Journal of the Korean Society of
Tobacco Science Vol. 27, No. 1, 11~18 (2005)
Printed in Republic of Korea

Identification of Nicotine Converter Plants in Burley Tobacco KB9118 (KB108)

Suk-Hun Jung*, Yun-Hwa Chung, Wan-Soo Keum, Yue-Gyu Kang,
Seung -Ku Shin, Chun-Joon Jo[1] and Sang-Ju Choi
KT&G Central Research Institute, Agro-tech. Research Group,
R&D Planning Office[1]
(Received May 31, 2005)

ABSTRACT : The nicotine converter genotypes of burley tobacco (*Nicotiana tabacum* L.), which convert nicotine to nornicotine, contain a high amount of nornicotine that degrades tobacco quality and smoking taste. Elimination of nicotine converter plants before seed harvesting is required for breeding nicotine low-converter lines and for increasing their seed production. This study aims to develop a rapid and convenient method of identifying nicotine converter plants of burley breeding lines of KB9118(KB108) using thin-layer chromatography (TLC) and isatin coloration method. Out of 223 plants in 10 lines harvested at maturity in 2002, 102 plants (45%) were identified as nicotine converters by TLC of tobacco leaves air-cured. For 16 lines selected as low-converters in 2002, 148 plants grown in the field in 2003 were tested by the isatin coloration method using two detached leaves at the flowering stage thoroughly sprayed with 1% $NaHCO_3$ solution and cured in conditioned chambers for the early identification of nicotine to nornicotine conversion. From these samples, 46 plants (31%) in 4 lines were identified as nicotine converters, indicating that the ratio of converters significantly decreased by one time selection. Mean percent conversion of non-screened lines was 14% higher than that of following generation. Therefore in the burley tobacco, a rapid and convenient means of identifying and removing nornicotine converter plants by the isatin coloration method during growth in the greenhouse or field were effective in reducing the converter plants in the following generation.

key words **: *Nicotiana toabacum* L., nornicotine, sodium bicabonate, nicotine converter, isatin**

Nornicotine, a secondary alkaloid primarily formed from nicotine in commercial tobacco (*Nicotiana tabacum* L.), degrades tobacco flavor, quality and smoking taste, and its high level directly contributes to the formation of N-nitrosonornicotine (NNN), an important tobacco-specific nitrosamine harmful to human health. Nicotine converter genotypes, which convert nicotine to nornicotine, are especially more frequently found in commercial burley tobacco cultivars in which nicotine is the predominant alkaloid compared to flue-cured ones. Breeder seeds of releasing cultivars containing high quantities of nornicotine (nicotine converter plants) must be screened periodically and discarded to obtain high quality breeding lines.

Griffith et al. (1955) determined that the

*연락저자 : 441-480 경기도 수원시 권선구 당수동 434, KT&G 중앙연구원 원료연구소
*Corresponding author : *Agro-tech. Research Group, KT&G Central Research Institute, 434 Dangsu-dong, Gwonsun-gu, Suwon 441-480, Korea*

conversion of nicotine to nornicotine in tobacco was controlled by gene at one or two loci. Mann et al. (1964) have demonstrated that each of the progenitor species of *N. tabacum* possesses a single dominant gene capable of initiating the conversion of nicotine to nornicotine. A recessive allele of tobacco is highly unstable, and genetically changes to a form that completes the metabolic pathway leading to nicotine demethylation (Wernsman et al., 2000). Nicotine to nornicotine conversion, which is mediated by nicotine demethylase, begins in green leaves and increases during the post harvested air-curing. The conversion activity peaks during the first 3 weeks of air-curing in burley tobacco, when the process of leaf senescence is accelerated.

In most converter tobacco cultivars, individual plants contain appreciable quantities of nornicotine after air-curing. However, stimulation of nicotine to nornicotine conversion before air-curing is required to identify and remove the converter plants from the population more easily and as early as possible. A few researchers reported the stimulating effect of ethylene and $NaHCO_3$ on demethylation of nicotine to nornicotine. Therefore, the objective of this study was to investigate a more effective way to identify and remove nicotine converters at early plant growth stage for the development of low nornicotine tobacco varieties and the high quality tobacco production.

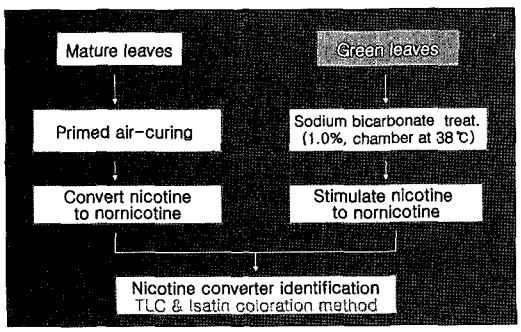

Fig. 1. Rapid method of nicotine converter genotypes identification.

Materials and Methods

In 2002, 10 burley tobacco breeder seed lines of KB9118 named as KB108 (Jung et al.,1994) with the genetically stable traits were screened for identifying nicotine converter genotypes by the thin layer chromatographic (TLC) method (Wernsman et al., 1971). Among these lines tested in 2002, 16 low-converter and 3 converter lines were used in 2003 for the identification of nicotine converter plants. All plants were grown in standard plots with 40 cm spacings between plants and 120 cm distances between plots at the Agro-tech. Research Group, KT&G Central Research Institute. Cultural practices including fertilization, cultivation, sucker and pest controls followed the standard practices recommended for burley tobacco production in Korea by the Agro-tech. research group.

For detecting converter plants by TLC method, the upper leaves from the top of each individual plant were detached and cured in a conventional steel frame house. The standard air-curing method of burley tobacco in the KT&G Central Research Institute is as follows. The harvested leaves were loaded in plastic house and sun shading cloth for 4 weeks until the end of curing. A modified method of Sato et al. (1982) was used to identify nicotine converter plants at the early plant growth stage by isatin coloration method(Fig. 1). The upper green leaves from the top of the each individual plant were detached and sprayed with 1% $NaHCO_3$ aqueous solution, and cured in a chamber at 38℃ and RH 80% for 4 days, keeping leaves from drying too rapidly. The method of Gundiff and Markunas (1964) was used to determine the alkaloid contents.

Results and Discussion

Agronomic characteristics of the tested 10 burley tobacco breeder seed lines of KB9118 are shown in Table 1. There were significant differences among the lines tested at P = 0.05 by the least significance difference test in plant height, leaf no. per plant, and largest leaf width. No significant differences were found in largest leaf length and days to flower, and all were resistant to *Potato virus Y* (PVY).

The 223 of 440 plants from the ten lines of KB 9118 (Table 2) were selected in the disease nursery that soil was highly infested with the tobacco bacterial wilt, and tested for detecting nornicotine by TLC method (Fig. 2). From these lines, 25%-56% of the tested plants were apparently determined as converter genotypes to give an average of 45% (a total of 102 plants out of 223). The percentage of converters relative to the total plants in KB 9118-2 was the least, while the KB 9118-19 was the highest 56% (18 converters out of 32). These results are similar to the findings of Bush et al. (1999) who observed elevated conversion of nornicotine if the converter plants were not eliminated prior to increase seed of a pure line cultivar.

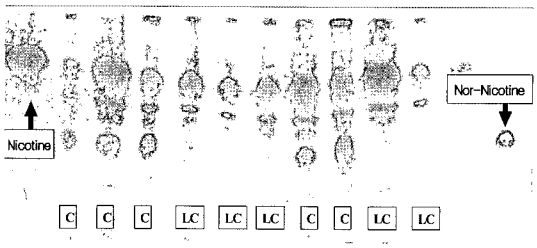

Fig. 2. A thin-layer chromatography (TLC) method for identifying nicotine converter genotypes. C : nicotine converter genotype, LC : low converter.

Seeds of the promising and low converter 16

Table 1. Comparison of agronomic characteristics among burley tobacco breeder seed lines of KB9118 tested in 2002

Breeder seed lines	Plant height (cm)	Leaves per plant (no.)	Largest leaf		Days to flower	Reaction to PVY
			Length (cm)	Width (cm)		
KB9118-2	173	23.0	66.9	30.1	66	R[1)]
KB9118-4	171	23.9	67.2	30.2	66	R
KB9118-5	173	22.9	67.1	30.2	67	R
KB9118-6	168	24.0	66.4	29.0	66	R
KB9118-7	177	24.5	65.0	28.1	68	R
KB9118-19	170	25.0	68.2	29.2	66	R
KB9118-20	163	22.1	66.2	28.5	67	R
KB9118-21	163	24.1	66.4	27.8	66	R
KB9118-22	173	23.5	66.4	30.4	66	R
KB9118-23	169	24.0	67.8	27.7	68	R
L.S.D. 5%	2.5	1.7	NS[2)]	2.0	NS	-
1%	NS	NS	NS	NS	NS	-
C.V. (%)	3.7	3.4	2.7	3.1	1.4	-

[1)]R : Resistance, [2)]NS : Non significant.

Table 2. Selection of nicotine converter type in burley tobacco breeder seed lines of KB9118 by thin layer chromatographic (TLC) method in 2002

Breeder seed lines	No of applied plants	No. of selected plants	No. of selected plants		Percent of converter plants
			Low converter	Converter	
KB 9118-2	40	12	9	3	25
KB 9118-4	40	15	11	4	27
KB 9118-5	40	14	7	7	50
KB 9118-6	40	33	16	17	51
KB 9118-7	40	19	9	10	53
KB 9118-19	40	32	14	18	56
KB 9118-20	40	9	5	4	44
KB 9118-21	80	49	29	20	41
KB 9118-22	40	20	10	10	50
KB 9118-23	40	20	11	9	45
Total	440	223	121	102	45

Table 3. Conversion of nicotine to nornicotine in burley tobacco breeder seed lines of KB9118 with a normally air-cured leaf by isatin method in 2003

Breeder seed lines	Genotype	No. of applied plants	No. of selected plants		Percent of converter plants
			Low converter	Converter	
KB9118-2-1	LC[1)]	8	8	0	0
KB9118-2-2	LC	6	6	0	0
KB9118-2-3	LC	6	6	0	0
KB9118-2-4	LC	6	5	1	17
KB9118-4-1	LC	4	4	0	0
KB9118-4-2	LC	5	1	4	80
KB9118-4-3	LC	7	5	2	29
KB9118-4-4	LC	8	8	0	0
KB9118-19-1	LC	8	8	0	0
KB9118-19-2	LC	5	4	1	20
KB9118-19-3	LC	5	5	0	0
KB9118-19-4	LC	7	7	0	0
KB9118-21-1	LC	7	7	0	0
KB9118-21-2	LC	5	5	0	0
KB9118-21-3	LC	7	7	0	0
KB9118-21-4	LC	6	6	0	0
Total		100	92	8	8

[1)] *LC : nicotine low converter type.*

lines selected from the 121 plants were produced by self-fertilization, and the progeny plants were tested to identify nicotine converters in the next generation. At maturity, two leaves from each plant were harvested and air cured. Lamina samples of 5 mm in diameter were punched and screened by the isatin coloration method. The tested plants were classified as nicotine converters or low converters by estimating the color levels as for the relative proportions of nornicotine in cured leaves of each plant. Four of 16 lines of KB9118 (Table 3) were classified as nicotine to nornicotine converters, comprising 8% of the total plants tested, showing that a significant number of converter plants arose from low converter plants clearly by genetic instability in the next generation as suggested by Wernsman et al. (2000). The fact that converter plants were emerged from the low converter in the following generation also suggests that the low converter genotypes should be heterozygous C_{TCT} for the C_T gene and segregated genetically. In KB9118-4-2, 80% of the total plants were identified as converters, probably resulting from misidentification of probable converter genotypes. However, in 12 lines of KB9118, all plants were low converters, suggesting that they should have low converter homozygotes (c_{TCT}). Based on

Table 4. Frequency of converter plants arising from low-converter plants by treatment of tobacco green leaves with sodium bicarbonate

Breeder seed lines	Genotype	No. of Applied plants	No. of selected plants		% converter plants[3]
			Low converter	Converter	
KB9118-2-1	LC[1]	9	4	5	56
KB9118-2-2	LC	7	4	3	44
KB9118-2-3	LC	9	4	5	56
KB9118-2-4	LC	9	8	1	11
KB9118-4-1	LC	8	5	3	38
KB9118-4-2	LC	10	4	6	60
KB9118-4-3	LC	10	8	2	20
KB9118-4-4	LC	10	7	3	30
KB9118-19-1	LC	9	4	5	56
KB9118-19-2	LC	10	9	1	10
KB9118-19-3	LC	10	10	0	0
KB9118-19-4	LC	10	6	4	40
KB9118-21-1	LC	9	5	4	44
KB9118-21-2	LC	10	9	1	10
KB9118-21-3	LC	9	9	0	0
KB9118-21-4	LC	9	6	3	33
Total		148	102	46	31
KB9118-2-5	C[2]	9	0	9	100
KB9118-2-6	C	9	0	9	100
KB9118-4-8	C	9	0	9	100
Total		27	0	27	100

[1] LC : nicotine low converter type, [2] C : nicotine converter type,
[3] converter/(converter + low converter) x 100.

these aspects, a significant number of converter plants might arise from low converter plants if air-cured leaves are used for detecting converters.

Sixteen lines of KB9118 comprising 148 low converter plants were grown in the field and also tested for nornicotine to identify nicotine converters at early stage of plant growth using both $NaHCO_3$ treatment and the isatin coloration method (Fig. 3). Out of 16 lines of KB9118, 14 lines had converter plants with 10%-60% frequencies, a total of 46 plants (31% in average) (Table 4). In this paper, the converter ratio also decreased compared to the parent generation, indicating that the TLC method with air-cured leaves should be effective in selecting out the converters. On the other hand, detecting ability for the converters increased from 8% to 31% by $NaHCO_3$ treatment. These results are similar to the finding of Shi et al. (2001) who observed elevated nicotine conversion in leaf samples of converter plants treated with $NaHCO_3$. Three

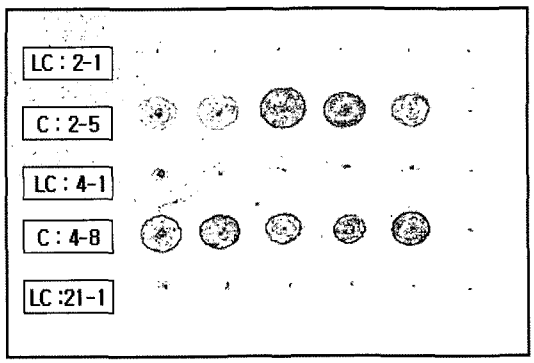

Fig. 3. KB9118 lines showing the degree of coloration formed by the reaction of isatin and nornicotine. LC : Nicotine low converter, C : Nicotine converter genotypes.

Table 5. Comparison of percent nicotine conversion in air-cured tobacco leaves between low converter and converter genotypes in the burley tobacco breeder seed lines of KB9118

Breeder seed lines	Genotype	Nicotine (%)	Nornicotine (%)	% nicotine conversion[3]
KB9118-2-1	LC[1]	2.19	0.13	6
KB9118-2-2	LC	1.46	0.27	15
KB9118-2-3	LC	2.04	0.40	16
KB9118-21-1	LC	1.61	0.13	7
KB9118-21-3	LC	1.61	0.27	14
KB9118-21-4	LC	1.31	0.27	17
Mean		1.70	0.24	12
KB9118-2-5	C[2]	0.37	0.93	72
KB9118-2-6	C	0.95	1.07	53
KB9118-2-7	C	0.95	0.40	30
KB9118-4-6	C	0.95	1.60	62
KB9118-4-7	C	0.66	1.20	65
KB9118-4-8	C	0.66	1.60	71
Mean		0.67	1.13	63

[1] nicotine low converter type, [2] nicotine converter type,
[3] nornicotine/(nicotine + nornicotine) x 100.

lines of KB9118-4-2 were classified as nicotine converter to nornicotine was also identified as converter plants in the next generation, a probable homozygous genotype for the converter gene, C_TC_T.

Analysis of alkaloid contents showed that nornicotine contents in low converter genotype lines were much lower than those in converter genotype lines although there were some variations (Table 5). This data clearly shows that low converter genotypes have low nicotine conversion (below 20%). Two lines of KB9118-2 and KB9118-21 identified as low converter lines by isatin coloration method with air-cured leaves had low nicotine conversion ranging from 6% to 17%. All the converter lines tested also had nicotine conversion higher than 60% in average.

The isatin coloration method is a significant way to identify converter or low converter genotype plants. Moreover, the $NaHCO_3$ treatment stimulates nicotine conversion in converter plants to the maximum level to make it possible to identify them early at the growing stage and with ease. The isatin coloration method with $NaHCO_3$ treatment was found to be effective in identifying nicotine to nornicotine converters in green tobacco leaves while the plants are still growing. If the elimination of converter genotypes in the field are conducted every year by this method, the percentage of nicotine conversion will be much lower than that of the previous generation. This is especially useful during the breeding process, when the converters should be identified and removed before flowering to ensure the production of pure low converter seeds. If genotypes with nicotine demethylation capability could be completely removed from breeder seed of a inbred line, an aliquot of clean breeder seed would be used to increase seed of a pure line cultivar for the production of commercial male sterility F_1 hybrids.

Conclusion

This study was conducted to select the nicotine converter plants of burley tobacco breeder seed lines of KB9118 by the rapid and convenient method. The results are as follow.

Out of 223 plants in 10 lines harvested at maturity in 2002, 102 plants (45%) were identified as nicotine converters by TLC of burley tobacco leaves air-cured. Among lines selected as low converters in 2002, 148 plants grown in the field in 2003 were tested by the isatin coloration method for the early identification of nicotine to nornicotine conversion. From these samples, 46 plants (31%) were identified as nicotine converters, indicating that the ratio of converters significantly decreased by one time selection. Mean percent conversion of non-screen lines was 14% higher than that of following generation. Lines of KB9118 indentified as low converter had nicotine conversion of 12% in average. The converter genotype lines tested had nicotine conversion ranging from 30% to 72%.

References

Burk, L.G. and Jeffery, R.N. (1958) A study of the inheritance of alkaloid. *Tob. Sci.* 2:139-141.

Bush, L.P., Zhan, Y., Yang, H., Shi, H., Fannin, F.F. and Burton, H.R. (1999) Time of nornicotine formation in burley tobacco. Presented at CORESTA, Suzhou, *China, Abstract* AP37.

Fannin, F.F. and Bush, L.P. (1992) Nicotine demethylation in *Nicotiana. Med. Sci. Res.* 20: 867-868.

Griffith, R.B., Valleau, W.D. and Stokes, G.W. (1955) Determination and ingeritance of nicotine to nornicotine conversion in tobacco.

Science 121 : 343-344.

Gundiff, R.H. and Markunas, P.C. (1964) Abberviatel techniques for determination of alkaloids in tobacco using the extraction procedure. *Tob. Sci.* 8: 136-137.

Jung, S. H., Choi, S. J., Jo, C.J., Kim, D.S., Jo, M.J. and Lee, S.C. (1994) Development of KB 108, a new burley tobacco (*Nicotiana toabacum* L.) variety and its agronomic characteristics. *J. Kor. Soc. Tob Sci.* 16(1): 57-63.

Legg, P.D., Chaplin, J. F. and Collins, G.B. (1969) Inheritance of percent total alkaloids in *Nicotiana tabacum* L. Population derived from cross of low alkaloid lines with burley and flue-cured varieties. *J. Herd.* 60: 213-217.

Mann, T.J., Weybrew, J.A., Matzinger, D.F. and Hall, J.L. (1964) Inheritance of conversion of nicotine to nornicotine in varieties of *Nicotiana tabacum* and related amphiploids. *Crop Sci.* 4: 349-353.

Miller, R.D., Bush, L.P., Burton, H.R. and Shi, H. (2001) Screening for nornicotine conversion in burley tobacco. Presented at the 55th Tobacco Science Research Conference, Greensboro, NC.

Sato, M. and Asane, K. (1982) Studies on the accumulation of nicotine-converting mutants in tobacco. Ⅲ. A Cotyledon-test method for the detection of nicotine-converting mutants. *Iwata* 14: 17-21.

Shi, H., Fannin, F.F., Burton, H.R. and Bush, L.P. (2000) Factors affecting nicotine to nornicotine conversion in burley tobacco. Presented at the 54th Tobacco Science Research Conference, Nashville, TN, Abstract 5451.

Shi, H., Fannin, F.F., Burton, H.R. and Bush, L.P. (2001) Identification of nicotine to nornicotine converters in burley tobacco. Presented at the 55th Tobacco Science Research Conference, Greensboro, NC.

Wernsman, E.A. and Hall, J.L. (1971) A thin-layer chromatographic method for indentifying nicotine converter genotypes. *Tob. Sci.* 101: 933.

Wernsman, E.A. (2000) Genetic instability at nicotine to nornicotine locus in burley tobacco and its consequences on secondary amine alkaloids and TSNA's. 2000 CORESTA Plenary paper APST 1.