Development of pale yellow dark burley:
An additional approach to reducing TSNAs in Kentucky burley

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Rationale

The recently published Surgeon General's 2014 Report is extremely critical of the burley tobacco component of cigarettes. This report, entitled *The Health Consequences of Smoking—50 Years of Progress*, concludes that " … the use of burley tobacco in U.S.-style blended cigarettes contributes substantially to the differences in tobacco-specific nitrosamines (TSNAs) between the U.S.-style cigarettes and those of Canada and Australia" (p. 155), and then attributes the increase of certain lung cancers in the United States to this difference (p. 178). Most of the data that supports this argument is based on the Massachusetts Benchmark Study (http://legacy.library.ucsf.edu/tid/yek21c00) which was completed in 1999.

To date, there is no published goal for the acceptable level of TSNAs, but during the past two decades and subsequent to the Massachusetts Benchmark Study, considerable effort has been made by the industry to reduce the level of TSNAs in burley. The approach to most of this work has been to reduce conversion of nicotine to nornicotine, which is the precursor to the TSNA NNN. The most dramatic reduction in TSNA levels has been achieved by the introduction of seed screening to eliminate plants with a high potential to convert nicotine to nornicotine resulting in low converter (LC) varieties (http://www.uky.edu/Ag/Tobacco/Pdf/LC-Protocol.pdf). Another on-going approach is the major effort to modify the current commercial varieties to include the demethylase mutant genes which will reduce the TSNAs in cured burley leaf even further.

There is still another approach that can be used to reduce TSNA levels even further that has not received much attention: the use of green tobaccos that have a much lower propensity for conversion of nicotine to nornicotine than in burley.

This may be a direct result of the higher nitrogen rates, about four times the amount than for green tobacco, required to produce an acceptable yield and quality of burley because burley is genetically chlorophyll-deficient and therefore requires this higher nitrogen to compensate for the low concentration of chlorophyll. This high level of N fertilization, however, does result in higher levels of alkaloids, and consequently a greater potential for nornicotine and NNN accumulation. Introducing a green gene into burley (Fig. 1.) would further reduce the propensity of burley to produce NNN. In a test in 2010 in Kentucky, the TSNAs in a traditional burley-style line were dramatically reduced from 3.2 ppm to 1.1 ppm by adding a green gene to that burley line.

Another distinct advantage of this approach is the reduction in production cost by approximately $80/acre because only one quarter to one third of the amount of nitrogen fertilizer need be applied to this green style of tobacco.

There are many differences in leaf chemistry between burley and flue-cured (Adam *et al.* 2005) but Legg *et al.* (1977) demonstrated that most of these differences are the result of genetic diversity between varieties, regardless of the class of tobacco (burley or flue-cured), and not the result of the chlorophyll status of the plant. In Legg's study, there was often considerable overlap of the values between the analytes in flue-cured and burley, despite significant differences between the means of these analytes for each of the tobacco types. Selection of typical burley leaf chemistry in a green plant is therefore possible.

The manufactures' requirement of burley is that it is able to absorb flavorings, but the underlying physiological reason for this characteristic of burley is not well defined. This desirable burley characteristic is not necessarily linked to the chlorophyll status because Maryland (MD) tobacco is green but can be used as a burley substitute. There are also differences in the intensity of the green, and therefore presumably the concentration/density of chloroplasts, between green tobacco types (compare dark air-cured, flue-cured and Maryland) and also between varieties within the same green tobacco type (compare Kutsaga Mammoth 10 to Kutsaga E1). There is, therefore, no evidence to suggest that a dark burley cannot have satisfactory burley manufacturing characteristics.

A potential risk of a green style of burley is that not all the chlorophyll will break down during curing, especially in unfavorable curing seasons, resulting in some green color in the cured leaf. This is highly undesirable to the manufacturers. However, a gene known as "pale yellow" causes the tobacco plant to lose its chlorophyll very quickly after harvest (Figs. 2 and 3) and is not associated in any way with the green genes that increase the chlorophyll content of the leaves. The opportunity, therefore, exists of developing a "dark" burley that has an increased level of chlorophyll and so requires less fertilizer, and also contains the pale yellow trait that will ensure that the green tobacco will still cure like a conventional burley, even in a less than optimum curing environment.

Preliminary work to develop a "pale yellow dark burley" was started in 2012: KTRDC funded initial work to introduce the green genes from Maryland tobacco in burley, and the Burley Tobacco Growers Cooperative Association funded the initial work on developing markers to identify the pale yellow gene.

The starting point for this program comprises of an elite burley line developed by the Kentucky Tennessee Tobacco Improvement Initiative (KTTII) and currently used as the parent for most of the newer commercial KT burley hybrids. This parent already has resistance to blackshank race 0, PVY and TMV and contains two of the demethylase genes. The green color will be sourced from Maryland tobacco because this tobacco can be used as a burley substitute. The pale yellow gene will be extracted from the dark air-cured variety KY PY171 which has been tested for use in western Kentucky in recent years.

The initial crosses between Maryland and the KTTII elite line (MD x Bu) and between KY PY171 and the same KTTII elite line (PY x Bu) were done in 2012. In 2013, seed from these two F1 populations were grown in a heavily infected blackshank field. The surviving plants that have some resistance to blackshank were intercrossed to combine the pale yellow gene into the green burley background as well starting to accumulate increased resistance to blackshank race 1. The Maryland x elite burley line has been backcrossed four times in preparation of the male sterile parental lines.

Procedures:
The original protocol for this stage of the dark pale yellow burley development program was to screen for the genetic markers of the most important genetic characters, including various disease traits as well as the e4 and e5 demethylase mutant genes, in 1000 plants of the F_2 generation and set 300 of the most promising lines into a field infected with blackshank race 1 which cannot be screened using markers. The most agronomically acceptable plants that survive the blackshank were to be intercrossed between each other, and the resulting seed screened again in 2015 and re-tested in a blackshank-infected field. Funding for the 2014 test could not cover the cost of both the marker screening and the blackshank test. Of these two parts of the variety development program, it was most expedient to continue with the field screening of blackshank resistance because, although not ideal, marker screening of future generation of seed can still eliminate plants with the least of the desired characteristics.

Results:
A field with a history of blackshank race 1 on a farm in Fayette County was located with the considerable assistance of the Fayette County Extension Office and the lease agreement to use 0.8 acres was finally signed with the grower in May 2014. The KY14xL8 crop in this field in 2012 suffered severe losses, and soybeans were grown in this field in 2013. The field was set on June 19 (Fig. 1).
Six hundred and thirty plants of the R_0 population of (MDxBu) x (PYxBu) cross were set in the field, and 546 plants of S_1 PYxBu. A row of TN 90 was set between each four rows of the test lines as the check against which to compare the disease level. Five rows of Ky14 x L8, which only has resistance to blackshank race 0, were set through the field to gauge the severity of the blackshank.
The level of blackshank was very much lower than would have been expected after only one year of rotation after the reported losses in 2012. Selection of individual plants that would be intercrossed and from which seed would be saved was delayed as long as possible, until September 26, to allow more plants to succumb to blackshank. This low blackshank pressure is illustrated in Fig. 1 which compares the 2013 Christian County survival rate with that in the 2015 Fayette County plot: even after weight weeks after setting, only one percent of the KY14xL8 plants had died from blackshank and only 50% by October 23 at which date only half of one percent of the TN90 plants were infected.
In the absence of a meaningful level of disease pressure, individual plants were selected based on agronomic type. On September 26, the flower heads of 62 selections of R_0 (MDxBu) x (PYxBu) and 81 of S_1 PYxBu were bagged (Fig. 3) and the seed was collected on October 23.

Conclusions:
Progress in the development of a pale yellow dark burley is being made, although for a variety of logistical and financial reasons, this progress has been slower than expected. The development of a dark burley that contains the pale yellow gene that reduces the propensity for burley to produce high levels of TSNA remains a viable option. The including of the pale yellow gene in conventional burley may also reduce the amount of leaf that cures with a hard green color as occurred in a considerable portion of the crop in Kentucky in 2014 (Fig. 4).

References:

Chaplin, J. F. 1969. Inheritance and possible use of pale yellow character in tobacco. Crop Science 9:169-172

Legg, P. D. 1995. Registration of PY KY 171 and PY KY 160 tobacco germplasm lines with the pale-yellow trait. Crop Science 35(2):602

Legg, P.D., Chaplin, J.F., Williamson, R.E. 1977. Genetic diversity in burley and flue-cured tobacco. Crop Science 17 (6): 943-947

Figures:

Fig. 1. Fayette County blackshank field, July 25, 36 days after setting

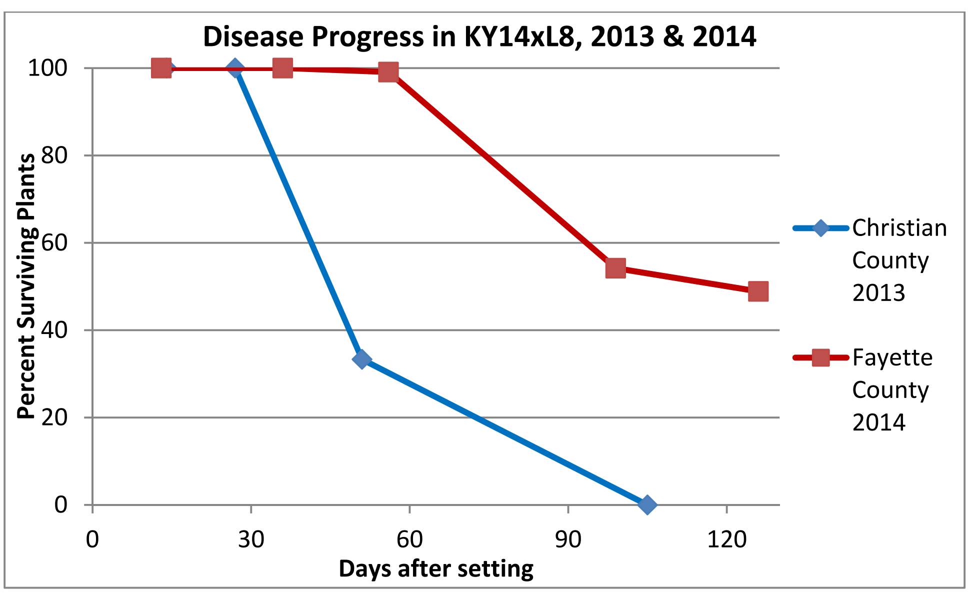

Fig. 2. Survival rate of KY14xL8 in 2013 Christian County and 2014 Fayette blackshank nurseries.

Fig. 3. The flower heads of selected plants were bagged to prevent natural cross-pollination.

Fig. 4. A considerable portion of the 2014 Kentucky burley crop cured with an undesirable green color. The pale yellow gene should reduce this problem.