Comparison of Genetic Variability among Anther-derived and Ovule-derived Doubled Haploid Lines of Tobacco

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This study was conducted to obtain information on the causes of variation of anther-derived doubled haploid lines from an inbred cultivar of tobacco (*Nicotiana tabacum*). From a spontaneous doubled haploid line of androgenetic origin of tobacco, an experimental series of doubled haploid lines was raised through anther culture or induced parthenogenesis. The field test of 38 anther-derived and 12 ovule-derived doubled lines along with 17 lines of the selfed parent revealed considerable variations among the anther-derived lines both in quantitative and qualitative characters. On the other hand, all the ovule-derived lines, some of which were doubled by colchicine treatment while the others were doubled spontaneously, were not different from the selfed lines of the parental plant for the characters measured. No cytoplasmic effects were detected in the reciprocal crosses between anther-derived doubled haploid lines possessing variations and the parental line. These results indicate that the variations observed among doubled haploid lines of androgenetic origin were due to the mutations of nuclear genes in the course of androgenesis.

KEY WORDS : *Nicotiana tabacum*, anther culture, induced parthenogenesis, genetic variation, doubled haploid.

Introduction

Haploid breeding method based on the application of anther culture technique has been playing an important role in shortening the time required for developing new tobacco varieties (NAKAMURA *et al.* 1975, YAMADA *et al.* 1978, OKAMURA *et al.* 1978, HAMAMURA *et al.* 1981). Although the haploid breeding method has been proven to be very efficient, a number of investigators reported that genetic variations were induced among the doubled haploid lines obtained from anther of an inbred line of tobacco and that many of those were agronomically and chemically inferior to their parental sources (OINUMA and YOSHIDA 1974, BURK and MATZINGER 1976, ARCIA *et al.* 1978, BROWN *et al.* 1983). These results appeared applicable also to the doubled haploid lines from hybrid sources (SCHNELL *et al.* 1980).

Several hypotheses to explain the cause of the variations have been put forth by these investigators, which include 1) residual heterozygosity existed in the parental source for anther culture, 2) mutations induced during anther culture, 3) mutational effects of colchicine, and 4) cytoplasmic effects due to culturing microspores. No definite explanation, however, has ever been advanced for the occurrence of genetic variations among anther-derived doubled haploid lines of an inbred tobacco.

In this study, these hypotheses for the occurrence of variation among anther-derived doubled haploid lines were investigated. First, the hypothesis that variations among tobacco doubled haploid lines were a result of the residual heterozygosity in the ancestral plant

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was tested by serving a doubled haploid line which can be considered completely homozygous as a source plant for another series of anther culture, and by comparing the induced doubled haploid lines with the selfed lines of the parental source plant. The second hypothesis that a prolonged haploid state are liable to accumulate variations was tested by comparing anther-derived doubled haploid lines with ovule-derived doubled haploid lines. Lastly, reciprocal F_1 hybrids between lines exhibiting variations and the ancestral plant were compared for their agronomic performances to determine whether variations among doubled haploid lines were the results of nuclear or cytoplasmic alterations.

Materials and Methods

Source plants for haploid induction

From anthers of a single plant of a flue-cured tobacco variety, *N. tabacum* cv. Bright Yellow 103, more than 100 doubled haploid lines were obtained through anther culture and subsequent chromosome doubling. Among those of which the chromosome complement was doubled spontaneously, one doubled haploid line designated as N34 was selected because it was free from any detectable changes in a previous field test.

Establishment of anther-derived doubled haploid lines

Anthers at uni- or binucleate stage of pollen were collected from N34 plants and placed on solidified NAKATA's basal medium (1972) supplemented with 1 mg/l of IAA and 3 g/l activated carbon (NAKAMURA and ITAGAKI 1973). Cultures were maintained at 25°C under continuous illumination. After about 40 days' culture, emerged plantlets were immersed in 0.2% aqueous colchicine solution for 48 hrs at 25°C (KUMASHIRO and OKA 1978), and then transplanted into soil. Out of about 150 plantlets treated with colchicine, 38 plants were successfully doubled. These doubled haploid lines originated from pollen are hereinafter referred to as A-DH, and served as the experimental materials for the field test I.

*Induction of parthenogenesis by pollinating X-irradiated *N. alata* pollen*

Procedures for inducing ovule-derived haploid plants were essentially the same as those described by TAKENAKA and TANAKA (1956). *N. alata*'s anthers were collected just before their dehiscence, kept in glass vials, and maintained dry over silica gel. After the dehiscence, glass vials were irradiated with X-rays at a dose of 18,000R with a dose rate of 225R/min. Irradiated pollen was pollinated onto N34 plants which were emasculated previously and isolated in an insect free greenhouse.

Establishment of ovule-derived doubled haploid lines

Pollination of X-irradiated *N. alata* pollen onto *N. tabacum* produced normal seed pods in all the cases, but the germination rate of the obtained seeds was low, and many of the germinated seedlings died. About ten plants per pod grew very slowly to maturity and besides these, plants having morphological similarity to *N. tabacum* were found at a very low rate. Chromosome count revealed that the formers were hybrids between *N. alata* and *N. tabacum* and the latter was a maternal *N. tabacum*. Out of about 50 pollinations of the X-irradiated *N. alata* pollen onto N34, nine maternal haploids and nine maternal diploids were obtained. Axillary shoot meristems of the nine maternal haploids were colchicine-treated with the results of successful diploidization of three haploids. Together with the spontaneously doubled lines of parthenogenetic origin, the doubled lines obtained

in the above procedures were referred to as P-DH and served as the experimental materials for the field test I.

Comparison of A-DH with P-DH families (Field test I)

For the field test I, three families which included 17 lines of the selfed parental plants (N34S), 38 lines of A-DH, and 12 lines of P-DH were grown in the field and were evaluated for their agronomic performances. All these lines were arranged in a fashion of a complete randomized block design with three replications. Each plot consisted of nine plants. At flowering, the following characters were measured for all normally growing plants: days to flower, plant height, number of leaves per plant, leaf length, and leaf width. Yield of cured leaves was given on a single plant base.

Comparison of the reciprocal hybrids between doubled haploid lines with variations and the parent (Field test II)

From doubled haploid lines which were found to be different from the selfed lines of the parental plants for agronomic characters in the field test I, four lines were randomly selected. Reciprocal crosses were made between these varied lines and one of the selfed lines of the ancestral plant, N34S, in a greenhouse. For each of the four lines with variation, the doubled haploid line itself, the parental line, and the reciprocal hybrids were designated as a family and were evaluated as a whole plot in a randomized split-plot design with four replications. Sub-plots were single row consisting of nine competitive plants. At flowering, the same growth characteristics as recorded in the field test I were measured.

Measurement of total alkaloids and nicotine content

For determination of the percent total alkaloids, dried leaf sample from each plot was analyzed according to HARVEY's method (1967), which consisted of the extraction by hydrochloric acid and activated carbon. The total alkaloid fraction was distilled further to give nicotine fraction. By subtracting the nicotine portion from the total alkaloids, nicotine content was determined.

Chromosome count

Root-tips were exised, pretreated with 2mM 8-hydroxy quinolin for 3 hrs at 20°C, fixed in CARNOY's solution. Fixed root tips were stained according to FEULGEN's method. At least three plants were examined for the identification of chromosome number of a doubled haploid line. All the lines served in this study showed a normal somatic chromosome number of *N. tabacum* ($2n=48$).

Results

Comparison for agronomic performances between A-DH and P-DH

Table 1 shows average performances for seven agronomic characters in each family and the results of variance analyses. Among A-DH lines, there are significant differences for all the characters except for leaf width. On the contrary, P-DH and N34S families exhibited only a few significant within family differences. For P-DH family, differences of the mean performances between parthenogenetic haploid and diploid were small and for all the characters measured no significant differences were detected. Growth of progenies in the A-DH family was generally less vigorous than that of other two families; namely,

Table 1. Mean performances of each family for the seven agronomic characters and the results of variance analyses

Family	Days to flower	Plant height cm	Number of leaves per plant	Leaf length cm	Leaf width cm	Yield g/plant	Total alkaloids %
A-DH	63.2	163.7	27.9	61.9	28.1	124.3	3.35
P-DH	65.3	177.7	30.2	62.5	27.7	140.0	2.75
Diploid	65.2	177.6	29.9	62.6	27.8	140.4	2.78
Haploid	65.6	178.0	30.7	62.0	27.6	138.8	2.50
N34S	65.3	175.9	30.0	62.8	28.0	139.6	2.79
F-test							
Within Family							
A-DH	**	**	**	*	NS	**	**
P-DH	NS	NS	NS	NS	NS	**	NS
Diploid vs.							
Haploid	NS	NS	NS	NS	NS	NS	NS
N34S	NS	NS	NS	NS	*	NS	NS
Between Families							
A-DH vs. P-DH	**	**	**	NS	NS	NS	**
A-DH vs. N34S	**	**	**	NS	NS	**	NS
P-DH vs. N34S	NS	NS	NS	NS	*	*	NS

**, * : Significantly different at 0.01 and 0.05 probability levels, respectively. NS : No significant differences.

A-DH flowered earlier, had fewer leaves, shorter stature, and lower yield.

By using mean variance of the error component within N34S family, the number of significantly different lines from the mean value of N34S was determined for each character (Table 2). The result shows a sharp contrast between A-DH and P-DH : in A-DH family a considerable number of lines were significantly different from the parental line, but none of P-DH line was. Another supportive evidence on the variable nature of the A-DH family was provided by the results of nornicotine analysis of dried leaves. Out of 38 lines of A-DH family, three lines were found to have nornicotine as a major alkaloid, but neither of P-DH nor N34S lines contained any nornicotine type lines.

Table 2. Relative performances of individual doubled haploid lines compared with N34S for the agronomic characters¹⁾

	Days to flower	Plant height	Leaves /plant	Leaf length	Leaf width	Yield	Total alkaloids	Nornicotine ²⁾
A-DH Family								
Greater	0	0	0	0	0	0	4	3
Not Different	24	26	19	38	38	27	34	35
Less	14	12	19	0	0	11	0	—
P-DH Family								
Greater	0	0	0	0	0	0	0	0
Not Different	12	12	10	12	12	12	12	12
Less	0	0	0	0	0	0	0	—

¹⁾ Significant difference of means at 0.05 probability level. Means of whole N34S family and w values by TURKEY's method were used to judge the significance.

²⁾ Based on the results of chemical analysis of nornicotine, a line which has a high level of nornicotine is classified as "Greater".

Table 3. Genotypic correlations among characteristics in A-DH family

	Plant height	Leaf number	Leaf length	Leaf width	Yield	Total alkaloids
Days to flower	.855*	.963*	.339	-.576	.890*	-.765*
Plant height		.801*	.380	-.255	.944*	-.765*
Leaf number			.403	-.719*	.836*	-.642*
Leaf length				-.038	.585	-.260
Leaf width					-.115	-.038
Yield						-.785*

* Covariance twice as large as its standard error

In A-DH family which exhibited a great variability, genotypic correlations between characters were computed (Table 3). Correlations among days to flower, plant height, number of leaves, and yield are all positive and most of them are larger than 0.5. Total alkaloids are negatively correlated with all the other characters measured and covariances between total alkaloids and the characters listed above are greater than twice its standard error.

Comparison for agronomic performances between the reciprocal hybrids

From 38 A-DH lines, four lines which were found to possess variations in agronomic characters were randomly selected. Reciprocal crosses were made between these lines and the parental line. Table 4 shows the growth characteristics of the selected doubled haploid of androgenetic origin, the parental lines, and their reciprocal hybrids. These four doubled haploid lines designated as DH exhibited an early maturity, shorter stature, and lower yield as they did in the field test I. The reciprocal hybrids showed nearly the same mean performances each other, irrespective of the direction of crosses, and the differences between

Table 4. Mean performances of doubled haploid lines, selfed parental lines (N34S), and their reciprocal F₁ hybrids

Parent F ₁ hybrid (♀ × ♂)	Days to flower	Plant height cm	Number of leaves per plant	Leaf length cm	Leaf width cm
N34S	66.1	162	34.3	55.4	27.5
DH 3	61.6	131	29.6	50.8	24.8
F ₁ (DH 3 × N34S)	65.0	156	33.3	53.9	26.6
F ₁ (N34S × DH 3)	64.7	153	32.9	54.3	26.8
N34S	66.1	152	34.0	52.3	25.8
DH 21	60.8	138	28.3	55.8	28.2
F ₁ (DH 21 × N34S)	65.8	152	33.2	53.4	25.8
F ₁ (N34S × DH 21)	66.8	156	33.4	53.7	25.9
N34S	65.6	152	33.5	54.5	26.5
DH 30	56.8	119	27.4	50.6	25.4
F ₁ (DH 30 × N34S)	62.5	152	32.1	52.3	25.9
F ₁ (N34S × DH 30)	64.1	151	33.2	51.8	26.4
N34S	65.4	161	33.2	53.1	27.0
DH 32	58.2	124	27.3	51.4	25.9
F ₁ (DH 32 × N34S)	64.5	154	32.8	52.8	26.7
F ₁ (N34S × DH 32)	64.3	155	32.8	55.6	27.0

Each value is the average over four replications.

Table 5. Results of variance analyses on cytoplasmic effects on the variations

	Degree of freedom	Days to flower	Plant height	Leaves /plant	Leaf length	Leaf width
DH Family	3	**	*	**	NS	NS
Replication	3	NS	**	*	*	NS
Pedigree within Family	12	**	**	**	*	*
Parents vs. F ₁ s	4	**	**	**	NS	NS
N34S vs. DH line	4	**	**	**	**	**
Reciprocal	4	NS	NS	NS	NS	NS

**, * : Significantly different at 0.01 and 0.05 probability levels, respectively. NS : No significant differences.

the reciprocal hybrids and the N34S were also small. Analysis of variance detected no significant differences between the reciprocal hybrids (Table 5), indicating no cytoplasmic effects on the variations. The observed recovery of the varied characters in both of the reciprocal hybrids indicates that the varied characters were controlled by recessive genes.

Discussion

Anther-derived doubled haploid lines from a number of pure-line cultivars of tobacco have shown considerable variability among lines and lower productivity than their parents (OINUMA and YOSHIDA 1974, BURK and MATZINGER 1976, ARCIA *et al.* 1978). This phenomenon was confirmed also in this study. Variability of anther-derived doubled haploid lines has been hypothetically attributed to residual heterozygosity in a source plant for anther culture (DEATON *et al.* 1982), mutagenic effects of colchicine used to double haploids (DEPAEPE *et al.* 1977) and mutations during anther culture (OINUMA and YOSHIDA 1974).

As the source plant for anther culture, BURK and MATZINGER (1976) used a single plant which was extensively inbred to S₁₅ generations to eliminate residual heterozygosity, and yet they found reduced vigor of doubled haploid lines. Although DEPAEPE *et al.* (1981) reported the segregation after a few generations of selfing in doubled haploid lines of *N. sylvestris*, it is generally accepted that, in *N. tabacum*, the lines derived through selfing a doubled haploid plant are extremely uniform in growth and other characters (COLLINS and LEGG 1980). We used a doubled haploid line for the source plant of haploid induction to assure the complete homozygosity. However, as stated before, the significant variations were observed for leaf width among the selfed progenies of a source plant. It is not clear whether the variation is due to the mutation in the source plant or due to the error in the field experiment.

The variation range of anther-derived doubled haploid lines was larger than that of 17 lines of N34S for all the characters for which N34S lines were not significantly different each other. In addition, most of A-DH lines exhibited reduced productivity. These results indicate that the main causal factor for the variation in A-DH family could not be the residual heterozygosity in the source plant for anther culture. BROWN *et al.* (1983) compared the agronomic performances of the second cycle doubled haploids with the source cultivar and the first cycle doubled haploids, and found reduction in vigor in each cycle of anther culture. They stated that these results could not be explained by the existence of residual

heterozygosity in the original source cultivars, which is in good agreement with our observation.

Pollination of X-irradiated *N. alata*'s pollen onto *N. tabacum* produced both parthenogenetic-haploid and -diploid, as observed in the recent PANDEY's (1982) study. Although the mechanism by which parthenogenetic diploid gave rise was not certain, parthenogenetic diploid can be considered to have followed a relatively similar developmental process to that of normal fertilized ovules, especially in terms of ploidy. On the other hand, the parthenogenetic haploids which were doubled artificially with colchicine have remained in haploid stage for a longer period. With regard to the agronomic performances, these two types of P-DH lines did not differ each other as confirmed by the results of variance analysis between them and all the P-DH lines showed stable performances as the selfed lines of the parental plant did (Table 1).

No significant difference between parthenogenetic-diploids and haploids which were doubled their chromosome complement by colchicine treatment would provide us with several keys for the occurrence of the variation among anther-derived doubled haploid lines; on the effects of colchicine and on the hypothesis that a prolonged haploid state is liable to mutation.

Colchicine has been reported to induce mutations of quantitatively inherited characters (FRANZKE and ROSS 1957) and to enhance somatic crossing over (VIG 1971). BURK and MATZINGER (1976) observed that doubled haploid lines which had not been treated with colchicine in their haploid stage exhibited as much variability as those that had been, indicating little contribution of colchicine in inducing variation. Stable performances of parthenogenetic haploids which were later doubled by colchicine treatment would imply that colchicine plays little role, if any, in inducing variations among doubled haploids from a pure line of tobacco.

The parthenogenetic haploids remained in haploid state much longer than the androgenetic ones, since the colchicine treatments were made after flowering for the former, and during the seedling stage for the latter. Yet, only the doubled haploids of androgenetic origin showed great variability. This would indicate that a prolonged haploid state itself is not always linked with the high susceptibility to genetic alteration. Since the number of P-DH lines was not large, the conclusions we reached in this experiment should be confirmed in another experiment.

Among doubled haploid lines of androgenetic origin, observed variations were not restricted to quantitative characters, namely, three lines of A-DH were found to have nornicotine as a major alkaloid. Spontaneous mutation rate of the conversion from nicotine to nornicotine has been reported very high when compared with that of other traits (WERNSMAN and MATZINGER 1970). Nornicotine is converted from nicotine, and this nicotine to nornicotine conversion is controlled by a single dominant gene (MANN *et al.* 1964). Taking these into account, we can reasonably infer that variations both in qualitative and in quantitative characters are caused by some mutational events during anther culture. Furthermore, regarding the quantitative characters, the reciprocal F_1 hybrids between doubled haploid line with variations and the parental line exhibited similar performances, thus indicating that cytoplasmic factors have not played any role in inducing the variations.

BROWN and WERNSMAN (1982) also showed that genetic differentiation between doubled haploid lines and their source cultivar is largely, if not exclusively, nuclear origin. Therefore, the genetic variations among androgenetic doubled haploid lines of tobacco seem to be caused by mutations in nuclear genes during anther culture.

Doubled haploid lines of parthenogenetic origin were found to be superior to those of androgenetic origin from the point of view to raise the doubled haploid lines free from the mutations in inferior direction. However, as the frequencies of inducing both parthenogenetic haploid and diploid plants remain very low, they can not be used in a large scale breeding program at present. Hence, new techniques to induce parthenogenetic haploid or diploid plants at a high rate, or to control the unfavorable genetic changes of the androgenetic haploids are needed to improve the haploid method of breeding which has a great potential for the development of new varieties of tobacco.

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葯由来と胚珠由来のタバコ半数体倍加系統における遺伝的変異性の比較

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タバコの純系品種を葯培養した場合, 得られた半数体倍加系統間に高い頻度で遺伝的変異が生じることが報告されている. この変異の生因に関しては, 葯培養に供する親品種に存在しているわずかなヘテロ性の反映, 染色体倍加に用いるコルヒチンにより誘起された突然変異, 葯培養中に誘起された突然変異, あるいは花粉細胞質における突然変異などの仮説が提起されているが, 明確な結論は得られていない. 本実験では葯培養によって得られた半数体倍加系統間にみられる遺伝的変異の生因に関するいくつかの仮説を検討した.

実験材料として, 黄色種タバコ (*Nicotiana tabacum* cv. Bright Yellow 103) の葯培養とそれにつづく染色体の自然倍加によって得た1個体の半数体倍加個体 (N34) を供試した. N34 の自殖系統の圃場における生育諸特性は親品種の Bright Yellow 103 と同等であり, 系統内での分離は認められないことを予備実験で確認した.

N34 の自殖個体より2つの異なる方法, すなわち, 葯培養あるいはX線を照射した *N. alata* の花粉を用いた単為生殖によってそれぞれN34の半数体を誘導し, これらをコルヒチンで処理して半数体倍加系統を育成した. このようにして得た葯由来, および胚珠由来の半数体倍加系統を対照のN34の自殖系統とともに圃場に栽培し, 生育諸特性を比較した. なお, 供試した半数体倍加系統はすべて正常な *N. tabacum* の体細胞染色体数 ($2n=48$) を示した.

葯由来の38系統の半数体倍加系統中には諸形質に関してN34の自殖系統と有意に異なる系統が高い頻度で出現し, それらはいずれも早生化, 小型化の傾向を示した. また, 優性一因子に支配されるニコチンからノルニコチンへの転換に関しても, 3系統がノルニコチンを主アルカロイドとする変異系統とみなされた. 完全なホモ接合体と考えられる半数体倍加系統を葯培養の素材としたにもかかわらず, 量的形質および質的形質に高い頻度で変異が生じたことから, このような変異を葯培養の素材として用いる母木に存在していた一部のヘテロ性で説明するのは困難である.

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N. alata のX線照射花粉を N34 に交配したところ、胚珠由来の2倍体、および半数体がそれぞれ9個体出現した。半数体についてはコルヒチン処理を行い、3個体の倍加個体を得た。胚珠由来の12系統の倍加系統の圃場における生育特性はN34の自殖系統と同様に安定しており、対照との間に有意差を示す系統、あるいはノルニコチンを主アルカロイドとする変異系統は認められなかった。また、胚珠由来の系統をその倍加様式のちがい、すなわち、自然倍加あるいはコルヒチンによる倍加によって分類した場合にも、両者の間の差は検出されなかった。このことは、染色体倍加に用いるコルヒチン処理が変異を誘起する主要因ではないことを示唆するとともに、長期間にわたる半数体世代が必ずしも変異を集積する傾向を持たないことを示している。

葯由来の半数体倍加系統の中で変異を伴うと判断された系統と親系統であるN34との正逆交配を行い、得られた正逆 F_1 と両親との間で生育特性を比較した。変異系統で見られた早生化、小型化の傾向は正逆 F_1 では顕著ではなく、N34に近い特性を示した。また、正逆間の差は小さく、分散分析の結果もこの傾向を裏付けている。したがって、葯由来の半数体倍加系統にみられる変異は細胞質因子の突然変異によるものではなく、核遺伝子に生じた劣性方向への突然変異によりもたらされたものと考えられる。

これらの知見から、葯培養と染色体の倍加操作によって得られたタバコの半数体倍加系統間に生じる遺伝的変異は、葯培養時に誘起された核遺伝子の突然変異によるものと推察される。