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Further yearly analyses of spontaneous pink mutant events in the stamen hairs of *Tradescantia* clone BNL 4430 cultivated in the NSC growth chamber

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In order to confirm the results obtained in the previous 1-year-term (December 12, 1998, through December 10, 1999) scorings and analyses of spontaneous pink mutant events (PMEs) in the stamen hairs of *Tradescantia* clone BNL 4430 cultivated in a nutrient solution circulating (NSC) growth chamber, similar scorings and analyses were continued for another 52-week period from December 11, 1999, through December 8, 2000. The environmental conditions were not changed, except for a minor modification in the method of supplying the nutrient solution used. During the scoring period, 732,128 stamen hairs with an average cell number of 24.90 cells were observed, and 2,368 PMEs were detected. The overall spontaneous somatic mutation frequency was 1.35 ± 0.03 PMEs per 10^4 hair-cell divisions, which was significantly lower than the value of 1.56 ± 0.03 determined in the previous 52-week period, and the frequencies were lower during April through September than in other months, the period showing lower frequencies lasting 1-month longer than in the previous year. The present results reconfirmed the occurrence of a clear seasonal variation in the spontaneous mutation frequency in the NSC growth chamber, and the lower overall frequency, probably related to the minor modification in supplying the nutrient solution, is helpful for conducting mutagenicity tests at low levels, offering a lower background level. The analyses of the sectoring patterns of all these PMEs showed that the most of the 203 cases of multiple (two to five) pink sectors observed in the same stamen hairs (scored as 253 PMEs for calculating mutation frequency) were the results of events involving somatic recombinations occurred in single cells or cell lineages, rather than those of two or more independent somatic mutations occurred in different cells, agreeing with our previous study, and the significance of somatic recombinations in causing single PMEs was also reconfirmed.

INTRODUCTION

The *Tradescantia* stamen-hair system, selected by the International Program on Plant Bioassays as one of the most suitable testers for detecting genotoxic hazards in the environment, has been used successfully to detect the genetic effects of ionizing radiations and various chemicals at low levels, as reviewed earlier (Underbrink et al., 1973; Ichikawa, 1981b, 1992; Schairer and Sautkulis, 1982; Schairer et al., 1983; Ma et al., 1994). The system has also been shown to be suitable for studying the variation in spontaneous somatic mutation frequency (Takahashi and Ichikawa, 1976; Schairer and Sautkulis, 1982; Schairer et al., 1983; Ichikawa 1984, 1992, 1994; Imai et al., 1991; Sanda-Kamigawara et al., 1991, 1995; Ichikawa et al., 1995, 1996a, 1996b; Shima et al., 1997; Ichikawa and Wushur, 2000). The use of the young inflorescence-bearing shoots with roots of clone BNL 4430 cultivated in a recently developed nutrient solution circulating (NSC) growth chamber (Shima and Ichikawa, 1994; Ichikawa et al., 1995; Shima et al., 1997; Ichikawa and Wushur, 2000) has been shown to be especially efficient for detecting mutagenic synergisms (Shima and Ichikawa, 1994, 1995, 1997; Xiao and Ichikawa, 1995, 1996, 1998a, 1998b) or antagonisms (Xiao and Ichikawa, 1995, 1996, 1998a, 1998c) among various chemicals and X-rays, making it possible to determine spontaneous background mutation

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frequencies with higher accuracy than before (Ichikawa et al., 1995; Shima et al., 1997; Ichikawa and Wushur, 2000).

The present study was carried out in order to confirm and extend the results obtained by the previous 1-year-term (December 12, 1998, through December 10, 1999) scorings and analyses of spontaneous pink mutant events (PMEs) in the stamen hairs of clone BNL 4430 cultivated in the NSC growth chamber (Ichikawa and Wushur, 2000). Namely, the seasonal variation in spontaneous somatic pink mutation frequency as well as the involvement of somatic recombinations in producing multiple and also single pink sectors in the stamen hairs found in the previous study were re-examined, because no such obvious seasonal variation had been observed in 1992–1995 in the NSC growth chamber (Shima et al., 1997), and also because the sectoring patterns of spontaneous PMEs in the stamen hairs of clone BNL 4430 were analyzed for the first time in the previous study (Ichikawa and Wushur, 2000). The scorings of spontaneous PMEs were therefore continued also for 52 weeks starting on December 11, 1999, on a slightly larger scale than in the previous year, and the sectoring patterns of all the PMEs detected were analyzed, differing from the previous study, in which only about 70% of the PMEs detected were analyzed (Ichikawa and Wushur, 2000).

MATERIALS AND METHODS

The materials used. The young inflorescence-bearing shoots with roots of *Tradescantia* clone BNL 4430 described earlier (Shima and Ichikawa, 1994; Ichikawa et al., 1995; Shima et al., 1997; Ichikawa and Wushur, 2000) were used. This clone is a diploid hybrid ($2n = 12$) between a blue-flowered *T. hirsutiflora* Bush and a pink-flowered *T. subacaulis* Bush (Emmerling-Thompson and Nawrocky, 1980), and thus is a blue/pink heterozygote. This clone has frequently been used in studies of somatic mutations in the stamen hairs, as reviewed earlier (Schairer and Sautkulis, 1982; Schairer et al., 1983; Ichikawa, 1992), and is very suitable for propagating and growing the young inflorescence-bearing shoots with roots because of its characteristics, i.e., many new shoots constantly emerging from the basal nodes one after another and its short height (Shima et al., 1997).

Preparation and cultivation of the materials. New shoots emerged from the basal nodes were divided after they bore their own new roots, and were cultivated in a simplified NSC system as described earlier (Shima et al., 1997) until they started to form inflorescences (Ichikawa and Wushur, 2000). The very young inflorescence-bearing shoots with roots were then transferred to the NSC growth chamber for experimental use (Shima et al., 1997; Ichikawa and Wushur, 2000) at least 1 week before starting flowering (Ichikawa and Wushur, 2000). The simplified NSC system and the NSC growth chamber have capacities to cultivate 144 and 216 such shoots with roots, respectively (Shima et al., 1997).

The environmental conditions in the simplified NSC system were 23.0 ± 1.0°C and 16-h day length with the light intensity of 7.5 klx from white fluorescent tubes, and those in the NSC growth chamber were 22.0 ± 0.5°C during the 16-h day with the same light intensity and source as the above, and 20.0 ± 0.5°C at night. The nutrient solution used in the both facilities was a 1/2,500 Hyponex solution, and the solution was exchanged every 2 weeks. During the 2-week periods between the exchanges, Hyponex solution at a lower concentration (1/4,000) was added every day and every 2 days in the latter and former facilities, respectively, to keep the amount nearly constant, as the only modification in the environmental conditions compared to those in previous studies (Shima and Ichikawa, 1994, 1995, 1997; Xiao and Ichikawa, 1995, 1996, 1998a, 1998b, 1998c; Ichikawa et al., 1995; Shima et al., 1997; Ichikawa and Wushur, 2000).

Scoring methods. During the 52-week period of December 11, 1999, through December 8, 2000, 15 to 20 flowers were randomly collected every morning from those opened on the young inflorescence-bearing shoots with roots which had been cultivated in the NSC growth chamber longer than 2 weeks, and as many as possible of them were observed after storing them in a refrigerator. The spontaneous mutation frequency was determined using the scoring method described earlier in detail (Ichikawa, 1992), which enables to calculate the frequency of PMEs per hair-cell division, as in the previous 52-week scorings (Ichikawa and Wushur, 2000). Briefly, the numbers of stamen hairs and PMEs were scored on each of six stamens, and the number of hair cells was also counted on ten representative hairs each of two oppositely located stamens per flower (Ichikawa and Takahashi, 1977, 1978; Ichikawa and Ishii, 1991; Ichikawa, 1992) to estimate the average number of cells per hair for calculating the mutation frequency per hair-cell division. A PME has been defined to represent the result of a single mutation (Ichikawa, 1981a, 1992). Two or more entirely pink hairs on the same stamen, regardless of their number, were regarded as a single PME, since they are most likely the results of a mutation occurred in an epidermal cell of the stamen filament (Ichikawa, 1992). Pink sectors in the same hair each separated by a single blue cell were also regarded as a single PME, since they most likely result from an event involving somatic recombination occurred in a cell or a cell lineage, rather than two or more independent mutations in different cells (Ichikawa, 1981a, 1992, 1994; Sanda-Kamigawara et al., 1995).

Mutation frequency. The data obtained were pooled for every 2 weeks for calculating the somatic mutation fre-

quencies as in the previous study (Ichikawa and Wushur, 2000), considering that the nutrient solution was exchanged every 2 weeks and also that discarding older inflorescences and selecting fully developed young inflorescences were carried out each time the nutrient solution was exchanged. The data were also pooled for every month as another way of detecting any seasonal variation in the mutation frequency. Somatic mutation frequency was expressed as the number of PMEs per 10^4 hair-cell divisions, and the differences among the 26 frequencies for the 2-week periods, among the 12 monthly frequencies, and between each of these frequencies and the overall frequency were examined statistically by the chi-square test, as in the previous study (Ichikawa and Wushur, 2000).

Analysis of sectoring patterns. The sectoring patterns of all the PMEs detected were analyzed, differing from the previous study (Ichikawa and Wushur, 2000). The patterns were classified into six categories as will be shown below in Table 1, recording not only the multiple pink sectors in the same hair each separated by a single blue cell as described above, but also those separated by two or more blue cells, as in the previous study. The observed frequencies of multiple entirely pink hairs on the same stamen as well as those of the multiple pink sectors in the same hair separated by a single blue cell or by two or more blue cells were compared with the frequencies expected from the occurrences of two or more independent somatic mutations, and the differences between the observed and expected frequencies were examined by the chi-square test, if needed (Ichikawa and Wushur, 2000).

RESULTS

Spontaneous mutation frequency. During the 52-week scoring period, 732,128 stamen hairs in total with an average cell number of 24.90 were observed, and 2,368 PMEs were detected. The overall spontaneous somatic pink mutation frequency was calculated to be 1.35 ± 0.03 PMEs per 10^4 hair-cell divisions.

The 26 spontaneous mutation frequencies determined for every 2 weeks are shown in Fig. 1, together with the overall mutation frequency for comparison. The frequency varied between 1.08 ± 0.13 (April 1–14) and 1.82 ± 0.19 PMEs (December 11–24) per 10^4 hair-cell divisions. The frequencies from April to September were mostly relatively lower, whereas higher frequencies were often observed in the other months. When the differences among the 26 frequencies (325 combinations in total) were tested, significant differences were found in 63 combinations (26, 12 and 25 at the 1, 2 and 5% levels, respectively), and the above lowest and highest frequencies were significantly lower and higher than seven and 15 other frequencies, respectively. Compared with the overall mutation

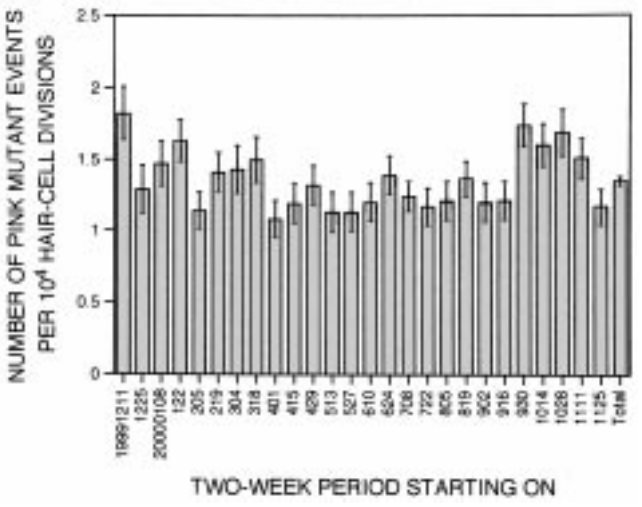

Fig. 1. Variation of the 26 spontaneous somatic pink mutation frequencies in the stamen hairs determined for every 2 weeks and the overall mutation frequency for comparison. The standard errors are shown on all columns.

frequency, the highest and the second-highest (1.74 ± 0.15 in September 30–October 13) frequencies were significantly higher at the 1% level, and the third-highest frequency (1.69 ± 0.16 in October 28–November 10) at the 5% level. The other 23 frequencies were not significantly different from the overall frequency.

The data obtained were also pooled for every month in order to further examine the seasonal variation in the spontaneous mutation frequency, and the results are shown in Fig. 2, also together with the overall mutation frequency for comparison. In this figure, the data obtained for December 11–31, 1999, and those for December 1–8, 2000, are pooled in order to enable comparisons

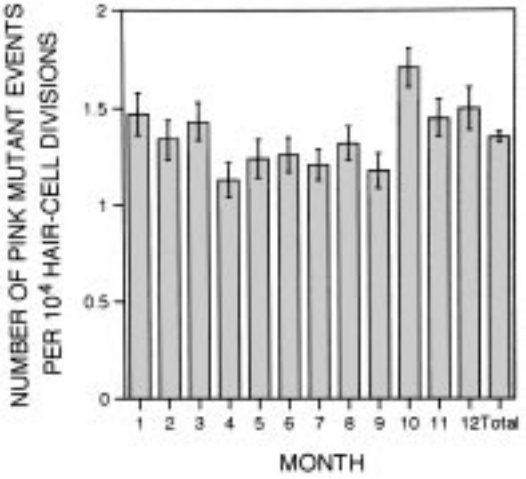

Fig. 2. Variation of the 12 monthly spontaneous somatic pink mutation frequencies in the stamen hairs and the overall mutation frequency for comparison. The data for December 11–31, 1999, and for December 1–8, 2000, are pooled (see text). The standard errors are shown on all columns.

Table 1. Frequencies of six different sectoring patterns in four 3-month periods (the numbers observed are shown as those of PMEs)

Period	Single terminal	Single interstitial	I/T ratio[1]	Whole hair[2]	Multiple type1[3]	Multiple type2[4]	Complex sectors[5]	Total
Jan.-Mar.	177	311	1.76	12	42	13	8	563
(%)	(31.4)	(55.2)		(2.1)	(7.5)	(2.3)	(1.4)	
Apr.-Jun.	143	278	1 94	18	30	18	3	490
(%)	(29.2)	(56.7)		(3.7)	(6.1)	(3.7)	(0.6)	
Jul.-Sep.	232	295	1.27	23	49	18	7	624
(%)	(37.2)	(47.3)		(3.7)	(7.9)	(2.9)	(1.1)	
Oct.-Dec.[6]	207	402	1.94	17	37	19	9	691
(%)	(30.0)	(58,2)		(2.5)	(5.4)	(2.7)	(1.3)	
Total	759	1,286	1.69	70	158	68	27	2,368
(%)	(32.1)	(54.3)		(3.0)	(6.7)	(2.9)	(1.1)	

[1] The ratio of the number of single interstitial PMEs against that of single terminal PMEs.
[2] Two to seven entirely pink hairs on the same stamen were scored as a single PME.
[3] Two and three pink sectors in the same hair each sparated by a single blue cell were scored as a single PME.
[4] Two and three pink sectors in the same hair each separated by two or more blue cells were scored as two and three PMEs, respectively.
[5] Three to five pink sectors partly separated by single and partly by two or more blue cells were scored as the same or different PMEs, depending on the separations.
[6] Data for December 11–31, 1999, and for December 1–8, 2000, are pooled (see text).

among months. As seen in this figure, the frequencies in January to March and in October to December were higher than those in April to September, and, especially, the highest frequency (in October) was significantly higher than those in seven other months at the 0.1–2% levels, and the lowest frequency (in April) was significantly lower than those in five other months at the 0.1–5% levels. The highest and lowest frequencies in October and April were also significantly higher and lower than the overall mutation frequency at the 0.1 and 5% levels, respectively.

Analyses of sectoring patterns. Analyses of the sectoring patterns of pink mutant cells occurred spontaneously in stamen hairs were carried out on all the 2,368 PMEs detected (in 2,335 hairs) during the 52-week scoring period. The frequencies of six categories of sectoring pattern are shown in Table 1, pooling the data for every 3 months, i.e., two 3-month periods each showing higher and lower mutation frequencies, in order to examine whether or not there were any seasonal differences in the sectoring patterns.

The numbers of single terminal and single interstitial PMEs were 759 and 1,286, respectively, as shown in Table 1. The number of cells per single terminal PME ranged

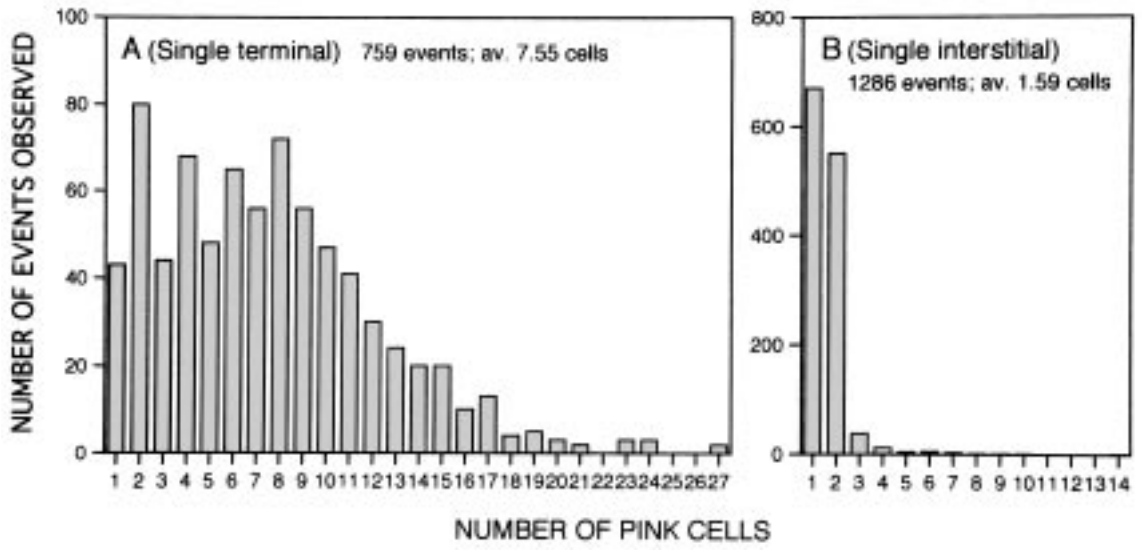

Fig. 3. Distribution patterns of the number of pink cells per single terminal (A) and single interstitial (B) PMEs occurred spontaneously in the stamen hairs. Note the eight-times different scales of the Y axis in A and B.

from one to 27, averaging 7.55, and that per single interstitial PME varied between one and 14, averaging 1.59, as shown in Fig. 3. The ratio of the number of single interstitial PMEs against that of single terminal PMEs (I/T) was 1.69, as seen in Table 1. The I/T value in July to September was lower (1.27) than in the other 3-month periods, January to March (1.76), April to June (1.94) and October to December (1.94).

Besides these single PMEs, 87 entirely pink hairs were observed on 70 stamens (thus 70 PMEs for calculating mutation frequency; Table 1). The numbers of patterns of double and triple pink sectors in the same hair each separated by a single blue cell were 146 and 12, respectively (thus 158 PMEs in total; Table 1). On the other hand, the numbers of patterns of double and triple pink sectors in the same hair each separated by two or more blue cells were 31 and two, respectively (thus 62 and six PMEs, respectively, for calculating mutation frequency; Table 1). More complex patterns with three to five pink sectors separated partly by single blue cells and partly by two or more blue cells were also observed in 12 hairs, and 14 and 15 positions between pink sectors in these complex patterns were separated by single and two or more blue cells, respectively (thus 12 + 15 = 27 PMEs for calculating mutation frequency; Table 1). Including such complex patterns, the separations of pink sectors by two or more blue cells were counted to be 50 ($31 + 2 \times 2 + 15$), and the separations by two, three, four, five, six, eight and nine blue cells were 28, six, eight, two, two, three and one, respectively.

DISCUSSION

Seasonal variation in spontaneous mutation frequency. The overall spontaneous somatic pink mutation frequency of 1.35 ± 0.03 PMEs per 10^4 hair-cell divisions during the scoring period of 52 weeks determined in the present study is based on 2,368 PMEs detected from 732,128 stamen hairs observed which were composed of 24.90 cells on the average. In other words, this mutation frequency is based on a large denominator of about 17.5 million hair-cell divisions, i.e., $732{,}128 \times (24.90 - 1)$. This mutation frequency is 13.5% lower than the frequency of 1.56 ± 0.03 PMEs per 10^4 hair-cell divisions determined in the previous 52-week period in 1998–1999, also based on a comparable sample size of about 17 millions (Ichikawa and Wushur, 2000), and the difference is highly significant at the 0.1% level. The frequency is also lower than those obtained in 1992–1995 by cultivating young inflorescence-bearing shoots with roots in the same NSC growth chamber, which ranged from 1.50 ± 0.04 to 1.56 ± 0.04 PMEs per 10^4 hair-cell divisions (Shima et al., 1997). The only modification in the environmental conditions between the present and earlier studies (Shima and Ichikawa, 1994, 1995, 1997; Xiao and Ichikawa, 1995, 1996, 1998a, 1998b, 1998c; Ichikawa et al., 1995; Shima et al., 1997; Ichikawa and Wushur, 2000) was a minor change in the method of supplying the nutrient solution used, i.e., modified to keep the amount nearly constant, and it does not seem unlikely that this modification resulted in the significantly lower spontaneous mutation frequency. The lower background mutation frequency is helpful especially for conducting mutagenicity tests at low levels.

The 26 spontaneous mutation frequencies determined for every 2 weeks showed a variation between 1.08 ± 0.13 (April 1–14) and 1.82 ± 0.19 PMEs (December 11–24) per 10^4 hair-cell divisions (Fig. 1). The frequencies during April through September were mostly relatively lower, while higher frequencies were often observed from December to March and October to November. The pattern of the variation was similar to that observed in the previous similar study (Ichikawa and Wushur, 2000), but the period of lower frequencies lasted longer in the present study, continuing until September. When the differences among the 26 frequencies were tested, significant differences at the 1–5% levels were detected in 63 out of 325 combinations, and the above lowest and highest frequencies were significantly lower and higher than seven and 15 other frequencies, respectively. Compared with the overall mutation frequency, on the other hand, the highest and the second- and third-highest frequencies (1.74 ± 0.15 in September 30–October 13 and 1.69 ± 0.16 in October 28–November 10) were found to be significantly higher at the 1–5% levels. The other 23 frequencies for 2-week periods were not significantly different from the overall frequency. The differences among the 12 monthly mutation frequencies (Fig. 2) were also tested, and the highest (1.71 ± 0.10 in October) and lowest (1.13 ± 0.09 in April) frequencies were found to be significantly higher and lower than those in seven and five other months, respectively, at the 0.1–5% levels. The highest and lowest monthly frequencies were also significantly higher and lower than the overall mutation frequency at the 0.1 and 5% levels, respectively. The present results clearly reconfirm the seasonal variation in spontaneous mutation frequency observed during the previous 1-year-term scorings (Ichikawa and Wushur, 2000).

The present results and those in the previous study (Ichikawa and Wushur, 2000) do not agree with the earlier report of a seasonal variation in spontaneous mutation frequency in the stamen hairs of clone BNL 4430 with a consistent cycle showing higher frequencies in late summer months, with ambient air pollution as the suspected cause (Schairer et al., 1983). The present and the previous results also differ from those obtained in 1992–1995 in the same NSC growth chamber, which showed no obvious seasonal variation in spontaneous mutation frequency (Shima et al., 1997).

As for the reason why the spontaneous mutation fre-

quency was lower in April through September (or August; Ichikawa and Wushur, 2000) and higher in October (or September; Ichikawa and Wushur, 2000) through March in our studies, the only factor which can be suspected is the seasonal differences in natural environmental conditions to which the materials used were exposed at the time they were transferred from the simplified NSC system to the NSC growth chamber, which are located in different buildings (Shima et al., 1997). It has been shown that the spontaneous mutation frequency in stamen hairs decreases and increases at higher and lower temperatures, respectively, not only in temperature-sensitive mutable clones (Takahashi and Ichikawa, 1976; Ichikawa, 1984, 1992; Imai et al., 1991), but also in other non-mutable clones (Takahashi and Ichikawa, 1976; Ichikawa, 1981a, 1984, 1992; Ichikawa et al., 1996a, 1996b). Although the flowers were collected from the young inflorescence-bearing shoots with roots which had been grown in the NSC growth chamber longer than 2 weeks in the present study and also in the previous study (Ichikawa and Wushur, 2000), it is possible that the higher and lower temperatures at the time they were transferred between the two buildings (a distance of about 25 m) might have influenced. If this is true, the both NSC facilities should be set in the same air-conditioned room to keep a more stable background mutation frequency for mutagenicity tests.

Sectoring patterns analyzed. The average numbers of cells composing the 759 single terminal and 1,286 single interstitial PMEs observed (Table 1) were 7.55 and 1.59, respectively (Fig. 3). These two values are close to 7.75 and 1.52, respectively, obtained in the previous similar study (Ichikawa and Wushur, 2000). The value of 7.55 for single terminal PMEs is also close to 7.40 (Ichikawa, 1994) but is smaller than 8.63 (Sanda-Kamigawara et al., 1995), values obtained for spontaneous mutations in the mutable clone KU 20. It should be noted that clone KU 20 has a larger average number of cells per hair than clone BNL 4430 (Ichikawa, 1992). On the other hand, the value of 1.59 for single interstitial PMEs is considerably smaller than 1.97 (Ichikawa, 1994) and 2.02 (Sanda-Kamigawara et al., 1995) in clone KU 20, and the smaller value in clone BNL 4430 suggests more frequent occurrences of chromatid-type mutations in the subterminal cells of young stamen hairs, as described earlier (Ichikawa, 1981b, 1992, 1994; Ichikawa and Wushur, 2000), and/or less frequent divisions of the subterminal cells than the terminal cells (Ichikawa and Sparrow, 1967). It could also be due to chromatid-type mutations in interstitial cells which undergo cell divisions at earlier stage of hair growth (Mericle and Hazard, 1980) and occasionally even at later stage (Ichikawa and Sparrow, 1967). Occurrences of single interstitial PMEs composed of three or more cells (Fig. 3B) are the evidences of cell divisions of interstitial cells, but they could also be produced as the results of events involving somatic recombinations (Ichikawa and Wushur, 2000).

The distribution patterns of the number of cells per single terminal and single interstitial PMEs obtained in the present study (Fig. 3) are similar to those in the previous study (Ichikawa and Wushur, 2000). Namely, the single terminal PMEs composed of relatively small even numbers of cells (two, four, six and eight) occurred obviously more frequently than those with relatively small odd numbers of cells (one, three, five and seven), as seen in Fig. 3A, confirming the results obtained in the previous study (Ichikawa and Wushur, 2000), and these results are clearly different from those obtained in clone KU 20, which showed much smaller differences in frequency between the single terminal PMEs with even and odd cell numbers (Ichikawa, 1994; Sanda-Kamigawara et al., 1995). No evident differences in frequency were observed, however, between the single terminal PMEs with even and odd numbers of cells greater than nine (Fig. 3A). As for single interstitial PMEs, one-cell events occurred 1.22 times more frequently than two-cell events (Fig. 3B), resulting in the relatively small average cell number of 1.59 noted above, also differing from their comparable occurrences in clone KU 20 (Ichikawa, 1994; Sanda-Kamigawara et al., 1995). However, the difference between the frequencies of one- and two-cell events was smaller than that of about 1.5 times in the previous study (Ichikawa and Wushur, 2000).

The ratio of the number of single interstitial PMEs against that of single terminal PMEs, I/T = 1.69, obtained in the present study (Table 1) was lower than 2.02 in the previous study (Ichikawa and Wushur, 2000), mainly due to the lower I/T value in July to September (1.27; Table 1), but the ratio was still obviously higher than 1.35 (Ichikawa, 1994) and 1.14 (Sanda-Kamigawara et al., 1995) obtained in clone KU 20. The higher I/T ratios in clone BNL 4430 seem to indicate that either or both of the subterminal and/or interstitial cells of the stamen hairs divided more frequently than in clone KU 20, but somatic recombinations must also have contributed to the higher I/T ratio in clone BNL 4430 (Ichikawa and Wushur, 2000). The lower I/T value in July to September does not seem to be related to the seasonal variation in the spontaneous mutation frequency, because the highest I/T value (1.94; Table 1) was obtained in April to June, when mutation frequencies were nearly as low as those in July to September (Fig. 2).

In addition to these single PMEs, 70 whole-hair PMEs (87 entirely pink hairs on 70 stamens) were observed (Table 1). Namely, two entirely pink hairs each on five stamens and three, five and seven entirely pink hairs on one stamen each were found, besides a single entirely pink hair each on 62 other stamens. Compared with the frequency of such single entirely pink hairs in 732,128 hairs scored, the observed frequencies of two, three, five and

seven entirely pink hairs (on a stamen) far exceeded the expected frequencies of independent occurrences of two, three, five and seven entirely pink hairs on the same stamen, as shown in Table 2. These results prove again the validity of regarding two or more entirely pink hairs on the same stamen as a single PME (Ichikawa, 1992), as proven in the previous study (Ichikawa and Wushur, 2000).

The numbers of double and triple pink sectors in a hair each separated by a single blue cell were 146 and 12, respectively. Based on the frequency of single (terminal and interstitial) PMEs against 732,128 hairs scored times 23.90 (average cell number per hair minus one), the observed frequencies of such double and triple pink sectors in the same hairs (including 12 double and one triple pink sectors each separated by a single blue cell in more complex sectoring patterns; Table 1) also far exceeded the expected frequencies of independent occurrences of two and three mutations in the same hairs (Table 2). These results also confirm the validity of regarding two or more pink sectors in a hair each separated by a single blue cell as a single PME (Ichikawa, 1981a, 1992, 1994; Sanda-Kamigawara et al., 1995), as proven in the previous study (Ichikawa and Wushur, 2000).

On the other hand, the numbers of double and triple pink sectors in the same hairs each separated by two or more blue cells were 31 and two, respectively. More complex sectoring patterns with three, four and five pink sectors separated partly by single blue cells and partly by two or more blue cells were also observed in 12 hairs. Including these complex patterns, the separations of pink sectors by two, three, four, five, six, eight and nine blue cells were 28, six, eight, two, two, three and one, respectively. When the observed frequencies of the separations by two to nine blue cells were calculated, those of the separations by two to four blue cells were found to be 117 to 25 times higher than the expected frequencies of occurrences of independent mutations, as shown in Table 2, the differences being significant. As for separations by five or more blue cells, however, their incidences were too low to detect significant differences. These results are also similar to those obtained in the previous study (Ichikawa and Wushur, 2000).

Significance of somatic recombinations. As discussed above, the occurrences of almost all multiple pink sectors in the same hairs far exceeded the expectations based on independent mutations occurred in different cells of the same hairs. This was true not only for multiple sectors each separated by a single blue cell, but also for those separated by two to four blue cells (Table 2). The only difference from the previous study (Ichikawa and Wushur, 2000) was that the observed frequency of multiple sectors each separated by five blue cells (1.14×10^{-7}/cell divisions; Table 2) was lower than that in the previous study (4.20×10^{-7}), thus resulting in no significant difference from the expected frequency. Similar results have been obtained also for spontaneous mutations in the stamen hairs of clone KU 20 (Ichikawa, 1994; Sanda-Kamigawara et al., 1995).

The possibility of involvement of somatic recombinations in producing multiple pink sectors had been suggested earlier in the stamen hairs of clone BNL 02 (Mericle and Mericle, 1967, 1973). A similar conclusion had also

Table 2. The observed frequencies of multiple entirely pink hairs on the same stamens and of multiple pink sectors in the same hairs and their expected frequencies from independent occurrences

Category (Number observed)	Observed frequency	Expected frequency[1]	Observed/ Expected
Multiple entirely pink hairs			
2 entirely pink hairs (5)	6.83×10^{-6}/hair	7.17×10^{-9}/hair	9.53×10^{2}
3 entirely pink hairs (1)	1.37×10^{-6}	6.08×10^{-13}	2.25×10^{6}
5 entirely pink hairs (1)	1.37×10^{-6}	4.36×10^{-21}	3.14×10^{14}
7 entirely pink hairs (1)	1.37×10^{-6}	3.13×10^{-29}	4.38×10^{22}
Multiple pink sectors separated by a single blue cell[2]			
2 pink sectors (158)	9.03×10^{-6}/cell div.	1.37×10^{-8}/cell div.	6.59×10^{2}
3 pink sectors (13)	7.43×10^{-7}	1.60×10^{-12}	4.67×10^{5}
Multiple pink sectors separated by two or more blue cells[2]			
by 2 blue cells (28)	1.60×10^{-6}/cell div.	1.37×10^{-8}/cell div.	1.17×10^{2}
by 3 blue cells (6)	3.43×10^{-7}	1.37×10^{-8}	2.50×10
by 4 blue cells (8)	4.57×10^{-7}	1.37×10^{-8}	3.34×10
by 5 blue cells (2)	1.14×10^{-7}	1.37×10^{-8}	8.32
by 6 blue cells (2)	1.14×10^{-7}	1.37×10^{-8}	8.32
by 8 blue cells (3)	1.71×10^{-7}	1.37×10^{-8}	1.25×10
by 9 blue cells (1)	5.71×10^{-8}	1.37×10^{-8}	4.17

[1] See text.
[2] Those in complex multiple sectors are included.

been reported in a heterozygous tester of *Glycine max* (Vig, 1973). The occurrences of somatic recombinations were later confirmed in the stamen hairs and petals of clone BNL 2091 of *T. hirsuticaulis* Small, a blue/red heterozygous purple-flowered clone, which produces blue/red twin spots when a somatic recombination occurs (Christianson, 1975). It has been therefore considered that somatic recombinations, which yield pairs of homozygous blue and homozygous pink cells from blue/pink heterozygous cells in clones KU 20 and BNL 4430, are also involved in producing multiple pink sectors spontaneously in these clones (Ichikawa, 1994; Sanda-Kamigawara et al., 1995; Ichikawa and Wushur, 2000).

The present analyses of the sectoring patterns of spontaneous pink mutant cells in the stamen hairs of clone BNL 4430 were performed on a larger scale than in the previous similar study (Ichikawa and Wushur, 2000) and earlier studies in clone KU 20 (Ichikawa, 1994; Sanda-Kamigawara et al., 1995), and reconfirmed the involvement of somatic recombinations in producing multiple pink sectors, as well as in yielding the high I/T ratio, as mentioned above. As shown in the previous report (Ichikawa and Wushur, 2000), the occurrence of a single somatic recombination alone results in either a single terminal or interstitial PME, and does not produce multiple pink sectors, but occurrences of two somatic recombinations or of one somatic recombination plus one somatic mutation can produce double pink sectors. Additional occurrences of somatic recombination or somatic mutation may produce triple or more pink sectors. Somatic mutations may occur before or after somatic recombinations, but the combination of chromatid-type mutation before recombination produces multiple pink sectors more frequently (Ichikawa and Wushur, 2000).

Compared with the previous study (Ichikawa and Wushur, 2000), the frequencies of multiple pink sectors in the same hairs, especially of those each separated by two or more blue cells and of more complex multiple sectors separated partly by single and partly by two or more blue cells, were somewhat lower in the present study (Tables 1 and 2). The lower frequencies of multiple sectors in the present study might be related to the minor modification in the method of supplying the nutrient solution, resulting in to keep the amount more constant. While the absolute number of separations by two or more blue cells in such multiple sectors detected was smaller, being 50 (Table 2), than 60 in the previous study (Ichikawa and Wushur, 2000), the number of separations by single blue cells was larger, being 184 (Table 2), than 150 in the previous study (Ichikawa and Wushur, 2000). The total number of sectors in such multiple sectors observed in the present study was 437 (382 in the previous study; Ichikawa and Wushur, 2000), and they constituted 17.1% of the 2,552 pink sectors in total analyzed. Considering that this value is nearly as high as 19.0% in the previous study (Ichikawa and Wushur, 2000), and also that occurrences of single somatic recombinations merely result in single pink sectors (Ichikawa and Wushur, 2000), it can be concluded that somatic recombination plays significant roles, not only in producing multiple sectors, but also in causing single PMEs spontaneously in the stamen hairs of clone BNL 4430.

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