Efficient Gene Knockout in the Maize Pathogen *Setosphaeria turcica* Using *Agrobacterium tumefaciens*-Mediated Transformation

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ABSTRACT

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Setosphaeria turcica, a hemibiotrophic pathogenic dothideomycete, is the causal agent of Northern Leaf Blight of maize, which periodically causes significant yield losses worldwide. To explore molecular mechanisms of fungal pathogenicity and virulence to the host, an efficient targeted gene knockout transformation system using *Agrobacterium tumefaciens* was established with field collected strains. The starting ma-

terials, incubation time, induction medium type, *Agrobacterium* cell density, and method of co-incubation were optimized for deletion of 1,3,8-trihydroxynaphthalene reductase, a gene in the melanin biosynthesis pathway, as a test case. Four additional genes were deleted in two different *S. turcica* field isolates to confirm robustness of the method. One of these mutant strains was reduced in virulence compared with the wild-type strain when inoculated on susceptible maize. Transformation efficiency was $\approx 20 \pm 3$ transformants per 1×10^6 germlings and homologous recombination efficiency was 33.3 to 100%.

Additional keyword: ECP6.

Setosphaeria turcica, a hemibiotrophic pathogenic Dothideomycete, is the causal agent of Northern Leaf Blight (NLB) of maize, one of the most serious maize foliar diseases impacting yield in warm and humid regions (6,23). To date, at least seven races of the fungus have been identified. As with all hemibiotrophs (and biotrophs), gene-for-gene interactions between plant and pathogen are expected to be continually evolving and, as new fungal effector molecules (secreted disease factors) are deployed, the host responds with new or modified plant resistance genes. In recent years, new races of *S. turcica* have emerged which overcome common sources of NLB resistance, and NLB is once again in focus as a major constraint to worldwide maize production (6).

The genus *Setosphaeria* is an important group of species that includes pathogens of monocots such as *S. turcica* and the additional maize pathogen, *S. rostrata* (causal agent of rostratum leaf spot and apparent agent of the recent spinal meningitis outbreak). The genus *Setosphaeria* is sister to the related maize pathogen *Cochliobolus heterostrophus* and to the wheat pathogens *Pyrenophora tritici-repentis* and *Mycosphaerella graminicola*, all of which have been sequenced (<http://genome.jgi.doe.gov/programs/fungi/index.jsf>). Recently, *S. turcica* field strain Et28A (race 23N) was sequenced (<http://genome.jgi.doe.gov/Sett1/Sett1.home.html>), which provides an opportunity for in-depth understanding of virulence requirements of the fungus to its host, maize. Lacking however, until this report, are targeted gene-

deletion protocols for this fungus needed to assess candidate genes for involvement in mechanisms of pathogenicity to plants. This is especially critical for testing mutants of field isolates representing the various races of the fungus on susceptible and resistant plants.

Protoplast-polyethylene glycol (PEG)-based transformation and *Agrobacterium tumefaciens*-mediated transformation (ATMT) are two major methods for deleting fungal genes by homologous recombination. The protoplast-PEG-mediated transformation approach has been used with many important plant-pathogenic fungi (7,13,14,21,25,27,30); however, for some fungi, low yield and low viability of protoplasts are limiting (3,19). Our unsuccessful attempts at transformation of *S. turcica* (see Results) are likely attributable to this constraint. Targeted gene knockout by protoplast-PEG-based transformation has been reported in three thesis publications on *S. turcica* (4,11,31) but efficiency was <1%. ATMT has been used successfully with many fungi as an alternative to protoplasting (9,18). With ATMT, it is often possible to choose one or more developmental stages, such as germinating conidia, fresh mycelia, or fruiting bodies, as starting materials for transformation (28). Random insertion mutations have been reported for *S. turcica* using ATMT (8,15); however, high-efficiency transformation with targeted gene disruption has not been established to date.

In this study, we first attempted transformation by protoplasting several *S. turcica* field isolates representing different races but had no success, then focused on evaluating factors influencing efficiency of gene knockout by ATMT. For this, we choose the 1,3,8-trihydroxynaphthalene reductase (*3HNR*) gene as a target. *3HNR* reduces 1,3,8-trihydroxynaphthalene to vermeline, which is then dehydrated, dimerized, and polymerized to form 1,8-dihydroxynaphthalene-type melanin, making it a useful indicator of targeted gene knockout, because mutants can be easily identified by an alteration in pigmentation (29). Deletion of *3HNR* did not alter

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virulence of the corresponding mutant on susceptible maize. Upon success with transformation, we then confirmed the robustness of our method by using it to delete four additional genes in two different undomesticated strains. Deletion of the *ECP6* gene, encoding a lysine motif protein used as an effector by some fungi (2,17), caused a reduction in virulence to maize compared with the wild-type (WT) strain.

MATERIALS AND METHODS

Fungal strains, culture conditions, and harvesting conidia. *S. turcica* strains Et100-1A, Et86A, EtNY001, Et10A, Et28A, and 52B obtained from R. Nelson at Cornell University were tested for sensitivity to hygromycin B and ability to be protoplasted. Strain Et28A (race 23N) was chosen initially for transformation attempts, based on drug sensitivity (described in Results). Fungal strains were stored as single conidial cultures at -80°C in lactose casein (LC) medium (37.5 g of lactose, 3.0 g of casein, 0.5 g of MgSO₄, and 1.0 g of KH₂PO₄ per liter) in 25% glycerol. For each

experiment, an aliquot was recovered from glycerol and transferred to LC agar (LCA) for 12 to 14 days at 25°C in 12 h light and 12 h of darkness. To harvest conidia, sterile distilled H₂O was added to LCA plates, the surface was rubbed with a rubber policeman to dislodge conidia, and the resulting conidial suspension was filtered through two layers of cheesecloth to remove mycelial debris.

Construction of a plasmid for *Agrobacterium* transformation. The 2,113-bp *HPH* cassette for resistance to hygromycin B was isolated by digesting pUCATPH (16) with *Xba*I and cloned into the binary vector pZP100 (12), pre-cut with *Xba*I; the resulting plasmid was named pZPH. Portions of the *3HNR* gene, *3HNR*-5F (469 bp) and *3HNR*-3R (414 bp), were amplified by polymerase chain reaction (PCR) with primers *3HNR*-F₁/R₁ and *3HNR*-F₂/R₂, respectively, using Phusion DNA polymerase (New England Biolabs, Ipswich, MA). *Hind*III restriction enzyme recognition sites were added to the 5' ends of the *3HNR*-F₁/R₁ primers and *Kpn*I restriction enzyme recognition sites were added to the 5' ends of *3HNR*-F₂/R₂ primers. These two PCR products were then inserted, separately, into the TOPO vector (Life Technologies, Carlsbad, CA), giving rise to plasmids TOPO-5F and TOPO-3R. The *3HNR*-5F fragment was cut from TOPO-5F with *Hind*III and ligated into pZPH, pre-cut with *Hind*III, to generate pZPH5F. The *3HNR*-3R fragment was cut from TOPO-3R using *Kpn*I and ligated into pZPH5F pre-cut with *Kpn*I. The final plasmid was named pZPH3HNR (Fig. 1). Insertion orientation was determined by PCR using primers *3HNR*-F₁/TtrpCend and TtrpC/3HNR-R₂. pZPH3HNR was transformed into *A. tumefaciens* strain AGL-1 by the freeze-thaw method (1). Primer sequences are shown in Table 1.

Four additional *S. turcica* genes—*LAEL1*, encoding a transcriptional regulator of secondary metabolite genes; *PKS179218*, encoding a polyketide synthase; *ECP6*, encoding a lysine motif protein used as an effector by some fungi; and *FTR1*, encoding an iron permease—were deleted using the one-step construction of *Agrobacterium*-recombination-ready-plasmids (OSCAR) method to construct the transformation plasmids (21) which were transformed into AGL-1 by the same method mentioned below (1). Primers are listed in Supplementary Table 1 and lengths of 5' and 3' flanking sequences used in constructions for each gene are listed in Table 2.

Transformation of *S. turcica*. *A. tumefaciens* strain AGL-1, containing pZPH3HNR or the OSCAR plasmids, was grown at 28°C for 1 day in Luria broth (LB) (5 ml), supplemented with chloramphenicol (50 µg/ml) or spectinomycin (100 µg/ml) for OSCAR plasmids, and ampicillin (100 µg/ml). The culture was diluted with *Agrobacterium* induction medium (IAM) to an optical density at 600 nm (OD₆₀₀) = 0.15 in a final volume of 5 ml. IAM (5) is 10 ml of potassium phosphate buffer (1 M, pH 5.3), 20 ml of MN buffer (MgSO₄ · 7H₂O at 30 g/liter and NaCl at 15 g/liter), 1 ml of 1% CaCl₂ · 2H₂O, 10 ml of 0.01% FeSO₄, 5 ml of spore elements (ZnSO₄ · 7H₂O at 100 mg/liter, CuSO₄ · 5H₂O

Fig. 1. *Agrobacterium tumefaciens*-mediated transformation transformation plasmid for 1,3,8-trihydroxynaphthalene reductase (*3HNR*) gene knockout. Restriction map of pZPH3HNR, a binary vector for deletion of *Setosphaeria turcica 3HNR* gene. CAT = chloramphenicol acetyl transferase; CamR = chloramphenicol resistance gene for selection in bacteria. The *Xba*I site was used for insertion of the hygromycin B resistance cassette (*HPH*). PtrpC and TtrpC are the promoter and terminator regions of the *TrpC* gene from *Aspergillus nidulans*. *3HNR*-5F (upstream flanking sequence) was inserted as a *Hind*III fragment between the *HPH* cassette and the T-DNA right border (RB), while *3HNR*-3R (downstream flanking sequence) was inserted as *Kpn*I fragment between the *HPH* cassette and the left border (LB).

TABLE 1. Oligonucleotides used in targeted deletion of 1,3,8-trihydroxynaphthalene reductase (*3HNR*)

Name	Description ^a	Sequence (5'–3') ^b
3HNR-F ₁	Forward primer of <i>3HNR</i> upstream flank sequence	gccaaagcttATGGCCAACATCGAGCAAAC
3HNR-R ₁	Reverse primer of <i>3HNR</i> upstream flank sequence	gccaaagcttACTTCCATGCGCTTGTAGG
3HNR-F ₂	Forward primer of <i>3HNR</i> downstream flank sequence	gccgggtaccGTGGCCGCATCATCCTCA
3HNR-R ₂	Reverse primer of <i>3HNR</i> downstream flank sequence	gccgggtaccTGACCTTTCCGTTGACCCAG
3HNR-F ₃	Forward primer upstream of <i>3HNR</i> upstream flank	CGACCCAATCCCAACACAA
3HNR-R ₃	Reverse primer downstream of <i>3HNR</i> downstream flank	AGGCAGCACCCTCAATACCA
PtrpC	Reverse primer of <i>TrpC</i> promoter	GGTCGTTCACTTACCTTGCTTG
TtrpCend	Forward primer of <i>TrpC</i> terminator	GTGAATGCTCCGTAACACCCAATAC
NLC37	<i>HPH</i> gene	GGATGCCTCCGCTCGAAGTA
NLC38	<i>HPH</i> gene	CGTTGCAAGACCTGCCTGAA
M13R	Vector sequence in pUCATPH (16)	AGCGGATAACAATTTACACAGGA

^a *TrpC* is a tryptophan biosynthesis gene from *Aspergillus nidulans*.
^b Lowercase letters are restriction enzyme recognition sites added to the primers.

at 100 mg/liter, H_3BO_3 at 100 mg/liter, $\text{MnSO}_4 \cdot \text{H}_2\text{O}$ at 100 mg/liter, and $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$ at 100 mg/liter), 2.5 ml of 20% NH_4NO_3 , 10 ml of 50% glycerol (vol/vol), 40 ml of 1 M MES (pH 5.3), and 10 ml of 20% glucose for liquid medium (5 ml for solid medium) in a 1-liter volume (containing the same concentrations of antibiotics as LB), plus acetosyringone (AS) (200 to 500 $\mu\text{g}/\text{ml}$). Cultures were grown to $\text{OD}_{600} = 0.2$ to 0.6. An Et28A conidial suspension (5 ml at $1 \times 10^6/\text{ml}$) was germinated in 100 ml of Fries medium (23) in a 250-ml flask, for 0, 2, 6, 12, 18, or 24 h before mixing with an equal volume of induced AGL-1 cells in a 50-ml falcon centrifuge tube. The mixture (500 μl) was placed on a cellulose nitrate membrane (Whatman, 0.45 μm) on top of IAM agar (15 g/liter) or induction medium (IM) agar (15 g/liter) in the presence of AS (200, 300, 400, or 500 μM) and incubated at 25°C for 48 h. IM (8) (in 1 liter) is 10.5 g of K_2HPO_4 , 4.5 g of KH_2PO_4 , 1.0 g of NH_4SO_4 , 0.5 g of $\text{Na}_3\text{citrate} \cdot 2\text{H}_2\text{O}$, 0.2 g of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 1 mg of thiamine-HCl, 2 g of glucose, 40 mM MES, and 0.5% glycerol, pH 5.3 (containing the same concentrations of antibiotics as AS and IAM). Membranes were transferred onto LCA plates containing cefotaxime (200 $\mu\text{g}/\text{ml}$) and hygromycin B (100 $\mu\text{g}/\text{ml}$) to kill *Agrobacterium* cells and select for candidate transformants, respectively. Plates were incubated at 25°C for 7 to 12 days until conidiogenesis. Candidate transformants were purified by isolating single conidia and reselecting on LCA medium containing the same concentrations of hygromycin B and cefotaxime. Purified hygromycin B-resistant candidate transformants were stored in LC in 25% glycerol at -80°C.

DNA isolation and confirmation of gene deletion. For 3HNR, genomic DNA was extracted from candidate transformants grown on LCA plates using the Ultraclean Microbial DNA Isolation kit (MOBIO, Carlsbad, CA). PCR evaluation to confirm 3HNR gene

deletion was performed using GoTaq DNA polymerase (Promega Corp., Madison, WI). To verify insertion of the *HPH* gene at the native 3HNR locus, four primer pairs were used: primers NLC37/NLC38 to confirm the presence of *HPH*; TtrpCend/3HNR-F₃ and PtrpC/3HNR-R₃ to confirm correct insertion into the 5' and 3' flanks of 3HNR, respectively; and 3HNR-F₃/3HNR-R₃ to confirm expected size of insertion. Primer sequences are listed in Table 1.

In addition, Southern blot analysis of DNA from the WT and three of five confirmed 3hnr mutants was performed. For this, genomic DNA of mutant and WT strains was isolated from lyophilized mycelia. Mycelia were ground in liquid nitrogen using a mortar and pestle, resuspended in extraction buffer (150 mM EDTA, 50 mM Tris-HCl [pH 8.0], 1% sodium lauryl sarkosyl, and proteinase K at 300 mg/liter) and extracted once with an equal volume of Tris-saturated phenol, twice with phenol/chloroform/isoamyl alcohol (25:24:1), and twice with chloroform/isoamyl alcohol (24:1). DNA was precipitated with two volumes of cold 99.5% ethanol and 1/10 volume sodium acetate (3 M) and dissolved in 400 μl of Tris-EDTA (TE). Samples were treated with RNase (10 $\mu\text{g}/\mu\text{l}$) at 37°C for 1 h. A second round of DNA purification was done as above. Finally, DNA was precipitated with 20% (wt/vol) PEG 8000/2.5 M NaCl, spooled out, washed with 70% ethanol, air dried, and dissolved in an appropriate volume of TE.

For blotting, genomic DNA was digested with *Xho*I overnight at 37°C, separated by electrophoresis in a 1% (wt/vol) agarose gel, and transferred to a nitrocellulose membrane using standard protocols (26). A digoxigenin (DIG)-labeled probe was generated by PCR using primers NLC37/M13R to amplify the *HPH* gene and the membrane hybridized to the labeled DNA. Hybridizing bands were detected using the DIG High Prime DNA Labeling and Detection Starter Kit II Direct Labeling and Detection System according to the manufacturer's instructions (Roche, Mannheim, Germany).

LAE1, PKS179218, ECP6, and FTR1. For confirmation of deletion of *S. turcica* *LAE1*, *PKS179218*, *ECP6*, and *FTR1* genes, the same PCR primer strategies described above for 3HNR were used (Supplemental Figure 1).

Virulence assays. Three-week-old 'W64A-N' or 'Pa91' maize plants were used to evaluate virulence of *lae1* and *ecp6* mutants, respectively, as previously described (20). A spore suspension (2 ml of 5×10^3 spores/ml) was spray inoculated on each plant. Diseased leaves were photographed at 8 (for *lae1*) or 9 (for *ecp6*) days postinoculation.

TABLE 2. Transformation and targeted deletion statistics for five *Setosphaeria turcica* genes

Gene	Flanking sequence (bp)		Del/trans ^a	Efficiency (%) ^b
	5'	3'		
3HNR	469	414	5/15	33.3
LAE1	842	846	11/13	84.6
PKS179218	760	762	14/15	93.3
ECP6	921	983	6/6	100
FTR1	879	892	6/6	100

^a Number of deletants/transformants (D/T).

^b Homologous recombination efficiency.

Fig. 2. Transformation plates. **A**, Control wild-type (WT) strain Et28A and AGL-1 cells without plasmid pPZPH3HNR on hygromycin B (100 $\mu\text{g}/\text{ml}$) selection medium plus cefotaxime (200 μM). Note background growth. **B**, Example of a typical original transformation plate containing Et28A and AGL-1 cells carrying pPZPH3HNR plasmid on the same selection medium as in A, 7 days after plating. Note large, dark colonies (arrow) as well as background growth seen in control. **C**, Candidate 3hnr mutant (left) taken from a transformation plated and a WT strain (right) transferred to hygromycin B (100 $\mu\text{g}/\text{ml}$) selection medium. Note that the candidate grows well while the untransformed WT control does not.

RESULTS AND DISCUSSION

Strain selection. Strains Et28A (*MAT1-1*) and 52B (*MAT1-2*) were chosen from five candidate WT strains because they grew well, conidiated well, and were sensitive to hygromycin B. Level of sensitivity to hygromycin B was tested by growing strains on LCA plates supplemented with different concentrations of the antibiotic (50, 100, 150, 200, 300, 400, 500, 600, 800, or 1,000 µg/ml). Growth of Et28A and 52B was inhibited partly at 100 µg/ml and completely at 200 µg/ml. Other strains showed less sensitivity to hygromycin B. For selection of candidate transformants, 100 µg/ml was chosen.

Fig. 3. Phenotype of 1,3,8-trihydroxynaphthalene reductase (*3hnr*) mutants compared with the wild type (WT). WT and mutant **A**, on lactose casein (LC) agar and **B**, in LC liquid. Note that mutant is light brown, indicating a block in the melanin synthesis pathway.

Factors influencing ATMT efficiency in *S. turcica*. Germination conditions. Conidial germination rate was tested in triplicate in three different media—Fries (24), LC, and potato dextrose (29)—and Fries was the best. Conidia germinated for 0, 2, 6, 12, 18, or 24 h were tried as starting material for transformation; transformants were obtained only with the 24-h materials even though a number of parameters, including varying the induction medium (below), co-cultivation conditions (below), and concentration of AS, were tried as discussed below.

Induction medium. Two different inducing media, IAM and IM, were tested. Transformants were obtained only with IAM, although IM had been reported to work previously (8) for random insertion in a different strain of *S. turcica*. The reason for this difference is unclear but may be related to strain differences.

Fig. 5. Southern blot analysis of 1,3,8-trihydroxynaphthalene reductase (*3hnr*) mutants. Genomic DNA was cut with *Xho*I, a restriction enzyme that does not cut in *HPH*, and separated on a 1.0% agarose gel by electrophoresis. DNAs were transferred to a nitrocellulose membrane and hybridized using a digoxigenin-labeled 1.1-kb fragment of *HPH* as described in Materials and Methods. Lanes 1 to 3, DNA from transformants and unmarked lane is DNA from WT, wild type. Sizes of the 1-kb DNA ladder markers (kb) are indicated on the left. When homologous integration has occurred, a hybridizing band of ≈5 kb is observed due to integration of the selectable marker. A band of ≈10 kb was also observed in one of the mutants (lane 2) indicating that a copy of *HPH* also integrated somewhere other than at the *3HNR* locus. The WT lane doesn't have any hybridization signal due to lack of the *HPH* gene.

Fig. 4. Polymerase chain reaction (PCR) analysis of 1,3,8-trihydroxynaphthalene reductase (*3hnr*) mutants of *Setosphaeria turcica*. **A**, Schematic representation of the DNA used for transformation. 5F = *3HNR*-5F flanking sequence of *3HNR*, 3R = *3HNR*-3R flanking sequence of *3HNR*, PtpC = *Aspergillus nidulans* trpC promoter; *HPH* is the hygromycin B resistance gene and TtpC is the *A. nidulans* trpC terminator. *3HNR*-F3, TtpCend, NLC38, NLC37, PtpC, and *3HNR*-R3, are primers used for screening *3hnr* mutants (Table 1). **B** and **C**, PCR confirmation of *3hnr* mutants from three randomly selected transformants using primers **B**, *3HNR*-F3/TtpCend (lanes 1 to 5) and PtpC/*3HNR*-R3 (lanes 6 to 10) and **C**, *3HNR*-F3/*3HNR*-R3. Lane M, NEB 1-kb DNA ladder marker; lanes 1 and 6, H_2O ; lanes 2 and 7, DNA from wild type; lanes 3 to 5 and 8 to 10, DNA from transformants. Size of PCR product from the mutants matches the predicted size of *3HNR*-5F+*HPH* + *3HNR*-3R, indicating replacement of the *3HNR* gene by *hygB* resistance gene at the *3HNR* locus.

Agrobacterium cell density. Cell concentration (OD₆₀₀ value) of AGL-1 after induction was also a key factor; no gene knockouts were obtained when the OD value was <0.5, although it was evident that *HPH* was randomly inserted into the genome at lower cell densities (0.2 to 0.5 OD).

Plating conditions. The mixture of agrobacteria and fungal conidia was shaken in liquid medium for 48 h or plated immediately on a cellulose nitrate membrane. Transformants were obtained only when mixtures were plated immediately on membranes. Previous publications reported no success using this procedure (4). Varying the concentration of AS (200, 300, or

500 μ M) made no difference to transformation efficiency. Putative transformants grew faster than background growth on selection medium (Fig. 2). Overall transformation efficiency was $\approx 20 \pm 3$ transformants per 1×10^6 starting conidia.

We did not succeed with protoplast transformation of strain Et28A or 52B. At least 20 protoplast transformation attempts executed in triplicate for each set of conditions, including several cell-wall-degrading enzymes such as Snailase, Driselase, lysing enzymes, Rhozyme, Cellulase, and Glucanex, were made without success. In some experiments, we did generate low numbers of protoplasts but inconsistently. Initially, we speculated that it may

Fig. 6. Virulence phenotype of *Setosphaeria turcica* wild type (WT) and mutants on susceptible maize. **A**, Virulence of WT strain 28A and the *ecp6* mutant on maize 'Pa91'. WT caused large, cigar-shaped beige lesions whereas the mutant produced only small chlorotic flecks. **B**, Virulence of WT strain 28A and the *lae1* mutant on maize 'W64A-N'. Virulence of the mutant was similar to the WT.

be difficult to obtain sufficient numbers of protoplasts because the fungus melanizes very quickly. However, using ATMT, we have since created an albino strain deleted for the polyketide synthase gene, *PKS18*, required for melanin biosynthesis. Protoplasting attempts were no more successful with this mutant than with the pigmented strain. Thus, we speculate that there is something about the *S. turcica* cell wall composition that is recalcitrant to digestion by the battery of cell-wall-degrading enzymes we employed.

Verification of transformation and targeted integration. 3HNR. The *3HNR* gene in the melanin biosynthesis pathway was chosen as the initial target for deletion because mutants were expected to be brown and, thus, readily distinguishable from black WT colonies (Fig. 3). Hygromycin-B-resistant colonies obtained with ATMT were screened by PCR using primer pairs NLC37/NLC38, 3HNR-F₃/TrpCend, and PtpC/3HNR-R₃, which were expected to amplify products of 465, 810, and 550 bp, respectively, if the *3HNR* gene was deleted (Table 2). These three bands should be absent in the WT. Primer pair 3HNR-F₃/3HNR-R₃ should amplify a product of 3,055 bp in deletion strains and 942 bp in the WT. According to PCR assays, 5 of 15 transformants screened were *3hnr* deletants when flanking sequences of 469 and 414 bp were used for integration (Fig. 4). Thus, under these conditions, homologous recombination efficiency was 30%.

Southern blot analysis of three independent transformants, determined by PCR to be *3hnr* mutants, confirmed that the *HPH* gene had integrated at a single site in two of the three cases. In the two transformants, a single *Xho*I hybridizing band of ≈5 kb was detected in transformants but absent from the WT; in the third, the same band plus an additional band of 10 kb, perhaps due to a second site insertion of the *HPH* gene, were present (Fig. 5).

LAE1, PKS179218, ECP6, and FTR1. Verification of deletion of four additional genes was done by diagnostic PCR. Because the genome sequence of strain 28A was available at the time these deletions were made (unlike for *3HNR*), longer flanking sequences were readily available for constructions. Increasing the flanking region to >700 bp improved transformation efficiency dramatically from ≈30% to >80% (Table 2). Three of these genes were deleted from strain 28A while the fourth, *FTR1*, was deleted in strain 52B, all using the same protocols.

Thus, the ATMT method has proven to be robust for deletion of five different genes in two different field isolates and should be applicable to all field-collected and laboratory strains.

Virulence phenotypes on susceptible maize. The *3hnr*, *lae1*, and *ecp6* mutants and WT strain 28A were inoculated on susceptible maize W64A-N or Pa91 (for *ecp6*). Virulence phenotypes of the first two mutants were similar to those of the WT (Fig. 6). In contrast, inoculation with the *ecp6* mutant caused a significant reduction in virulence to maize. At 9 days postinoculation, the WT strain produced long beige cigar-shaped lesions; in contrast, inoculation with the mutant did not elicit this type of lesion but, instead, produced tiny chlorotic flecks. This matches results of studies of this gene in other fungi (e.g., *Cladosporium fulvum* and *Magnaporthe oryzae* (2,17)).

Conclusions. The final recommended protocol is as follows. AGL-1-containing vector is grown at 28°C for 1 day in LB. The culture is diluted with IAM to OD₆₀₀ = ≈0.1 to 0.15 and grown overnight to OD₆₀₀ = 0.6 to 0.7. An Et28A conidial suspension is germinated 24 h before mixing with an equal volume of induced *A. tumefaciens*. The mixture (500 µl) is spread on a cellulose nitrate membrane (Whatman 0.45 µm) on top of IAM agar in the presence of AS (200 µM) and incubated at 22 to 25°C for 48 h. Membranes are transferred onto LCA plates containing cefotaxime (200 µg/ml) and hygromycin B (100 µg/ml). Plates are incubated at 22 to 25°C for 7 to 12 days until conidiogenesis. Candidate transformants are purified by picking single conidia and reselected on LCA medium containing the same concentrations of hygromycin B and cefotaxime.

To obtain high efficiency of targeted integration, flanking sequences that are >700 bp are suggested. This protocol appears robust, based on trials using the same transformation parameters and protocols with two different field isolates and five different genes and phenotypic screens, including virulence assays, for alterations from WT.

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