Genetic Variation of Colchicine Induced and Spontaneous Doubled Haploids Obtained by Anther Culture of an Inbred Tobacco

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Effects of colchicine used for chromosome doubling of haploid plants were examined as a possible cause for the genetic variation among anther-derived doubled haploid lines of an inbred tobacco (*Nicotiana tabacum* L.). Colchicine induced doubled haploid lines (C-DH) and spontaneous doubled haploid lines (S-DH) were developed and their agronomic traits were compared with those of the selfed lines of the parental plant used for the anther culture. S-DH exhibited as great quantitative variability within the family as C-DH did. In each case, the direction of the changes was towards low productivity; reduction of plant height and leaf size. Some doubled haploid lines were revealed to have nornicotine, another type of the alkaloids, whereas all of the selfed lines of the source plant for anther culture had normal nicotine as the major alkaloid. This change in the alkaloid type was found in the plantlets before the colchicine treatment was applied. In addition, colchicine treatment of seed-derived diploid seedlings did not produce the nornicotine-type plantlets. These findings indicate that the colchicine treatment contributed little to the genetic variation of doubled haploid lines.

KEY WORDS : *Nicotiana tabacum*, anther culture, doubled haploid, colchicine, spontaneous diploidization, mutation.

Introduction

The successful production of homozygous doubled haploid lines by means of anther culture offers an excellent procedure for plant improvement, especially in tobacco (NAKAMURA *et al.* 1975, HAMAMURA *et al.* 1981). However, a number of studies have presented evidence for genetic variability of doubled haploid lines derived from nearly homozygous sources (OINUMA and YOSHIDA 1974, BURK and MATZINGER 1976, SCHNELL *et al.* 1980, KUMASHIRO and OINUMA 1985). To this unexpected phenomenon, several hypotheses have been postulated. They are residual heterozygosity in a source plant for anther culture, effect of colchicine used to double chromosome complement of haploids, and mutation induced during anther culture, etc. (DePAEPE *et al.* 1981).

In the previous report (KUMASHIRO and OINUMA 1985), we examined some of the hypotheses. Colchicine was considered to have little effect on the induction of genetic variation among anther-derived doubled haploids, because no significant differences were detected between three lines of ovule-derived doubled haploid treated with colchicine and nine lines without the treatment. Effects of colchicine on the induction of genetic variations can be measured more precisely by comparing anther-derived spontaneous doubled haploid lines (S-DH) and colchicine induced ones (C-DH). There have been a limited number of reports dealing with agronomic performance of S-DH, due to low frequency of spontaneous doubled haploid plants in tobacco (TAKAKA and NAKATA 1969, KASPERBAUER and COLLINS 1972). BURK and MATZINGER (1976) stated that the performances

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of five lines of S-DH were distributed throughout the ranges of 41 lines of C-DH, although the formers were in a somewhat narrower range.

The purpose of the present study was to determine if colchicine was a possible causal factor for inducing genetic variations among anther-derived doubled haploids of tobacco. A large number of S-DH and C-DH were obtained by anther culture of a single inbred plant, and they were compared each other for agronomic traits.

Materials and Methods

Source plant for the anther culture: A single plant of an inbred flue-cured tobacco (*N. tabacum*) cv. Bright Yellow 103 (BY 103) was served as the source plant for haploid induction. The procedure for the anther culture was the same as described previously (KUMASHIRO and OINUMA 1985).

Establishment of doubled haploid lines: More than 2,500 plantlets obtained by the anther culture were grown in a greenhouse. At flowering, spontaneous doubled haploid plants which possessed a normal quantity of pollen were selected and bagged individually to prevent out-crosses. As a result, a total of 38 spontaneous doubled haploid plants were obtained. Inflorescence of the remaining haploid plants was immersed in 0.2% aqueous solution of colchicine for 48 hrs. Out of about 200 haploid plants treated, 65 plants were successfully doubled. As a control, the single plant which was used as the source plant for the anther culture was selfed. 100 plants in S_1 generation were grown in a greenhouse and selfed to give S_2 lines.

Field experiment: From each of three families, i.e., S-DH, C-DH and the selfed lines, 30 lines were randomly chosen. All of the lines used for the field test were normal as to the somatic chromosome number ($2n=48$). A total of 90 lines were arranged in the fashion of the complete randomized block design with three replications. Each plot consisted of 10 competitive plants with two border plants at the edge of a row.

Characters measured for doubled haploid lines: The following characters were measured for all the normally growing plants at flowering; days to flower, plant height, number of leaves, leaf length and width. All the leaves were harvested at their optimum maturity and cured. Dried leaf weight per plot was measured and expressed by a single plant base. Total alkaloid content of the cured leaves was analyzed according to the procedure described by HARVEY *et al.* (1967). Nornicotine content of the doubled haploid lines was measured by the distilling procedure as previously described (KUMASHIRO and OINUMA 1985).

Nornicotine detection in the colchicine treated plantlets: Anther-derived small plantlets (2~3 leaves stage) of the same BY 103 S_1 line as used for developing the above mentioned doubled haploids were treated with 0.2% aqueous colchicine solution for 48 hrs, and grown in a greenhouse. As a control, seed-derived plantlets of similar size from the same BY 103 line were treated in the same manner. Besides these, both anther- and seed-derived plantlets without colchicine treatment were grown. Four weeks after the transplantation, all the plants were decapitated to enhance the alkaloid accumulation in the leaves. Three weeks later, two leaves from each plant were

Fig. 1. Distributions of mean days to flower of selfed lines, S-DH and C-DH of BY 103.

Fig. 2. Distributions of mean plant height of selfed lines, S-DH and C-DH of BY 103.

Table 1. Variance analysis

Source of variation	d. f.	Days to flower	Plant height	Number of leaves	Leaf length	Leaf width	Yield	Total alkaloids
Replication	2	**	NS	*	NS	NS	NS	NS
Family	2	**	NS	NS	**	*	**	NS
Within								
Self	29	*	NS	NS	NS	NS	NS	NS
S-DH	29	**	**	**	**	**	**	NS
C-DH	29	**	**	**	**	**	*	*

*, **: Significantly different at 0.05 and 0.01 probability levels, respectively.

NS : not significantly different.

harvested and cured. Alkaloid types of the dried leaf sample were determined by gas-chromatography (ARAKAWA and MAEKAWA 1978).

Results

Figures 1~3 show the frequency distributions of days to flower, plant height and yield of S-DH, C-DH, and the selfed lines. The distributions indicate that two doubled haploid families had a wider range of variation compared with the selfed family. Both doubled haploid families showed variations in both directions for days to flower, while for plant height and yield they deviated unidirectionally towards a

Table 2. Field performances of spontaneous doubled haploid lines with presumably mutated traits

Line No.	Days to flower	Plant height	Number of leaves	Leaf length	Leaf width	Yield	Total alkaloids
31		—		—		—	
35	+						
48				—			
49				—			
50		—		—	—	—	
51		—	—				
52	+		+				
53					—	—	
54	+		+	—	—	—	
57	+			—	—	—	
59				—			
60	+						

+ and — : indicate lines having significantly greater or less value than $\bar{X} \pm 3\sigma$ of selfed lines, respectively.

Table 3. Field performance of colchicine induced doubled haploid lines with presumably mutated traits

Line No.	Days to flower	Plant height	Number of leaves	Leaf length	Leaf width	Yield	Total alkaloids
61	+						
62	+	—	+	—	—		
63		—		—	—	—	
64	+						
66		—	—	—	—	—	
70	—	—	—	—			
71	+					—	
72	+		+				
76				—			
77	+		+				
78				—			
81	+		+				
82						—	
83				—	—	—	
84		—	—	—	—	—	
85				—			
86		—	—	—		—	
87		—		—	—		+
88	—	—					

+ and — : indicate lines having significantly greater or less value than $\bar{X} \pm 3\sigma$ of selfed lines, respectively.

lower value from those of the selfed family.

Table 1 shows the results of variance analysis. Among the selfed lines, no significant difference was detected except for days to flower. Within both doubled haploid families, however, significant differences were detected in almost all the traits measured.

Fig. 3. Distributions of mean yield of selfed lines, S-DH and C-DH of BY 103.

Mean value of performance of each doubled haploid line was checked for the difference from the mean value of the selfed family based on the error variance of the selfed family. The results are shown in Table 2 and 3. Out of 30 lines, 12 and 19 lines exhibited significant differences in at least one trait in S-DH and C-DH, respectively.

For each of doubled haploid lines and the selfed lines, alkaloid type was determined by chemical analysis of the dried leaves. While all the 30 lines of the selfed family possessed nicotine as the major alkaloid, two of the S-DH and seven of C-DH contained large amounts of nornicotine, another type of tobacco alkaloids. Thus, they were regarded as mutants (Table 4).

The alkaloid types of the plantlets derived from anthers of BY 103 were determined. Irrespective of the colchicine treatment, the alkaloid type of all of the seed-derived plants of BY 103 was normal nicotine. However, nine plants out of 147 anther-derived plants treated with 0.2% colchicine for 48 hrs were regarded as nornicotine type. Even when the plantlets were not treated with colchicine, nine anther-derived plants out of 219 had nornicotine as the major alkaloid (Table 5).

Discussion

The origin of spontaneous doubled haploid plants of tobacco has not been fully understood. Participation of the anther wall cells in plantlet formation, therefore, cannot be completely neglected. A number of studies have shown that in anther culture of *N. tabacum* plantlets were directly induced from embryoids, not from calli

Table 4. Alkaloid type of anther-derived doubled haploids of BY 103

Family	Number of lines	
	Nicotine	Nornicotine
Self	30	0
S-DH	28	2
C-DH	23	7

Table 5. Alkaloid type of anther-derived haploid plants and seed-derived plants of BY 103

Origin	Colchicine ^{a)} treatment	Number of plants	
		Nicotine	Nornicotine
Anther	—	210	9
Anther	+	138	9
Seed	—	234	0
Seed	+	149	0

Alkaloid type was determined by gas-chromatography.

^{a)} 0.2% colchicine for 48 hrs at seedling stage

(MIL 1973), and that isolated pollen grains of *N. tabacum* were successfully cultured to give a whole plant (NITSCH 1974, IMAMURA *et al.* 1982). These findings would suggest little participation of anther wall cells to plant regeneration. Furthermore, NITSCH (1972) described that anther culture of a heterozygous plant for leaf color yielded only two homozygous types of regenerates but not heterozygous type. Therefore, it is likely to suppose that the spontaneous doubled haploid plants of tobacco originate from pollen grains.

Doubled haploid lines which had not been exposed to colchicine (S-DH) exhibited as great variability in quantitative traits as those treated with colchicine (C-DH) did. The direction of variability was towards reduction of vigor; smaller plant stature and lower yield, as observed in several cases (OINUMA and YOSHIDA 1974, BURK and MATZINGER 1976, KUMASHIRO and OINUMA 1985). Number of lines which showed significant differences from mean values of the selfed family was greater in C-DH than in S-DH. These findings might imply that the mutagenic effect of colchicine could not be completely neglected.

Besides quantitative variation, both doubled haploid families had segregated lines with changes in qualitative traits, such as the alkaloid type. Such changes in qualitative traits were also found among anther-derived haploid plantlets, irrespective of the colchicine treatment. In addition, when seed-derived plantlets were treated with colchicine, all of the plants were of the normal nicotine type. Since the conversion from nicotine to nor nicotine is controlled by a single dominant gene (MANN *et al.* 1964), one can detect the mutation for this trait, even if diploid materials are used. Thus, the unaltered alkaloid type of the colchicine treated diploid seedlings, which is not in agreement with the case of the quantitative traits described above, would indicate that the effects of colchicine on the induction of mutation is, if any, very little.

These findings suggest that a considerable portion of genetic variation observed in anther-derived doubled haploids of tobacco was induced either during the culture process or before anthers were placed on the medium. As *in vitro* system itself is likely to generate genetic variations (LARKIN and SCOWCROFT 1981), this case can be a sort of somaclonal variations. On the other hand, as a major source of preexisted genetic variations among pollen, the residual heterozygosity of the source plant for anther culture was examined in many studies. The majority of experimental data indicate that it was not substantial for the observed variations (BROWN *et al.* 1983, KUMASHIRO and OINUMA 1985). Nevertheless, the genetic variability among pollen grains may exist in another form. In the anther culture of tobacco, instead of generative nuclei which always participate in the production of the next generation, vegetative nuclei have been reported to preferentially undergo cell divisions to form embryoids (SUNDERLAND 1974). DHILLON *et al.* (1983) indicated that doubled haploid lines of tobacco had a larger amount of DNA, especially in the heterochromatic regions, than normal seed-derived tobacco. They discussed the relationship between the vegetative nuclei and the observed genetic variation. The similar increase in DNA values has been reported in pollen-derived doubled haploids of *N. sylvestris* (DEPAEPE *et al.* 1982). Therefore, to elucidate the nature of variations among anther-derived doubled haploids of tobacco,

we should examine not only the mechanism of somaclonal variation, but also the role of vegetative nuclei in the observed genetic variations.

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タバコ半数体のコルヒチン倍加系統および自然倍加系統における遺伝的変異

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薬培養によって得られたタバコの半数体倍加系統に見られる高頻度の遺伝的変異の成因の一つとして, コルヒチン処理による染色体数倍加過程の影響を検討した。

黄色種タバコ品種 Bright Yellow 103 (BY 103) の1個体から薬培養によって約 2,500 個体の植物体を誘導した。この中から, 自然に染色体を倍加したと思われる 38 個体の稔性個体を得, これらを自殖して自然倍加系統とした。残りの半数性植物体約 200 個体について, 花序浸漬法によるコルヒチン処理 (0.2%, 48 時間) を行ない, 65 個体で倍加種子を得た。一方, 1 個体の薬培養母木を2回自殖して, 100 系統の自殖系統を作成した。これら3種類の系統群, すなわち, 自然倍加, コルヒチン倍加および自殖系統からそれぞれ 30 系統を任意に選抜して, 圃場試験の供試材料とした。合計 90 系統を3回反復, 乱塊法により配置し, 開花期に全個体について, 開花日数, 草丈, 全葉数, 葉長および葉幅, 全系統について収量およびアルカロイドタイプに関する調査を実施した。

自然倍加系統およびコルヒチン倍加系統はともに自殖系統に比較して各形質で変動幅が大きい傾向を示した (Fig. 1~3, Table 1)。少なくとも一つの形質で自殖系統の平均値から有意に変異している系統数は, 自然倍加系統, コルヒチン倍加系統でそれぞれ 12, 19 であった。開花日, 全葉数に関しては半数体倍加系統は両方向に変異していたが, 草丈, 葉の大きさおよび収量ではいずれも低い方向に変異していた (Table 2, 3)。

このような量的形質に関する変異のほかに, 二種類の半数体倍加系統にはアルカロイドタイプのような質的な形質に関する変異も認められた。すなわち, 2 系統の自然倍加系統, そして7 系統のコルヒチン倍加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。しかし, 自殖系統は 30 系統ともニコチンを主アルカロイドとする正常な系統であった (Table 4)。

一方, BY 103 の薬培養によって誘導したコルヒチン処理前の半数性を主とする植物体集団にもアルカロイドタイプに関する変異個体が出現していた。しかし, BY 103 の種子由来の幼植物に同様のコルヒチン処理を施した場合, すべての個体は正常なアルカロイドタイプを示した (Table 5)。

このようにコルヒチン処理を受けていない自然倍加系統においてもコルヒチン倍加系統と同様に量的, 質的形質の変異が認められたこと, さらに, コルヒチン処理前の半数性植物においてアルカロイドタイプの変異がすでに見られたことから, 染色体数の倍加に用いるコルヒチン処理は薬由来の半数体倍加系統に見られる遺伝的変異の主な要因ではないと考えられる。

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