.3
C	1.0	Tungsten	0.4	50	1.0	Ubiquitin	4.7 ± 1.7
C	1.0	Tungsten	1.1	50	1.0	Ubiquitin	26.3 ± 2.7
C	1.0	Tungsten	1.5	50	1.0	Ubiquitin	16.0 ± 1.5
C	1.4	Tungsten	0.4	50	1.0	Ubiquitin	10.3 ± 7.2
C	1.4	Tungsten	1.1	50	1.0	Ubiquitin	32.1 ± 6.8
C	1.4	Tungsten	1.5	50	1.0	Ubiquitin	21.1 ± 3.3
D	1.0	Tungsten	1.1	25	1.0	CaMV 35S	6.7 ± 2.9
D	1.0	Tungsten	1.1	50	1.0	CaMV 35S	9.3 ± 2.1
D	1.0	Tungsten	1.1	25	1.0	Ubiquitin	13.0 ± 5.0
D	1.0	Tungsten	1.1	50	1.0	Ubiquitin	11.3 ± 1.4

^a Parameters varied per experiment are indicated in bold; CaMV = cauliflower mosaic virus; GUS = β-glucuronidase.

in inoculated leaves prompted us to establish an expression control for all test genes used, which consisted of test gene::GFP fusion constructs (Fig. 1). Thus, in every experiment, additional leaves were bombarded with the test gene::GFP fusion construct corresponding to the respective test gene. One half of the leaves bombarded with the test gene::GFP fusion construct were left noninoculated whereas the second half were inoculated with *E. graminis* f. sp. *tritici*. Table 3 shows that, for all test genes presented here, the expression control suggested reproducible expression levels that were not influenced by the pathogen. For *GOX*, which has reporter gene activity, no GFP fusion construct was made (Table 3). The reason for the drop of transgene expression of the *germin*-like gene family is still not clear. For test gene::GFP constructs with apoplastic fusion partners, it was essential to remove the N-terminal signal peptide, otherwise GFP fluorescence strongly decreased or became undetectable (Fig. 5). This might be due to reduced GFP fluorescence at the acidic pH in the apoplast (Bokman

Table 2. Influence of leaf bombardment on penetration efficiency of *Erysiphe graminis* f. sp. *tritici*

Leaf treatment	Benzimidazole in phyto-agar	Bombarded with pUbiGUS	β -glucuronidase (<i>GUS</i>) expression	PE (%) ^a
On intact seedling	No	No	No	86 $\pm$ 6
Detached, on phytagar	No	No	No	85 $\pm$ 3
Detached, on phytagar	Yes	No	No	87 $\pm$ 2
Detached, on phytagar	Yes	Yes	No ^b	14 $\pm$ 4
Detached, on phytagar	Yes	Yes	Yes ^c	35 $\pm$ 6

^a Penetration efficiency. Mean $\pm$ SD.

^b Determined on type A, B, and C cells not expressing *GUS*, at least two rows of cells separated from *GUS*-expressing cells (see Figure 2G).

^c Determined on type A, B and C cells expressing *GUS* (see Figure 2G).

Fig. 4. Rate of transient *germin gf-2.8* (syn. *OXOX*) gene expression compared with early fungal development. Leaves were bombarded with tungsten particles coated with pGermin. At times indicated after bombardment, leaves were infiltrated with *OXOX* staining solution, a treatment that kills leaves and thus reveals only levels of transient *germin* (*OXOX*) expression before the infiltration. Data of appressorial germ tube (APG) formation were taken from Schweizer et al. (1989). Leaves were challenge inoculated 4 h after bombardment.

and Ward 1981). The concentration of GFP fluorescence at the border of cells expressing a construct that encodes a β -1,3 glucanase::GFP fusion protein containing the N-terminal signal peptide may indicate correct targeting of the fusion protein to the apoplast, although proof thereof is lacking (Fig. 5B).

Transgene effects.

The transient assay system described here was tested with a number of transgenes, as shown in Table 4. In general, the system produced reproducible data, making the distinction between ineffective and effective transgenes an easy task. The transgenes *WIR1* and *WIR2* (encoding a thaumatin-like protein) and a β -1,3-glucanase gene did not reduce the PE of

Table 3. Control of test gene expression in inoculated leaves

Plasmid ^b	Transgene-expressing cells per shot ^a		<i>n</i>
	Control	Inoculated ^c	
pWIR1GFP	22.4 $\pm$ 4.0 ^d	24.6 $\pm$ 4.4	5
pWIR2GFP	23.8 $\pm$ 3.5	25.6 $\pm$ 5.0	5
pWIR3GFP	13.0 $\pm$ 4.8	13.8 $\pm$ 4.0	6
pWCI5GFP	27.8 $\pm$ 3.6	25.6 $\pm$ 5.2	5
pGlucGFP	9.6 $\pm$ 2.5	10.0 $\pm$ 1.8	5
pCHI26GFP	17.3 $\pm$ 1.7	12.5 $\pm$ 0.9	4
pGOX	19.7 $\pm$ 3.7	17.0 $\pm$ 2.9	4

^a Only cell types A, B, and C (Figure 2G) were counted.

^b For details see Figure 1.

^c Leaves were inoculated with *Erysiphe graminis* f. sp. *tritici* 4 h after bombardment.

^d Mean $\pm$ SE from *n* independent experiments.

Fig. 5. Control of test gene expression by test gene::green fluorescent protein (*GFP*) fusion constructs. **A**, Expression of a construct encoding a β -1,3 glucanase-GFP fusion protein lacking the N-terminal signal peptide for apoplastic localization. **B**, Expression of a construct encoding a β -1,3 glucanase-GFP fusion protein containing the N-terminal signal peptide for apoplastic localization. Expression in noninoculated leaves is shown. For plasmid constructs see Figure 1. Scale bar = 50 μ m.

E. graminis f. sp. *tritici*. The transgenes *CHI26* (encoding a barley seed chitinase) and *WCI5* had a marginal effect, whereas *GOX* (encoding glucose oxidase) from *Aspergillus niger* (Frederik et al. 1990) as well as *WIR3* (encoding a putative peroxidase) clearly enhanced the penetration resistance of transformed cells. *GOX* has been reported to confer disease resistance to transgenic plants (Wu et al. 1997). The effect of the *WIR3* gene was especially strong: The degree of protection (70%) was comparable to the observed fidelity of co-expression of the *GUS* and the test gene (73%). Therefore, it may be assumed that transient expression of the *WIR3* gene conferred almost complete protection. We are currently characterizing the *WIR3* protein in more detail. Similar transgenes were also tested in a barley coleoptile system (Bushnell et al. 1998). In contrast to our findings, genes encoding a thaumatin-like protein, a glucanase, and a chitinase had a stronger antifungal effect. The genes tested in the coleoptile system were not identical to the genes tested in the study presented here, and therefore it remains unclear whether the differences in the two transient assay systems or the differences in the transgenes caused these conflicting results. We analyzed the expression of defense-related genes in wheat coleoptiles and found that, depending on the growth conditions, many defense-related genes were strongly expressed in a constitutive manner (R. Bruggmann, R. Dudler, and P. Schweizer, *unpublished observation*). This may influence results obtained in a coleoptile-based system.

In summary, we present a method for rapid, functional assessment of transgenes encoding putative antifungal proteins that produced reproducible data. Per week, two experiments can be carried out and evaluated by a single experimenter. The extent of a standard experiment is shown in Figure 3. Per test gene, five repetitions of independent experiments were found to be sufficient to produce statistically highly significant data. The transient assay system may not only be valuable as a pre-screening system for the generation of transgenic wheat lines but may also permit a functional insight into the interaction of wheat with *E. graminis* f. sp. *tritici*.

Table 4. Effect of transient expression of transgenes on penetration efficiency by *Erysiphe graminis* f. sp. *tritici*

Transgene ^a	Series	PE (%) ^b	rel PE ^c	Exp ^d	Int ^e	P (t test)
GUS	A	28 ± 3	100	5	577	...
GUS + WIR1	A	36 ± 3	135 ± 17	5	582	0.05
GUS	B	35 ± 3	100	5	335	...
GUS + WIR2	B	33 ± 3	97 ± 12	5	300	0.35
GUS	C	29 ± 4	100	5	492	...
GUS + WIR3	C	9 ± 3	30 ± 9	5	303	0.005
GUS	D	38 ± 1	100	4	307	...
GUS + WCI5	D	31 ± 1	81 ± 3	4	224	0.0025
GUS	E	30 ± 4	100	5	438	...
GUS + Gluc	E	33 ± 5	114 ± 12	5	352	0.35
GUS	F	35 ± 3	100	5	384	...
GUS + CHI26	F	26 ± 2	76 ± 10	5	252	0.05
GUS	G	33 ± 3	100	5	548	...
GUS + GOX	G	20 ± 3	63 ± 10	5	452	0.01

^a For constructs and references see Figure 1.

^b Penetration efficiency. Mean ± SE from 4–5 independent experiments.

^c Relative penetration efficiency, compared with β -glucuronidase (*GUS*)-expressing controls.

^d Number of independent experiments.

^e Total number of observed interactions on GUS-stained cells.

MATERIALS AND METHODS

Plant and fungus.

Seven-day-old seedlings of winter wheat (*Triticum aestivum* cv. Fidel) were used for all experiments. Plants were grown in a growth chamber (16 h light period at 26°C; 8 h dark period at 12°C; 60% relative humidity). For challenge inoculation, a Swiss field isolate of *E. graminis* f. sp. *tritici* that was maintained on wheat cv. Kanzler by weekly transfer to fresh plants was used. This isolate is highly virulent on cv. Fidel, resulting in 86 and 67% PE on the adaxial and abaxial leaf surface, respectively.

Plasmid constructions.

With the exception of pUbiGUS, all constructs were derived from the pUC18-based plant expression vector pGY1 (Shinshi et al. 1990) that contains a 540-bp fragment of the CaMV 35S promoter and the CaMV 35S terminator separated by a multiple cloning site. Constructs obtained by polymerase chain reaction (PCR) were not verified by sequencing. Instead, proofreading *Pwo* polymerase was used, and all constructs were functionally tested (GFP fluorescence or GOX activity; see below).

The reporter-gene constructs were obtained by inserting the coding regions of one of four reporter genes into the multiple cloning site of pGY1. The *uidA* gene (*GUS*) with an improved plant translation initiation consensus (GTGACCATGGTC) that was derived from the expression vector pRAY275 (EMBL accession no. U02456) was inserted as a *Sall/EcoRI* fragment. A genomic fragment of the wheat *germin gf-2.8* gene was excised from pPH100 (Elf Sanofi 1994) and inserted as a *BamHI/PstI* fragment. A PCR fragment derived from the glucose oxidase (*GOX*; E.C.1.1.3.4) gene of *Aspergillus niger* (Frederick et al. 1990) was inserted into the *BamHI* site of pGY1. The *GOX* PCR fragment was amplified from *A. niger* genomic DNA with the primers 5'-TATTATGGATCCCCCATCTGCCCATCATGCAGACT and 5'-ATATATGGATCCTACCACTCACTGCATGGAAGCATA. The ends of the PCR fragment of 1,836 bp were digested with *BamHI* prior to ligation. An *EcoRV/NaeI* fragment of pGFPemd-b containing GFPemerald (Packard, Meriden, CT), the GFP of *Aequorea victoria*, was inserted into the *SmaI* site of pGY1.

pUbiGUS contains a transcriptional fusion between a 1,850-bp fragment of the maize polyubiquitin1 promoter including an intron in the 5' nontranslated leader (Christensen et al. 1992) and the coding region of the *GUS* gene followed by the CaMV 35S terminator (J. Fütterer, ETH Zürich, Switzerland, *unpublished*).

The test gene constructs pWIR1, pWIR2, pWIR3, pWCI5, pGluc, and pCHI26 were obtained by inserting cloned, near-full-length cDNAs encompassing the AUG translation initiation codon into the multiple cloning site of pGY1. The cDNAs were inserted as the following fragments: *WIR1*, *SmaI/XhoI*; *WIR2*, *BamHI/XhoI*; *WIR3*, *BamHI/XhoI*; *WCI5*, *BamHI/XhoI*; *Gluc*, *SmaI/XhoI*; *CHI26*, *PstI*. With the exception of *CHI26*, all cDNAs were derived from Lambda ZAPII, which means that the *XhoI* site lies downstream of the cloned part of the poly(A) tail. The *PstI* fragment of *CHI26* including the CaMV 35S transcriptional terminator was excised from pGluChi (Bliffeld et al. 1999) and extends from 31 bp upstream of the AUG initiation codon to 138 bp downstream of the stop codon (for references see Figure 1).

The test gene::GFP fusion constructs were obtained by inserting PCR fragments derived from the corresponding test genes into a pGFP derivative where the 5' nontranslated leader and the AUG initiation codon had been replaced by a *Bst*EII site. The following PCR primers were used: pWIR1GFP, 5'-TTAATTCCCGGGACCAGCATGGCGCTTGGAGCCGCCG TCC and 5'-ATTTAAGGTGACCGGCTGGCTACCCGCC GG; pWIR2GFP, 5'-TTAATTGGATCCCCAGCAATGGCG GCCACCTTCAACATCA and 5'-TATATAGGTGACCGG ACAGAAGGTGATCTG; pWIR3GFP, 5'-GAAACAGCTA TGACCATG and 5'-TATTATGGATCCCTAACCATGGCC CAGCTGTCACCGACCTT; pWCI5GFP 5'-TTAATTGGAT CCCGCACGATGAAGGTGACGTTTCGACGCGA and 5'-TA TATAGGTGACCCCTGGGGCTGGGGGTA; pGluGFP, 5'- TTAATTGGATCCGCAGCAATGGCGATCGGCGTGTG CTACG and 5'-AATTAAGGTGACCTTGAACCTGGATGT TGT; pGluC(+Sig)GFP, 5'-TTAATTGGATCCGCAGCAAT GGCGACAAAGG and 5'-AATTAAGGTGACCTTGAACCT GGATGTTGTA; pCHI26GFP, 5'-TTAATT-GGATCCCGC ACAATGAGAAGCGTGTCTCCATCGT and 5'-TATATAG GTGACCGCGAAGGGTCTCTGGCT. The ends of the PCR fragments were digested with *Bam*HI (pWIR1GFP:*Sma*I) and *Bst*EII prior to ligation.

Transient gene expression.

The apical 4 cm of primary leaves was cut and placed inside 5-cm-diameter petri dishes onto 0.5% (wt/vol) water-phytoagar (Gibco BRL, Paisley, UK) containing 20 mg liter⁻¹ benzimidazole as senescence inhibitor (Leath and Heun 1990). A Teflon stencil (1 mm thick) with a rectangular window of 2.5 × 3.0 cm in the middle was placed onto the leaf segments and fixed with adhesive tape. This setup allowed simultaneous bombardment of six segments. The petri dishes were placed in a particle inflow gun chamber at a distance of 15 cm from the particle accelerator with a nylon mesh of 0.2 mm in between. The particle inflow gun was constructed in house according to Finer et al. (1992). After evacuating the chamber to 0.2 × 10⁵ Pa, leaf segments were bombarded at 1 MPa He₂ pressure with plasmid DNA-coated tungsten 1.1-μm-diameter particles (Bio-Rad, Hercules, CA). One microgram of plasmid DNA and 125 μg of particles were delivered to the leaf segments per shot. Tungsten particles were coated with supercoiled plasmid DNA by the method of Perl et al. (1992), with 1 M Ca(NO₃)₂ at pH 10 as the precipitating agent. Bombarded leaf segments were incubated in closed petri dishes on the bench (indirect daylight). If room temperature exceeded 26°C, leaves were incubated in a climatized Plexiglas hood at 22°C.

For GFP expression, leaf segments were observed by blue-light incident fluorescence microscopy (excitation filter 450 to 490 nm; bypass filter 515 to 565 nm) 24 h after bombardment. For GUS, *germin*, or GOX expression, leaves were infiltrated with the respective staining solutions under vacuum 24 h after bombardment and incubated overnight at 37°C. GUS-stained leaves were cleared in 70% (vol/vol) ethanol prior to observation. The GUS staining solution contained 0.1 M Na₂HPO₄/NaH₂PO₄ pH 7.0, 10 mM Na-EDTA, 5 mM concentrations of potassium hexacyanoferrat(II) and potassium hexacyanoferrat(III), 1 mg ml⁻¹ 5-bromo-4-chloro-3-indoxyl-β-D-glucuronic acid, cyclohexylammonium salt (X-Gluc; Biosynth, Staad, Switzerland), 0.1% (vol/vol) Triton X-100, 20% (vol/vol) methanol. The germin (OXOX) staining solution

contained 2.5 mM oxalic acid, 3.5 mM free EDTA, 0.6 mg ml⁻¹ 4-chloro-1-naphthol, 50 μg ml⁻¹ horseradish peroxidase (Boehringer Mannheim, Mannheim, Germany), and 20% (vol/vol) ethanol, and was titrated with Tris base to pH 4.0. The GOX staining solution contained 0.1 M sodium phosphate pH 6.0, 0.6 mg ml⁻¹ 4-chloro-1-naphthol, 20 mM D-glucose, 50 μg ml⁻¹ horseradish peroxidase, and 20% (vol/vol) ethanol. For double-staining experiments of GUS and OXOX activity in the same cells, leaves were bombarded with a 1:1 (wt/wt) mixture of pUbiGUS and pGermin. Each plasmid DNA (0.5 μg) was delivered to the leaves per shot. Twenty-four hours after the bombardment, the leaves were first incubated in GUS-staining solution for 8 h, followed by extensive washing in water (including vacuum infiltration), and subsequent vacuum infiltration of the OXOX-staining solution and incubation overnight.

Interaction of transformed cells with *E. graminis* f. sp. *tritici*.

Leaves were bombarded with a 1:2 (wt/wt) mixture of pUbiGUS and pGY1 empty vector (control), or with a 1:2 (wt/wt) mixture of pUbiGUS and pGY1 containing test gene constructs. Leaves were challenge inoculated with *E. graminis* f. sp. *tritici* on the adaxial, more susceptible leaf surface 4 h after the bombardment by shaking 1 to 3 pots of wheat seedlings with heavily sporulating fungus over the petri dishes placed inside an inoculation chamber. On average, this resulted in 150 to 250 conidia per mm² or 1 to 2 interactions per long epidermal cell. Variation of the inoculum density in the range of 50 to 250 conidia per mm² had no obvious effect on early fungal development (data not shown). Challenge-inoculated leaves were incubated inside the slightly opened petri dishes on the bench (indirect daylight) for 40 h. Petri dishes had to be opened in order to prevent droplet formation on the bombarded leaf surface, due to either the presence of condensation nuclei (tungsten particles) or exudation through small wounds. Staining for GUS enzymatic activity was carried out as described above, followed by staining of fungal structures with Coomassie blue (Schweizer et al. 1993). The interaction of GUS-expressing cells with *E. graminis* f. sp. *tritici* was observed by transmission light microscopy. Only cells attacked by primary but not by secondary appressorial lobes were evaluated. Sporadic isolated haustoria without associated epiphytic fungal structures, which apparently had been shared off during leaf treatments, were not counted. Nongerminated or only primary germ tube-forming spores lying on GUS-expressing cells were counted in order to calculate the spore germination rate. A susceptible interaction was characterized by a mature haustorium and elongating secondary hyphae. A resistant interaction was characterized by a fully matured appressorium with 1 to 2 appressorial lobes without haustorium or elongating secondary hyphae. The elongation rate of secondary hyphae was not evaluated.

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