Title: **Addition of Blue Mold Resistance to KTTII Burley Tobacco Varieties**

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Lay Summary: Blue mold is generally a sporadic disease in the United States but it can cause substantial economic damages when epidemics occur under ideal weather conditions. Even in seasons when a blue mold epidemic does not occur, the use of preventative fungicide sprays can cost tobacco growers thousands of dollars. Although the sporadic nature of the disease makes the release of a blue mold resistant variety that is inferior in terms of yield or other desirable disease resistance impractical, the addition of blue mold resistance to otherwise outstanding varieties that have high resistance to black shank and FW is highly desirable. The objective of this project is to use marker assisted selection techniques and an early flowering greenhouse procedure to add blue mold resistance to TN 90LC, KT 204LC, KT 206LC, KT 209LC, KT 210LC, KT 212LC, and KT 215LC. All of these varieties are well adapted, black shank resistant varieties that are already being utilized by burley growers. The successful completion of this project would greatly reduce the risk of catastrophic losses due to a blue mold epidemic, and eliminate the stress and economic burden of applying fungicides when a potential blue mold outbreak is forecast.

Introduction

Rationale

The objective of the current research project is to add blue mold resistance to several burley tobacco varieties previously released by KTTII. Blue mold is generally a sporadic disease of tobacco grown in the United States, but it can cause substantial economic damages when epidemics occur under ideal weather conditions. Even in seasons when a blue mold epidemic does not occur, the use of preventative fungicide sprays can cost tobacco growers thousands of dollars. Although the sporadic nature of the disease makes the release of a blue mold resistant variety that is inferior in terms of yield or other desirable disease resistance impractical, the addition of blue mold resistance to otherwise outstanding varieties that have high resistance to black shank and Fusarium wilt is highly desirable.

Breeding for blue mold resistance using traditional techniques is particularly difficult due to the interaction between the causal organism, *Peronospora tabacina*, and the tobacco host plant. Because *P. tabacina* is an obligate parasite, selection for resistance is typically done under naturally occurring field epidemics; artificial inoculation cannot be implemented because of the possibility of initiating or worsening an epidemic in surrounding commercial tobacco crops. Disease reactions due to natural infestations are greatly dependent on weather conditions and the physiological status and age of tobacco plants. This variability in disease pressure and the incomplete nature of genetic resistance often make field experiments highly variable and unpredictable, resulting in inadequate or inconsistent disease pressure to allow selection of plants having high resistance to blue mold. By using molecular markers, this environmental variability can be eliminated. DNA from a single plant can be analyzed for

the presence of markers linked to disease resistance and other desirable genes, greatly enhancing and expediting the breeding process. The goal of the current research project is to use marker assisted selection techniques and an early flowering greenhouse procedure to add blue mold resistance to existing KTTII burley tobacco parental lines and varieties.

Background

When this project was initiated, the original objective was to add blue mold resistance to "Zyvert" versions of KTTII parental lines and varieties. For that genetic material, KTTII had developed early flowering (FT) strains of elite parental lines used to develop commercial hybrid burley varieties. The FT trait can effectively shorten the "seed to seed" cycle in a breeding program from the normal 150 to 165 days to 70 to 75 days, greatly decreasing the time required for backcross introgression of desirable traits into breeding lines. These FT Zyvert lines also contained three alleles that minimized the conversion of nornicotine to nornicotine, and subsequently the formation of the associated tobacco specific nitrosamine, nitroso-nornicotine. At the time this blue mold resistance project was begun, it was assumed that within three to five years the entire tobacco industry would move to varieties containing Zyvert technology due to their reduced harm characteristics.

However, in July of 2016 a decision was made by Philip Morris International and Altria tobacco companies to not proceed with the commercial utilization of varieties containing Zyvert technology. This was due to the discovery that genetic engineering techniques had been utilized in the early stages of the development of the original source materials provided to KTTII for the initiation of the Zyvert project. Although genetically modified organisms (GMOs) are quite common among field crops in the United States, they are not accepted by several foreign countries, particularly within the European Union. In addition, since the FT early flowering technique utilizes a transgene (which is eliminated before the final varieties are commercially released) many of these same countries consider any variety that utilizes the FT trait during its development to also be a GMO product. As a result of the stigma of GMOs in these countries, and the international nature of the burley tobacco supply chain, it is not feasible to attempt to maintain two separate inventories of tobacco, one with and one without Zyvert technology. Because previous research conducted under this project involved adding blue mold resistance to Zyvert rather than LC versions of KTTII varieties, the decision to not utilize Zyvert varieties effectively means everything developed prior to June 2016 is non-usable. This project was re-initiated in July, 2016 utilizing only LC versions of breeding lines and non-FT methods of early floral initiation.

Summary of Progress

During July, 2015, a significant outbreak of blue mold occurred in the primary KTTII race 1 black shank nursery in Greeneville, TN. This represented the first chance in over five years to visually evaluate breeding materials that had putative blue mold resistance, based on the presence of neighboring genetic markers that are normally linked to the actual gene conferring resistance to blue mold, to confirm that they indeed possessed genetic resistance to the disease. The blue mold incidence was severe enough to clearly differentiate plants that had genetic resistance to blue mold versus those that were susceptible (**Photo 1**). Several breeding lines were identified that were genetically stable for blue mold resistance. A high level of resistance was verified both visually and through DNA analyses. These lines not only displayed good visual blue mold resistance, but moderate to high resistance to race 1 black shank. TKF 4028LC, one of the breeding lines that was identified as having high resistance to blue mold, is the male pollinator for hybrid variety KT 206LC.

The original objective of this project was to transfer blue mold resistance into previously released TN 90LC and KT hybrid varieties KT 204LC, KT 206LC, KT 209LC, KT 210LC, KT 212LC. Since the original

project was started, a new variety (KT 215LC) was released in 2015. KT 215LC has higher resistance to both race1 black shank and *Fusarium* wilt than any other commercially available burley variety. In addition, greenhouse inoculation studies indicate that KT 215LC also has a moderate level of resistance to bacterial wilt as well. Since the original blue mold project has to be restarted, resistance will also be added to KT 215LC. To accomplish this, it will be necessary to first introgress the blue mold resistance allele into each of the parental lines used in the development of KTTII hybrid varieties. Because TKS 2002 is the female parent for all KTTII hybrid varieties, the first step in this project is to introduce the blue mold resistance gene into the TKF 2002 and TKS 2002 parental lines. To restart the blue mold project, initial blue mold crosses were made in the black shank nursery during July and August, 2016. TKF 4028LC, one of the breeding lines that was verified in 2015 as having high resistance to blue mold, was chosen as the donor parent. This line was crossed onto male parental lines TKF 2002LC, TN 90LC, TKF 4024LC, TKF 6400LC, L8LC and TKF 2802LC. F_1 progeny seed from these crosses were collected and are currently seeded in the greenhouse. Because the early flowering FT trait can no longer be utilized to hasten flowering, a greenhouse procedure developed by KTTII that uses 20X the recommended rate of the fungicide Terramaster is being utilized to reduce the time required for flowering. This procedure will typically result in flowering within 85-90 days following seeding, compared to 40-45 days for the FT trait and 130-150 days for normal flowering in the greenhouse.

Plans for Future Work

Although this project will be continued until blue mold resistant KTTII varieties are commercially released, **due to having to restart the entire project the Council for Burley Tobacco will not be asked to provide additional funding**. To finish the project, early flowering plants will be selected in the greenhouse and molecular markers will be used to verify that individual plants used for the first backcross are heterozygous for the blue mold resistance marker. For each parental line family, five backcrosses will be made with the presence of blue mold resistance verified via molecular marker analyses each generation. Because the blue mold marker is not absolutely reliable in all cases, actual blue mold resistance will also be verified in plants containing the marker by inoculating at least three plants with the blue mold pathogen. The inoculations will be done in a biologically controlled growth chamber to prevent possible spread of the pathogen. Because the actual major blue mold resistance gene appears to be incompletely dominant, plants heterozygous for the trait should display disease symptoms intermediate to resistant and susceptible control plants.

To move the desired blue mold resistance trait into the male sterile version of TKF 2002 required for commercial seed production of all KT hybrid varieties, pollen from the backcross four (BC_4) generation of FT 2002 will be used to pollinate male sterile TKS 2002. This will be followed by an additional backcross with BC_5 pollen of TKF 2002. In the BC_5S_1 generations of TKF 2002 and BC_6 generation of TKS 2002, seed will be collected only from plants that are verified via marker analyses to be homozygous for the desired blue mold resistance allele. For the introgression of the blue mold resistance allele into each of the male parental lines that are pollinators for specific KTTII hybrid varieties, the F_1 progeny seed that are now growing in the greenhouse will be backcrossed to the appropriate parental line five times. Heterozygosity for blue mold resistance will need to be verified via marker analyses before each backcross generation for TN 90, TKF 4028, and TKF 4024 (pollinator lines for KT 204LC, KT 206LC, and KT 209LC, respectively). Because TKF 6400, L8, and TKF 2802 (pollinator lines for hybrid varieties KT 210, KT 212, and KT 215LC) do not currently possess the recessive allele that confers resistance to the PVY complex of viruses, segregating backcross generations will be screened for both the blue mold and the virus resistance alleles. This will allow the new versions of KT 210LC, KT 212LC, and KT 215LC to not only have added resistance to blue mold, but to the PVY complex as well.

Due to having to restart the blue mold breeding project, the estimated timeline projected in the initial funding request will be pushed back by approximately two to three years. By using the Terramaster protocol, it is estimated that introgression of the blue mold trait into the respective parental lines can be accomplished in 24 - 30 months. The actual experimental blue mold resistant hybrid varieties will be made using BC_5 parental lines; it is anticipated that this will occur in time to allow initial field testing in 2020. If this goal is met, resistant versions of ms TN 90LC, KT 204LC, KT 206LC, KT 209LC will be entered into the Regional Quality Test in 2021 or 2022. Because a maximum of four experimental lines can be entered into the RQT in a given year, KT 210LC, KT 212LC, and KT 215LC will be entered the following year. Assuming they meet the RQT minimum standards, the new varieties would be released for commercial seed production in 2022 or 2023, with seed available to growers the following year(s).

Figure 1. A comparison of a burley tobacco plant that has genetic resistance to blue mold versus one that is susceptible.