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DEVELOPMENT OF GRAY LEAF SPOT RESISTANT PERENNIAL RYEGRASS THROUGH BREEDING AND BIOTECHNOLOGICAL APPROACHES

Mark Farman[1], Tim Phillips[2], David Williams[2]
Departments of Plant Pathology[1] and Agronomy[2], University of Kentucky

Executive Summary: We are using two approaches to try and obtain perennial ryegrass plants that are resistant to infection by the gray leaf spot pathogen *Pyricularia grisea.* The first approach is based on the observation that strains of the fungus causing gray leaf spot of perennial ryegrass are unable to cause disease on rice. This suggested that rice may have disease resistance genes which recognize gray leaf spot strains , enabling it to resist infection. Introduction of such genes into perennial ryegrass may enable the grass to mount a defense to gray leaf spot pathogens. Work performed in the first 8 months of the USGA-funded project has shown that one variety of rice called CO39 is resistant to gray leaf spot because the fungus possesses a gene whose presence elicits a defense response in this host. Moreover, this defense response is controlled by a resistance gene that has been well characterized. We hope to introduce this resistance gene into perennial ryegrass within the duration of the project to determine if it will function to provide gray leaf spot resistance to this host plant.

The other approach we are taking is traditional breeding. Prior to starting this project, we had identified an annual ryegrass x tall fescue hybrid with high levels of resistance to gray leaf spot. This hybrid was crossed with both perennial ryegrass and tall fescue and a small amount of seed was obtained from each cross. During the past 8 months, we have used inoculation tests to examine gray leaf spot resistance in the progeny from these crosses. We have found that resistance was not transmitted to any of the progeny of a cross between the hybrid and perennial ryegrass. However, resistant progeny were identified when the hybrid was crossed with tall fescue. In the spring of 2001, we will attempt to introduce the hybrid's resistance into perennial ryegrass by performing additional crosses and by screening much larger numbers of progeny. We will also explore the possibility of introducing the resistance into perennial ryegrass by using tall fescue as an intermediary.

Research results:

Objective 1: **To evaluate *Pi-CO39*^{t}, a resistance gene from rice, for effectiveness against gray leaf spot.**

Rice cultivar CO39 is resistant to the *P. grisea* strains that cause gray leaf spot on perennial ryegrass. We hypothesized that this is because these fungal strains possess a copy of the avirulence gene *AVR1-CO39.*

In the current project year, we have performed experiments to determine if the *AVR1-CO39* gene homolog that is present in the gray leaf spot pathogens is effective at eliciting resistance in CO39 rice plants. The *AVR1-CO39* gene from one of the gray leaf

spot isolates (*AVR1-CO39^{Lp}*)was sequenced. This revealed that it was very similar to the previously characterized gene, having only 3 nucleotide (nt) substitutions and a 1 nt insertion. The gene was then excised from the cloning vector and transferred into the fungal transformation vector pCB1004. This vector contains a hygromycin B resistance gene, enabling transformants to be selected based on resistance to this antibiotic. The strain used as a recipient for transformation was ML33. This strain is virulent to CO39, with infection resulting in large leaf lesions which usually kill the leaf. The plasmid containing the *AVR1-CO39^{Lp}* gene (pAVR1-*CO39^{Lp}*) was introduced into protoplasts of ML33, using a PEG-mediated transformation procedure and over 40 transformants were generated.

Fifteen transformants were purified by single spore isolation and 5 were selected at random and used to inoculate rice cultivar CO39. Also inoculated was the control cultivar 51583, which lacks *Pi-CO39^{t}* and is susceptible to infection by strains possessing *AVR1-CO39*. These plants are used as controls to ensure that the transformants show cultivar-specific changes in infection types.

The results of the inoculation experiments are summarized in Table 1. The gray leaf spot isolate from which the *AVR1-CO39^{Lp}* gene was isolated was unable to infect either rice cultivar and produced only brown necrotic flecks (Figure 1A) . In contrast ML33 the recipient strain used for transformation produced many lesions on both 51583 and CO39 (Figure 1B). The ML33 transformants had lost the ability to infect CO39 but were still able to infect 51583 (Figure 1C). This is the pattern expected of strains that possess an avirulence gene recognized by cultivar CO39. Infection types of strain 6059-12-1, which expresses *AVR1-CO39* is shown Figure 1D for reference.

To confirm that the *AVR1-CO39^{Lp}* avirulence gene had been successfully introduced into the transformants, DNA was extracted from the fungal cultures and was analyzed by Southern hybridization using the *AVR1-CO39* gene as a probe. Also included in this analysis was the transforming plasmid (1st lane); DNAs from LpKY97-1A (lane 2), the original donor of *AVR1-CO39^{Lp}* and also from the recipient strain ML33 (lane 3). As shown in figure 2, strain LpKY97-1A yielded hybridization signals which correspond to the single *AVR1-CO39^{Lp}* gene resident in its genome (multiple hybridization signals are seen because the probe spans a *Kpn*I restriction site). ML33 does not contain a copy of the avirulence gene (hence its virulent phenotype). All five transformants examined possessed a copy of the *AVR1-CO39^{Lp}* gene. In transformants #2, #4, #5 and #6 there was at least one hybridizing *Kpn*I - *Sac*I fragment that was the same size as the gene fragment contained in the vector. This confirmed that these transformants possessed an intact copy of the gene. The hybridizing fragment was larger in transformant #1, indicating that the genic fragment had become altered during the integration process. However, the avirulent phenotype of this transformant indicated that gene expression was unimpaired by this rearrangement.

Conclusions: We conclude that the *P. grisea* isolates causing gray leaf spot possess a functional *AVR1-CO39* gene homolog. We hypothesize that possession of this gene prevents the gray leaf spot pathogens from infecting rice cultivar CO39 and, indeed, other rice cultivars possessing *Pi-CO39^{t}*. Furthermore, we hypothesize that expression of

Pi-CO39^t in perennial ryegrass will lead to recognition of the gray leaf spot pathogens by perennial ryegrass, resulting in the elicitation of resistance responses.

Dr. Sally Leong's laboratory that the University of Wisconsin has isolated rice BAC clones spanning a portion of the *Pi-CO39*^{t} genetic locus and one of these BACs contains sequences that resemble disease resistance genes. Therefore, it is probable that *Pi-CO39*^{t} has already been isolated.

Objective 2: **Introgression of gray leaf spot resistance into perennial ryegrass**

Prior to initiation of USGA funding, we had identified an annual ryegrass x tall fescue hybrid with resistance to gray leaf spot. This hybrid had been crossed with both perennial ryegrass and tall fescue. We had shown that the resistance had low heritability in the H#9 x *prg* cross, with only 4 of the 104 plants that were obtained showing resistance at the time of proposal submission. Unfortunately, none of the 4 plant showed resistance upon re-inoculation.

Four additional crosses were performed between hybrid #9 and perennial ryegrass cultivars Prizm I, Prizm II, Pennant I and Pennant II. Seeds from these crosses are being germinated presently.

We had also generated a population of H#9 x tall fescue progeny. A number of these progeny were also inoculated to determine if resistance was also poorly transmitted through this cross. In this case, we found that 72 out of 551 plants tested had inherited gray leaf spot resistance. The resistant phenotype of these plants was confirmed by re-inoculation. One gray leaf spot resistant progeny plant was obtained from a second hybrid #9 x tall fescue cross. This plant is of particular interest as it has a turf-type habit.

Conclusions: It appears that the resistance is heritable but the resistance trait may be poorly transmitted into a perennial ryegrass background. The ability to transfer resistance into tall fescue may enable the latter grass to be used as a "bridge" for introduction of resistance into perennial ryegrass.

Research Plan for Coming Year

Winter, 2000:

1. Perform crosses of fungal transformants to confirm cosegregation of the avirulence phenotype with the *AVR1-CO39*^{Lp} transgene.
2. Inoculate rice progeny segregating for *Pi-CO39*^{t} to confirm that *AVR1-CO39*^{Lp} is recognized by this gene.
3. Characterize resistance phenotype microscopically.

Spring, 2001:

1. We will attempt to transform perennial ryegrass using "naked" vector constructs and using published protocols (Dalton et al. 1999; van der Maas et al. 1994). We

anticipate that some *Pi-CO39*^{t} resistance gene constructs will be imminently available. Therefore, by the time we have established the transformation system, we expect to be in a position to start transforming with constructs containing Pi-CO39t.

2. We will perform several different types of crosses:

   a. *Hybrid #9 x perennial ryegrass:* We will cross hybrid #9 with several perennial ryegrass cultivars. To account for the low heritability we observed in the first cross analyzed, we will inoculate much larger numbers of progeny from the subsequent crosses to try and identify (what we expect to be) rare individuals that have inherited resistance.

   b. *F1#9, OP x perennial ryegrass:* The progeny from the H#9 x tall fescue cross will be crossed with several perennial ryegrass cultivars.

   c. *TF9-2 X perennial ryegrass:* The gray leaf spot resistant progeny from the second hybrid#9 x tall fescue cross will be crossed with perennial ryegrass. Resistant progeny will be screened using inoculation tests.

Summer, 2001:

1. We will inoculate progeny plants with *P. grisea* to screen for gray leaf spot resistance.

Fall, 2001:

1. Attempt transformation of perennial ryegrass with *Pi-CO39*^{t} resistance gene constructs.

References:

Dalton, S. J., Bettany, A. J. E. , Timms, E. and P. Morris (1999) Co-transformed diploid *Lolium perenne* (perennial ryegrass, *Lolium multiflorum* (Italian Ryegrass) and *Lolium temulentum* (darnel) plants produced by micrprojectile bombardment. Plant Cell Reports **18**: 721-726.

Valent, B., L. Farrall and F. G. Chumley (1991) *Magnaporthe grisea* genes for pathogenicity and virulence identified through a series of backcrosses. Genetics **127:** 87-101.

Van der Maas, H. M., De Jong, E. R., Rueb. S., Hensgens, L. A. M. and F. A. Krens (1994) Stable transformation and long-term expression of the *gusA* reporter gene in callus lines of perennial ryegrass (*Lolium perenne* L.) Plant Miolecu;ar Biology **24**: 401-405.

Table 1. **Infection types of ML33 transformants carrying the *AVR1-CO39^{Lp}* gene.**

FUNGAL STRAIN	INFECTION TYPE[a] ON 51583	INFECTION TYPE ON CO39
LpKY97-1A	1	1
6059-12-1	5	1
ML33	5	5
ML33 *AVR1-CO39^{Lp}* XF1	5	1
ML33 *AVR1-CO39^{Lp}* XF2	5	1
ML33 *AVR1-CO39^{Lp}* XF4	5	1
ML33 *AVR1-CO39^{Lp}* XF5	5	1
ML33 *AVR1-CO39^{Lp}* XF6	5	1

[a] Infection Types were scored according to the scale of Valent et al. (1991) as follows: 0=no symptoms; 1=brown necrotic flecks < 1mm dia; 2= small lesions with tan centers < 1 mm dia.; 3=lesions with pale centers and dark borders ~2 mm in length ; 4= intermediate eyespot lesions with larger pale centers 3-4 mm in length; 5= large lesions without dark borders > 5 mm length.

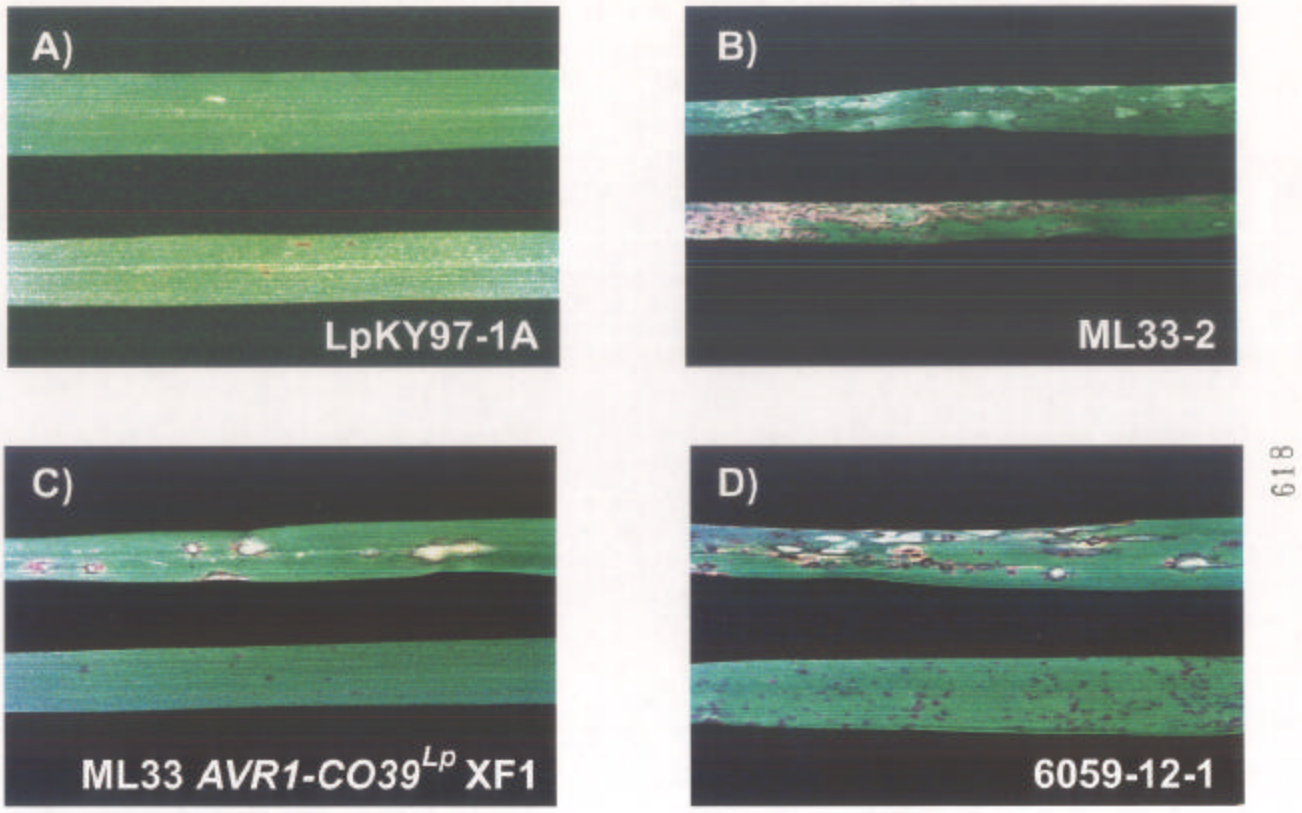

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Figure 1. Infection types of ML33 and a transformant containg the *AVR1-CO39*^{Lp} gene.

In each panel, the top leaf is from cultivar 51583 and the bottom one is from CO39. Also included are pictures of leaves inoculated with controls including a gray leaf spot isolate (LpKY97-1A) and a strain possessing *AVR1-CO39*.

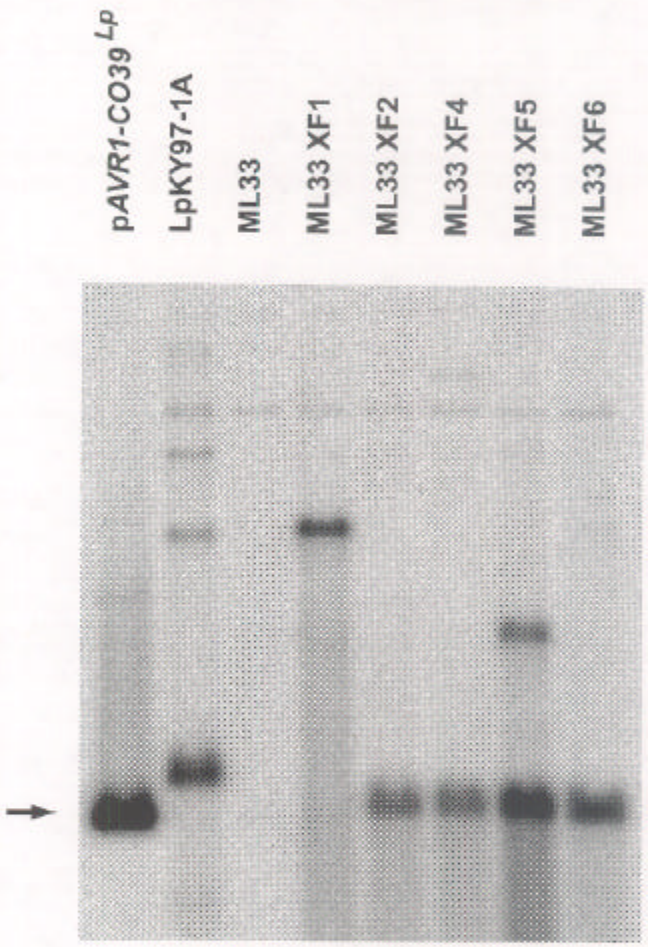

Figure 2. Presence of the *AVR1-CO39*^{Lp} transgene in transformants of ML33.

Two hundred nanograms of genomic DNA of each strain was digested with *Sac*I and *Kpn*I and electrophoresed in a 0.7% agarose gel. Also included was a lane containing the plasmid p*AVR1-CO39*^{Lp}, digested with the same enzymes. The gel was blotted to a membrane and the blot was probed with the *AVR1-CO39* gene. Shown is a phosphorimage. The identities of DNA samples are noted above the respective lanes. The position of the intact *AVR1-CO39*^{Lp} fragment is shown with an arrow.