TECHNICAL ADVANCE

A High-Throughput Gene-Silencing System for the Functional Assessment of Defense-Related Genes in Barley Epidermal Cells

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Large-scale gene silencing by RNA interference (RNAi) offers the possibility to address gene function in eukaryotic organisms at a depth unprecedented until recently. Although genome-wide RNAi approaches are being carried out in organisms like *Caenorhabditis elegans*, *Drosophila* spp. or human after the corresponding tools had been developed, knock-down of only single or a few genes by RNAi has been reported in plants thus far. Here, we present a method for high-throughput, transient-induced gene silencing (TIGS) by RNAi in barley epidermal cells that is based on biolistic transgene delivery. This method will be useful to address gene function of shoot epidermis resulting in cell-autonomous phenotypes such as resistance or susceptibility to the powdery-mildew fungus *Blumeria graminis* f. sp. *hordei*. Gene function in epidermal cell elongation, stomata regulation, or UV resistance might be addressed as well. Libraries of RNAi constructs can be built up by a new, cost-efficient method that combines highly efficient ligation and recombination by the Gateway cloning system. This method allows cloning of any blunt-ended DNA fragment without the need of adaptor sequences. The final RNAi destination vector was found to direct highly efficient RNAi, as reflected by complete knock-down of a cotransformed green fluorescent protein reporter gene as well as by complete phenocopy of the recessive loss-of-function *mlo* resistance gene. By using this method, a role of the t-SNARE protein HvSNAP34 in three types of durable, race-nonspecific resistance was observed.

Eight years after the first description of the cosuppression phenomenon in petunia resulting in silencing of sense transgenes (Napoli et al. 1990), RNA interference (RNAi) by double-stranded RNA was discovered simultaneously in plants and in *Caenorhabditis elegans* as the underlying mechanism (Fire et al. 1998; Waterhouse et al. 1998). This discovery opened up new possibilities of genome-wide, high-throughput functional genomics in eukaryotic organisms, including plants. Indeed, a series of milestone reports has been published since then, demonstrating that genome-wide gene silencing is feasible and yields valuable novel information about gene function in *C. elegans* and *Drosophila* spp. as well as human

(Boutros et al. 2004; Fraser et al. 2000; Gonczy et al. 2000; Paddison et al. 2004).

In plants, proof of concept for RNAi as tool of functional genomics has been obtained and, in a few cases, new gene function has been described (Azevedo et al. 2002; Christensen et al. 2004; Chuang and Meyerowitz 2000; Schweizer et al. 2000). Currently, the most advanced systems of medium- to high-throughput RNAi are based on virus-induced gene silencing (VIGS). VIGS has been described and employed first in *Nicotiana benthamiana* (Ruiz et al. 1998), then also in other plants, including barley (Burch-Smith et al. 2004; Holzberg et al. 2002; Lacomme et al. 2003).

Recently developed cereal systems that would, in principle, be amenable to high-throughput approaches are single-cell transient overexpression and transient-induced gene silencing (TIGS) systems in wheat and barley (Gubler et al. 1995; Leduc et al. 1994; Nelson and Bushnell 1997; Nielsen et al. 1999; Schweizer et al. 1999, 2000). These systems are based on biolistic bombardment of dissected grains, coleoptiles, young roots, or young leaves and result in a pattern of single transformed cells in the outermost cell layer, such as the aleuron or the epidermis. One of these systems based on transformed leaf epidermal cells has gained considerable attention during the last few years because it proved valuable for addressing key questions in plant biology related to molecular mechanisms of disease resistance or susceptibility (Panstruga 2004). The pathosystem used mostly for this purpose has been barley and the barley powdery mildew *Blumeria graminis* D. C. Speer f. sp. *hordei* Em Marchal. The single-cell transient expression system has been used successfully to show function for the map-based isolation of resistance-gene candidates *HvMla1*, *HvMla6*, *HvMla7*, *HvMla10*, *HvMla12*, *HvMla13*, and *TaPm3b* (Halterman et al. 2001, 2003; Halterman and Wise 2004; Shen et al. 2003; Yahiaoui et al. 2004; Zhou et al. 2001). Moreover, it enabled the functional confirmation of major defense-related genes such as *HvSGT1*, *HvBAX-inhibitor 1*, and *HvGLP4*, plus a number of pathogenesis-related genes (Azevedo et al. 2002; Christensen et al. 2004; Huckelhoven et al. 2003).

With the availability of the highly efficient, recombination-based Gateway cloning system, the TIGS assay may become amenable to high-throughput, reverse-genetic approaches for the discovery of novel, defense- or susceptibility-related genes in barley or wheat attacked by *B. graminis*. Here, we describe a combination of methods developed for high-throughput screening of genes required for nonhost resistance, *mlo*-mediated resistance, and basal resistance of barley.

RESULTS

High-throughput cloning procedure.

All 135,000 barley expressed sequence tag (EST) clones of the Institute of Plant Genetics and Crop Plant Research (IPK) collection that were planned to be used for RNAi studies are contained in conventional plasmid vectors, which are not compatible with the Gateway system. Therefore, we developed a cloning strategy for obtaining an inverted-repeat RNAi construct library based on polymerase chain reaction (PCR) fragments of selected EST clones, which were obtained by using one or two conventional, gene-specific primers without Gateway attB adaptor sequences (Fig. 1). The advantage of this cloning strategy is i) lack of poly(A) tails in the inverted repeats that might cause nonspecific side effects in plant cells and ii) a more regular repeat length of approximately 500 bp that might increase the overall stability of the RNAi constructs

in the library. In a first step, blunt-ended PCR fragments were ligated at room temperature into the *Swa*I site of pIPKTA38, a modified Gateway entry vector lacking the *ccdB* negative selection marker, in the presence of *Swa*I that had a temperature optimum of 25°C. The presence of this rare-cutting restriction enzyme producing blunt ends efficiently prevented re-ligation of the vector. The second step of the cloning procedure consisted of a standard Gateway LR reaction with the pUC18-based destination vector pIPKTA30N or pIPKTA30R, which differ in the orientation of first and second inverted repeat after recombination. This allowed us to perform strict control experiments regarding the absence of artifacts caused by spurious overexpression of test sequences. After recombination, the final RNAi construct contained the inverted repeats separated by the second intron of the wheat *RG2* gene, 313 bp in length, under the control of a single CaMV 35S promoter. The overall efficiency of the cloning procedure that was adapted to 96-well

Fig. 1. High-throughput cloning strategy of RNA interference (RNAi) constructs. Step I, polymerase chain reaction (PCR) amplification of cDNA fragments from expressed sequence tag clones by using one gene-specific and one universal primer. Step IIa, ligation of nonphosphorylated PCR fragment into the modified Gateway pENTR vector pIPKTA38 in the presence of blunt-cutting restriction enzyme *Swa*I. Step IIb, elimination of remaining re-ligated pIPKTA38 by *Swa*I after heat inactivation of the ligase. Step III, insertion of the PCR fragment into the final RNAi Gateway destination vector pIPKTA30N or pIPKTA30R (differing in the order of attR1 and attR2 sites relative to the direction of transcription) by LR clonase. Only restriction sites relevant for the cloning procedures are shown.

plate format (details discussed below) was found to be 90 to 98% (Fig. 2). Despite the fact that PCR fragments sometimes were too low in abundance to produce visible bands in agarose gels, final constructs were obtained, indicating that the ligation procedure was very efficient (data not shown). The design of the RNAi destination vector allowed easy monitoring of accuracy of the final constructs that were expected to produce a double-insert band. For example, constructs Nr. 1 and 3 (Fig. 2C) easily could be detected as problematic ones that might have resulted from incorrect recombination, construct instability in *Escherichia coli*, or the presence of additional restriction sites on the inverted repeats.

High-throughput TIGS system.

The RNAi constructs were used for TIGS in barley leaf epidermal cells as described previously (Schweizer et al. 2000); however, a few modifications were introduced (as specified below). Most importantly, the method of quantitative microscopy of interaction phenotypes was changed in order to enhance throughput. Before, microscopic analysis was based on appressoria attacking transformed cells, resulting in the calculation of penetration rates (Schweizer et al. 1999). That method was time consuming because it required accurate counting of all appressorial lobes that really attacked the transformed cell under investigation and not neighboring cells, in addition to counting of haustoria inside transformed cells. With the modified method presented here, only haustoria inside transformed cells were counted and related to the total number of transformed cells per bombardment, resulting in the haustorial index. Alternatively, the susceptibility index was calculated (fraction of transformed cells bearing at least one haustorium). This

Fig. 2. Example of the efficiency of high-throughput generation of RNA interference constructs. One-fourth of 96 reactions processed in parallel in a microtiter plate are shown. The final constructs (numbers 1 and 3) did not show the expected restriction fragments, which might be due to internal *EcoRV* sites of the polymerase chain reaction (PCR) fragments or to some instability of the inverted-repeat constructs. **A**, PCR fragments; **B**, recombinant pIPKTA38 digested with *EcoRI*; **C**, recombinant pIPKTA30N digested with *EcoRV*.

analysis method was dependent on a very regular inoculum density across all bombardments within an experiment, which was achieved by pinning down leaf segments for challenge inoculation (preventing curling) and by mixing leaf segments from all bombardments per experiment. In order to test whether the higher throughput interaction-scoring method produces reliable results, the effect of a test construct encoding a wheat peroxidase (pWIR3) (Schweizer et al. 1999) was compared by applying both analysis methods. Both methods produced very similar results and revealed enhanced resistance of those cells that transiently overexpressed the *TaPERO* gene (Table 1).

RNAi efficiency.

Efficiency of TIGS in barley was tested by using three constructs that should reproduce previously described phenotypes caused by mutation or RNAi (Fig. 3). First, double-stranded RNA of the *Mlo* gene was found to partially phenocopy the resistance phenotype mediated by recessive *mlo* alleles (Panstruga and Schulze-Lefert 2002). Second, a hairpin RNAi construct directed against *HvGLP4* was found to cause silencing of a cobombarded *HvGLP4::GFP* fusion construct (Christensen et al. 2004).

Knock-down of *Mlo* by TIGS caused a rapid and strong increase of resistance of barley epidermal cells of cv. Ingrid (*Mlo*), regardless of whether or not *Mlo* was transiently overexpressed in the same cells (Fig. 4). However, an incubation period of 4 days between bombardment and challenge inoculation was required for the RNAi knock-down to produce a complete phenocopy of genetically determined resistance of plants carrying null alleles of the *Mlo* gene. The time-course study shown in Figure 4 (control without *Mlo* overexpression) also demonstrates the general behavior of the barley TIGS system to build up enhanced penetration resistance after bombardment, an effect that could be blocked by coexpression of *Mlo*. Next, we tested whether the TIGS system would allow cosi-

Fig. 3. Plasmid constructs used for transient-induced gene silencing in barley epidermal cells. All constructs contain the CaMV 35S promoter (P) and terminator (T). The intron is derived from the RGA2 gene of wheat (accession number AF326781). B1 and B2 = attB1 and attB2 recombination sites of the Gateway system, respectively. Construct pIPKTA4 has been obtained by conventional two-step cloning, as described (Christensen et al. 2004) and served as control for influence of the attB sites and insert orientation on RNA interference efficiency.

Table 1. Comparison of the previous with the new, high-throughput method for scoring of transgene effects in the single-cell system

Method	Construct ^a	PE (%) ^b	HAU index ^c	Relative susceptibility (%)
Old ^d	pGY1 empty vector	35.7 ± 7.1	...	100
Old	pWIR3	10.2 ± 5.3	...	28.5 ± 11.9
New ^e	pGY1 empty vector	...	0.31 ± 0.15	100
New	pWIR3	...	0.09 ± 0.05	30.9 ± 16.6

^a pGY1, empty overexpression vector; pWIR3, wheat peroxidase TaPERO in pGY1. For constructs, see Schweizer and associates (1999).

^b Penetration efficiency (PE) of *Blumeria graminis* f. sp. *hordei* (number of appressoria associated with a haustorium divided by the total number of appressoria × 100). Mean ± standard deviation (SD) of three independent experiments.

^c Haustorial (HAU) index of *B. graminis* f. sp. *hordei* (number of haustoria in β-glucuronidase (GUS)-stained cells divided by the total number of GUS-stained cells. Mean ± SD of three independent experiments.

^d Method for determination of PE (Schweizer et al. 1999).

^e High throughput method for determination of HAU index.

lencing of several genes. RNAi of *HvGLP4* caused a complete suppression of the accumulation of an *HvGLP4::GFP* fusion protein after cobombardment of the corresponding constructs pIPKTA32 and pIPKTA19 (Fig. 5A). The RNAi efficiency started to erode in the presence of 12 additional RNAi “competitor” constructs targeted against randomly chosen, defense-related genes of barley. A similar experiment was carried out with pIPKTA36 that caused silencing of *Mlo* (Fig. 5B). In the case of this RNAi target gene, the system did not tolerate three additional “competitor” constructs and showed negligible RNAi effect. Therefore, the sequence of RNAi targets might influence overall TIGS efficiency via RNA secondary structure or other features, and no generally valid maximum construct-pool size can be specified.

We also tested whether the orientation of the inverted repeats (sense to antisense or antisense to sense) and absence or presence of *attB* sites in the final construct affected RNAi efficiency. Two RNAi constructs directed against *HvGLP4* caused equally efficient knock-down regardless of insert orientation or the absence or presence of *attB* sites (Table 2).

Discovery of new gene function by TIGS.

In an ongoing TIGS screening based on the method presented here for 193 genes that were found to be upregulated in *B. graminis* f. sp. *hordei*-attacked barley epidermis, one construct resulted in reproducible, partial breakdown of *mlo*-mediated resistance (Fig. 6; complete data for ongoing TIGS screening not shown). This construct was found to be directed against *HvSNAP34*, a t-SNARE encoding gene. Interestingly, the same member of a small multigene family recently has been described to cause partial breakdown of *mlo*-mediated resistance in barley (Collins et al. 2003). The authors of that study tested *SNAP* genes because the encoded proteins are predicted to interact with HvROR-2, a recently identified syntaxin required for *mlo*-mediated resistance. We also tested the impact of TIGS of *HvSNAP34* in nonhost resistance of barley against the wheat powdery mildew caused by *B. graminis* D. C. Speer f. sp. *tritici* Em Marchal and in modulation of basal

Fig. 4. Highly efficient gene silencing by the transient-induced gene silencing (TIGS) system in barley epidermal cells. RNA interference (RNAi) of the *Mlo* gene induced a phenocopy of *mlo*-mediated resistance. Leaf segments were cobombarded with pUbiGUS, pIPKTA36 (*Mlo* RNAi), and, where indicated, *Mlo* overexpression construct pUbiMlo (Shirasu et al. 1999) in order to prevent bombardment-induced partial resistance over time. As control, pIPKTA36 was replaced by the empty RNAi vector pIPKTA30N. Susceptibility of transformed cells was observed by microscopy after incubating leaf segments for different times between bombardment and challenge inoculation. Open triangles, control; closed triangles, *Mlo* RNAi; open squares, control with *Mlo* overexpression; closed squares, *Mlo* RNAi together with *Mlo* overexpression. The experiment was repeated twice with very similar results.

resistance in a susceptible interaction of cv. Golden Promise with *B. graminis* f. sp. *hordei*. All three types of race-nonspecific resistance in barley were found to be associated with the formation of papillae (Trujillo et al. 2004; Zeyen et al. 2002).

Fig. 5. Transient-induced gene silencing efficiency decreased with increasing number of cobombarded RNA interference (RNAi) constructs. **A**, pHvGLP4::green fluorescent protein (GFP) was cobombarded with pIPKTA32 (GLP4 RNAi) in the presence of an increasing number of competitor RNAi constructs. At 48 h postbombardment, the number of GFP-expressing cells was counted. Data were normalized to the number of anthocyanin-accumulating cells 6 days postbombardment due to the presence of pBC17 (Schweizer et al. 2000b) in the cobombarded plasmid mixture. **B**, pIPKTA36 (*Mlo* RNAi) was cobombarded with pUbiGUS in the presence of zero or three competitor RNAi constructs. Controls contained pIPKTA30N empty vector instead of pIPKTA36. Challenge inoculation with *Blumeria graminis* f. sp. *hordei* was done 4 days post-bombardments. **A** and **B**, Mean $\pm$ range of two parallel shots. The experiments were repeated twice with very similar results.

TIGS of *HvSNAP34* also caused a partial breakdown of non-host resistance and an increase in host susceptibility that were highly significant (Fig. 6). By contrast, RNAi of *HvSNAP34* did not attenuate *Mla9*-mediated resistance against *B. graminis* f. sp. *hordei*, which is in agreement with the previous observation that *HvSNAP34*-silenced cells retained *Mla1*-mediated resistance (Collins et al. 2003).

DISCUSSION

The combination of post-genomics methods presented here allows high-throughput screening for gene function in barley attacked by appropriate or inappropriate formae speciales of *B. graminis*. In addition, we believe that the TIGS system also may allow researchers to address gene function in other aspects of plant physiology or cell biology (e.g., stomatal regulation, response of epidermal cells to UV light, or epidermal cell-wall synthesis in elongating organs like the coleoptile). A prerequisite will be the cell-autonomous nature of the phenomenon studied, because only single cells will be transformed by the bombardment.

Our cloning pipeline for RNAi constructs is highly efficient yet economic due to the replacement of one Gateway recombination step by conventional ligation. Moreover, any piece of blunt-ended DNA can be cloned into the RNAi vectors. This makes the method also attractive for RNAi projects based on genome sequence tags (GSTs), because conventional, sequence-specific PCR primers can be used for generation of the GST fragments. In contrast, the Gateway entry system would require adapter primers of approximately 50 bp in total length. We currently are using the TIGS system for silencing approximately 200 and 900 candidate genes of barley in two host screenings and one nonhost screening, respectively, with the aim to identify genes that influence barley–*Blumeria* interactions. Per person-month, 1,536 RNAi constructs (i.e., 16 microtiter plates) can be produced and 160 can be tested in first-round TIGS screening. The observed RNAi efficiency in the TIGS system was high, thereby suggesting that it might be a sensitive tool for revealing gene function. However, it came as some disappointment that cobombardment of several RNAi constructs did not produce reliable silencing, at least not in the case of the *Mlo* gene. Therefore, we cannot recommend that TIGS screenings be carried out based on pools of RNAi constructs as a means to enhance throughput. This does not take away the possibility that cosilencing of two or three selected genes that are functionally linked can produce a synergistic effect. Indeed, this has been observed in TIGS experiments of *HvRar1* and *HvSGT1* genes (Azevedo et al. 2002). It might be speculated that preformed RNAi components such as the RISC complex or DICER became limiting for highly efficient silencing of several transcripts in one cell at the same time (Kusaba 2004).

As a first result of several ongoing TIGS screenings mentioned above, we identified a t-SNARE protein, *HvSNAP34*, as an important component of host or nonhost resistance. This result suggests that *mlo*-mediated, nonhost as well as host basal resistance, all known as race-nonspecific and durable, share functional components like *HvSNAP34*. This protein belongs to the family of SNAP25-like proteins, which are known to link vesicle-located v-SNAREs and target-membrane-located t-SNAREs in functional complexes, resulting in accurate docking of vesicles to target membranes (Salaun et al. 2004). The *mlo*-mediated basal as well as nonhost resistance is associated with papilla formation that requires targeted secretion of cell-wall material and that is characterized by the accumulation of vesicles around the site of attempted penetration (Collins et al. 2003). These vesicles eventually might fuse with the plasma-

lemma in order to release their cargo. Therefore, the observed RNAi-induced phenotype is in agreement with a proposed function of *HvSNAP34* in papilla formation. The fact that *Mla*-mediated resistance of barley is independent of *HvSNAP34* function and associated with hypersensitive cell death instead of papilla formation supports the view that race-specific and race-nonspecific durable resistance in barley are based on different molecular mechanisms.

MATERIALS AND METHODS

Plants and fungi.

A pair of near-isogenic lines of barley, cv. Ingrid (*Mlo*) and Ingrid BC₇ *mlo5* (Buschges et al. 1997), or barley cv. Golden Promise, were grown in pots of compost soil (from an IPK nursery) in a growth chamber (16 h of light from metal halo-

Table 2. RNA interference efficiency of hairpin constructs directed against *HvGLP4* is independent of insert orientation

Construct	HvGLP4::GFP expression ^a	Anthocyanin control ^b
pPKTA30N empty vector	94 ± 28	501 ± 22
pPKTA4	3 ± 3	478 ± 44
pPKTA32	0 ± 0	440 ± 109

^a GFP, green fluorescent protein; number of cells accumulating *HvGLP4::GFP*. Mean ± range of two parallel shots.

^b Number of cells accumulating anthocyanin in response to cobombarded pBC17 (Schweizer et al. 2000). Mean ± range of two parallel shots. This served as internal control for transformation efficiency. The experiment was performed twice with very similar results, and data from one experiment are shown.

Fig. 6. Transient-induced gene silencing of a gene encoding the t-SNARE protein *HvSNAP34* caused a weakening of three types of durable, race-nonspecific resistance in barley without attenuating *Mla9*-mediated resistance. Mean ± standard deviation of four to eight independent experiments per pathosystem. The *P* values from *t* test for differences between *HvSNAP34* RNAi and empty vector controls (to the left of the respective columns) are indicated. The effect of *HvSNAP34* silencing on *Mla9*-mediated resistance was tested in the presence of *Mlo* overexpression in order to enhance basal susceptibility of the system.

gen lamps, 8 h of dark, 70% relative humidity, and 20°C constant temperature). *B. graminis* f. sp. *hordei* (isolate 4.8 carrying *AvrMla9*) was maintained at 22°C and 16 h of light by weekly transfer to fresh barley cv. Golden Promise. *B. graminis* f. sp. *tritici* (Swiss field isolate FAL 92315) was maintained at 20°C and 16 h of light by weekly transfer to fresh wheat cv. Kanzler. To study the effect of *HvSNAP34* RNAi on *Mla9*-mediated resistance, barley cv. Roland (*Mla9*) was used. The control inoculation of Roland was done by using *B. graminis* f. sp. *hordei* isolate K1 (*AvrMla1*, *AvrMla3*, *AvrMla7*, *AvrMla22*, *AvrMla*, and *AvrMl(Ab)*).

Plasmid vectors.

The vector pIPKTA38 was used as Entry vector for the Gateway cloning system (Invitrogen, Karlsruhe, Germany). The vector is a pENTR1a derivative with excised *ccdB* gene and a new multiple cloning site (Fig. 1).

pIPKTA30N was developed as final Gateway destination vector (Fig. 1). The vector is based on a pUC18 backbone, CaMV 35S promoter and terminator, two Gateway cassettes containing attR sites, a *ccdB* gene and a chloramphenicol resistance gene. The two cassettes are oriented in an opposite direction and are separated by the second intron of the wheat *RGA2* gene (accession number AF326781).

This vector system allows a single-step transfer of two copies of the PCR fragment cloned in the Entry vector to the double-stranded RNAi vector by using Gateway LR clonase mix (Invitrogen) (Fig. 1). A second destination vector, pIPKTA30R, has been developed that is identical to pIPKTA30N except for inverted orientation of the attR1-*ccdB*-camR-attR2 cassettes (Fig. 1).

PCR and primer design.

EST sequences of the target genes were amplified by PCR. Purified DNA from the selected cDNA clones was used as template for the PCR reaction. Primers for the PCR amplification of the EST sequences were designed by the software package Primer3 (Whitehead Institute, Cambridge, MA, U.S.A.) in a batch-file mode by using 5' EST sequences. Typically, the EST sequences were amplified by using one universal forward primer targeted to the cDNA library vector and one reverse EST-specific primer. The EST-specific primers were designed so as to amplify PCR products of 400 to 700 bp in size (Fig. 1). Most of the designed primers were 20 to 22 bp in size and had a melting temperature of approximately 65°C. In some cases, when it was impossible to design EST-specific primers due to insufficient sequence information, two universal vector primers were used.

The PCR was carried out in 96-well plates by using DNA polymerase that produces blunt-end PCR fragments (Thermal-Ace; Invitrogen). PCR products were purified by a MinElute UF kit (Qiagen, Hilden, Germany) and eluted with 25 µl of water.

Ligation into Entry vector.

The PCR fragments were ligated into the *SwaI* site of pIPKTA38. The ligation reaction in 10 µl containing 40 mM Tris-Cl, pH 7.8, 10 mM MgCl₂, 10 mM dithiothreitol, 50 mM NaCl, and 0.5 mM ATP was carried out at 25°C in the presence of 2.5 U of T4 DNA ligase (Fermentas, St. Leon-Rot, Germany) and 5 U of *SwaI* per reaction. After 1 h, the reaction was stopped by heating to 65°C for 15 min. Then, an additional 5 U of *SwaI* (in 5 µl of 1× buffer O⁺; Fermentas) was added to the reaction in order to quantitatively eliminate religated plasmid. The *SwaI* buffer was supplemented with additional NaCl to a final concentration of 0.1 M in order to optimize NaCl concentration for *SwaI*. The samples were incubated further for 1 h at 25°C.

The resulting recombinant pIPKTA38_EST clones were used for transformation of chemically competent *E. coli* DH10B cells dispensed in 96-well PCR plates (5 µl of ligation reaction to 20 µl of competent cells), and spread on Luria-Bertani (LB) + kanamycin agar plates. One colony of each cloning reaction was picked and used to inoculate 1.2 ml of LB + kanamycin liquid cultures in 96-deep-well plates that then were covered with air-permeable foil and incubated at 37°C for 18 h on a plate shaker. The deep-well plates were centrifuged at 750 × *g* for 10 min and the pellets were used for plasmid DNA isolation with a NucleoSpin Robot-96 Plasmid Kit (Macherey-Nagel, Düren, Germany). Control digestion of each clone was performed by restriction digestion with *EcoRI*. The positive pIPKTA38 clones were used as a donor vector in the LR reaction of the Gateway system with pIPKTA30N.

LR reaction and preparation of RNAi constructs.

EST fragments in pIPKTA38 were cloned into the RNAi destination vector pIPKTA30N as inverted repeats by a single LR recombination reaction. The reaction volume was scaled down to 6 µl and contained 1 µl of the pIPKTA38 donor clone, 1 µl of pIPKTA30N destination vector (150 ng/µl), 0.8 µl of LR clonase enzyme mix, and 3.2 µl of H₂O. The reaction was incubated overnight at room temperature and 5 µl of it was used to transform 20 µl of chemically competent *E. coli* cells in 96-well PCR plates. Two 96-deep-well plates filled with 1.2 ml of LB plus ampicillin were inoculated in parallel with half the volume of the transformation reaction, covered with air-permeable foil, and incubated at 37°C for 24 h on a plate shaker. The deep-well plates were centrifuged at 750 × *g* for 10 min and the pellets of each two duplicates of each clone were combined and used for plasmid DNA isolation with NucleoSpin Robot-96 Plasmid Kit (Macherey-Nagel). The DNA yield typically consisted of 20 to 30 µg of DNA per clone.

Microprojectile bombardment and challenge inoculation.

Primary leaf segments of 7-day-old barley seedlings were placed onto 0.5% (wt/vol) phytoagar (Ducheva, Haarlem, Netherlands) in water containing benzimidazol at 20 ppm and bombarded with gold particles (diameter 1 µm) in a PDS-1000/He system (Bio-Rad, München, Germany) using the hepta adaptor with a helium pressure of 900 psi. Seven target leaves were used per bombardment. The particle coating by 0.5 M Ca(NO₃)₂ was done according to Schweizer and associates (1999), except that the stock of particle suspension contained gold at 25 mg ml⁻¹. The supernatant was completely removed after coating, and particles were finally resuspended in 30 µl of pure ethanol containing 2.18 mg of gold microcarrier per bombardment (310 µg per macrocarrier). At 4 h after bombardment, leaf segments were placed onto 1% (wt/vol) phytoagar (Ducheva) in water containing benzimidazol at 20 ppm in 20-by-20-cm plates and fixed at both ends with labeled pins. Moreover, leaves from all bombardments (each usually representing one gene to be tested or the controls) per experiment were mixed. These measures prevented artifacts caused by irregular inoculum density due to leaf curling or gradients of spore density across agar plates (Table 1). Leaf segments were incubated at 18 to 20°C and indirect day light (not supplemented with artificial light) in a climatized room. These incubation conditions were found to be optimal for delaying leaf senescence. Leaf segments were inoculated with *B. graminis* f. sp. *tritici* or *B. graminis* f. sp. *hordei* at 48 to 96 h postbombardment, as specified for each experiment. Plasmid pUbiGUS containing the *β-glucuronidase* (*GUS*) gene under the control of the maize *Ubiquitin* promoter was used as reporter gene for transformed epidermal cells (Schweizer et al. 1999), and leaf

segments were GUS stained at 40 h postinoculation, followed by destaining leaves in 7.5% (wt/vol) trichloroacetic acid and 50% methanol for 5 min. The GUS-staining solution was as described, except for reducing the concentrations of K-hexacyanoferrate(II) and K-hexacyanoferrate(III) to 1.4 mM each (Schweizer et al. 1999). Scoring of interaction phenotypes by light microscopy was carried out by counting GUS-stained cells and the number of haustoria inside these transformed cells per bombardment leading to the haustorial index. Alternatively, the fraction of GUS-stained cells bearing at least one haustorium was calculated per bombardment leading to the susceptibility index.

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AUTHOR-RECOMMENDED INTERNET RESOURCE

CR-EST: The IPK crop expressed-sequence-tag database:
pgrc.ipk-gatersleben.de/cr-est