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Morphological Characteristics and Their Inheritance in Colchicine-induced *Salvia* Polyploids

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Chromosome doubling by colchicine treatment, and the morphological characteristics of tetraploids and their inheritance were investigated in *Salvia*. Colchicine treatment of seeds had little effect on germination, but adversely affected true leaf development and the survival rate of seedlings. A 50% reduction in true leaf development was observed at 500 ppm colchicine in *S. coccinea* 'Coral Nymph', at 250 ppm in 'Lady in Red' and at 750 ppm in *S. patens* 'Blue Angel'. Of 50 seeds of *S. coccinea* 'Coral Nymph' treated with 500 ppm colchicine, 12 seedlings survived, of which five individuals were confirmed to be tetraploid, including two presumed chimeric polyploids. The tetraploid plants had larger, thicker leaves and enlarged inflorescences and flowers compared with diploid plants, which increased their ornamental value. Most of the tetraploid plants produced viable pollen and self-pollinated seeds were obtained from four tetraploid individuals. The 18 second-generation seedlings were confirmed to be tetraploids and their flower and leaf morphologies were identical to those of the tetraploid parents.

Key Words: colchicine, inheritance, morphology, polyploidy, *Salvia*.

Introduction

The genus *Salvia* L. (Lamiaceae) comprises about 900 species, mainly distributed in Central and South America. The genus contains a wide variety of flower colors, morphological characters and ecotypes. Some *Salvia* species have a long history of utilization as medicinal herbs, not only as ornamental plants. *S. splendens* Roem. & Schult. is a familiar species in cultivation and one of the most commercially important and famous bedding plants. Recently, numerous *Salvia* species and varieties have been introduced into Japan as ornamental plants. Their flowering and cultivation characteristics are being investigated. *S. coccinea* Juss., commonly called tropical sage, is widely distributed throughout tropical South America. Owing to its adaptability, self-sowing ability, and handsome appearance, it has been introduced and commended all over the world. *S. coccinea* bears small flowers, red, pink or white in color, and cultivated as bedding or pot plants (Clebsch, 2003).

Chromosomal studies of *Salvia* species have reported that the basic chromosome number is $x = 11$ and the ploidy level of cultivated *S. coccinea* is $2n = 2x = 22$ (Alberto et al., 2003). The induction of polyploidy is a means of improving desirable plant characteristics, such as growth habitat and leaf and flower size, and an effective breeding method in ornamental plants (Griesbach and Bhat, 1990; Ishikawa et al., 1999; Lindsay et al., 1994; Lu and Bridgen, 1997; Nakano et al., 2006; Takamura and Miyajima, 1996; Väinölä, 2000).

In the present study, the induction of polyploidy in *Salvia* by colchicine treatment of seeds was carried out to explore the possibility of generating new varieties with improved morphological characteristics. Variations in flower and leaf morphology of induced polyploids were investigated. The inheritance of morphological characteristics of the polyploids in second-generation progeny was also evaluated.

Materials and Methods

Effect of colchicine treatment of seeds on germination and seedling development in three Salvia cultivars

Seeds of the commercially available *S. coccinea* 'Coral Nymph' ($2n = 2x = 22$) (Sakata Seed Co., Japan) and 'Lady in Red' ($2n = 2x = 22$) (Sakata Seed Co.), and *S. patens* ($2n = 2x = 18$) 'Blue Angel' (Sakata Seed Co.) were soaked in 250, 500, 750, 1000, 1250, or 1500 ppm colchicine (Wako, Japan) for 24 h. The seeds ($n = 60$ per treatment) were washed in distilled water and sown in

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plug trays in a medium of Metro-mix: loam (2 : 1 v/v). The growth room temperature was ca. 24°C and the photoperiod was 24 h. The germination medium was kept wet by mist irrigation. The percentage germination, growth of seedlings, and true leaf development were assessed 16 days after sowing for each treatment.

Morphological characterization of polyploids of S. coccinea 'Coral Nymph'

Further investigation of the induction of polyploidy was conducted with *S. coccinea* 'Coral Nymph'. Fifty seeds were soaked in 500 ppm colchicine for 24 h and sowed as described above. True-leaf opened seedlings were transplanted into pots and maintained under minimum temperature ca. 15°C under greenhouse conditions with a conventional cultivation method. The growth and date of flowering of polyploid seedlings were observed and compared with control diploid plants grown under identical greenhouse conditions. At the same time, variations in leaf and flower morphology were also investigated. Pollen grains were observed by light microscopy after staining with aceto-orcein and the major and minor axes of about 10 grains were measured in each individual measured. Leaf stomata were observed by the SUMP (Suzuki's Universal Method of Printing; KENIS Ltd., Japan) method which makes celluloid leaf surface replica, and the size of 10 stomatas was measured with the aid of a light microscope.

Flow cytometry

Plant ploidy was analyzed by flow cytometry using young leaves. Approximately 5 mm^2 leaf material from each individual was chopped with a razor blade in HR-A nuclei exraction solution (Partec High Resolution Staining Kit, Partec GmbH, Germany) and filtered through a 50 µm CellTrics filter. The suspension of nuclei was stained with HR-B DAPI staining solotion (Partec High Resolution Staining Kit). The solution mixture of HR-A : HR-B (1 : 3 v/v) was analyzed with a Ploidy Analyzer (Partec Co.). At least five leaves from different diploid plants with 0 ppm colchicine treatment were used to determine the standard peak of diploid cells of cultivated *S. coccinea* ($2n = 2x = 22$) (Alberto et al., 2003). Naturaly obtained progeny seeds from four tetraploid individuals in a pollinator-free greenhouse were germinated and the inheritance of ploidy level and morphological traits in the second generation was also investigated.

Results and discussion

Effect of colchicine treatment of seeds on germination and seedling development in three Salvia cultivars

To identify the most effective concentration of colchicine to treat seeds of *Salvia* species, the effect of a range of colchicine concentrations on seed germination and seedling growth was investigated. The percentage of seed germination after colchicine treatments was approximately 80% in *S. coccinea* 'Coral Nymph' irrespective of colchicine concentration (Fig. 1). A similar result was obtained in *S. patens* 'Blue Angel'. High colchincine concentrations (1250 and 1500 ppm) had an adverse effect on germination in *S. coccinea* 'Lady in Red'. The percentage of true leaf development decreased with increasing colchicine concentration in all three cultivars. A 50% inhibition of true leaf development was observed at 500 ppm in *S. coccinea* 'Coral Nymph', 250 ppm in 'Lady in Red' and 750 ppm in *S. patens* 'Blue Angel'.

In this study, germination was indicated by splitting the seed coat and initiating root elongation. Colchicine inhibition for germination was not expressed until the initiation of root elongation; the development of some seeds was inhibited at this stage while the growth of some seedlings was aborted after emergence of cotyledons. Seedlings that failed to develop true leaves did not survive. In this regard, the effective colchicine concentration differed among cultivars. The concentration at which 50% inhibition of true leaf development was obtained, as mentioned above, would be considered

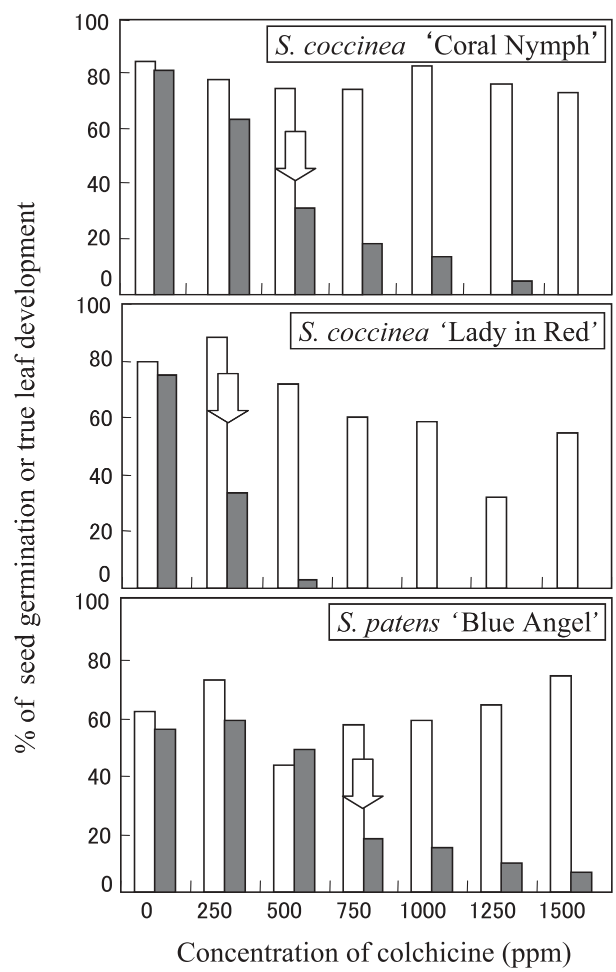

Fig. 1. Effect of colchicine treatment of seeds on germination and true leaf development in three *Salvia* cultivars. Arrows indicate 50% inhibition of true leaf development. □: germination, ■: true leaf development.

the median lethal dose of colchicine for each cultivar.

Morphological characterization of polyploids of S. coccinea 'Coral Nymph'

In the following study, *S. coccinea* 'Coral Nymph', one of the most popular salvia cultivar, was used as material. Fifteen *S. coccinea* 'Coral Nymph' seeds treated with 500 ppm colchicine were sown, of which 12 seedlings were still alive 3 months after germination. All 12 individuals flowered except for one deformed individual. From flow cytometry analysis, the standard peak of diploid plants appeared at about channel 45 of relative fluorescent intensity, and plants with a peak at about channel 90 of relative fluorescent intensity were regarded as tetraploids. Figure 2 shows the results of flow cytometric measurements with a leaf sample mixture of diploid (2x) and tetraploid (4x) plants. When two peaks of diploid (2x) and tetraploid (4x) could be recognized, the plants were regarded as chimeric.

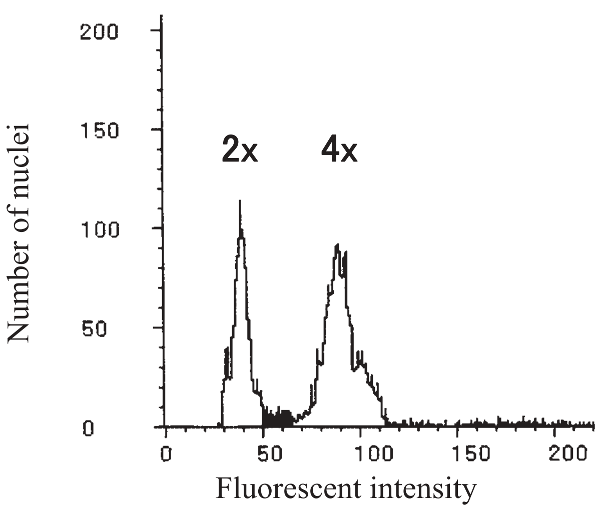

Fig. 2. Flow cytometric histograms of diploid (2x) and tetraploid (4x) plants of *S. coccinea* 'Coral Nymph'. This histogram was obtained from leaf sample mixture of diploid (2x) and tetraploid (4x) plants.

In the results, five individuals out of the 12 seedlings were regarded as tetraploid, and one tetraploid-octoploid sectorial chimeric plant and one diploid-tetraploid mixochimeric plant were detected (Table 1). Tetraploid plants had larger, thicker leaves and the total plant height at flowering tended to be higher than that of diploid control plants, although the total number of flowers per individual did not differ (Fig. 3 and Table 1).

The onset of flowering of tetraploids was delayed 10–30 days compared to diploid plants. Tetraploid plants had enlarged inflorescences and flower organs, as indicated by their larger corolla lobes and corolla tube than in diploid plants (Fig. 3 and Table 1). Similarly, in yellow-flowered cyclamen (Takamura and Miyajima, 1996), *Eustoma grandiflorum* (Griesbach and Bhat, 1990; Lindsay et al., 1994), *Buddleia globosa* (Rose et al., 2000), and *Tricyrtis hirta* (Nakano et al., 2006), enlargement of flower petals or inflorescences was observed in tetraploid plants. This enlargement of the *Salvia* flower is one beneficial effect of polyploidy and contributes to its increased ornamental value as a flowering pot or bedding plant.

The tetraploid-octoploid chimeric plant was recognized from the flow cytometric results which showed that leaves from one shoot indicated only a tetraploid peak and leaves from another shoot indicated only an octoploid peak at about channel 180 of relative fluorescent intensity. The octoploid shoot had malformed leaves and flowers (Fig. 4). In *Petunia hybrida* 'Mitchell', octoploid plants produced both deformed leaves and flower buds that aborted before opening (Griesbach and Kamo, 1996). Generally, high-level polyploid plants struggle to survive and to flower. This is true of the octoploid *Salvia* shoot, which produced neither viable pollen grains nor seeds.

Table 1. Ploidy level and characteristics of flower and leaf of *S. coccinea* 'Coral Nymph'.

Plant code	Ploidy level	Date of flowering	Flower (mm)				Leaf (mm)		
			Width of lip	Height of lip	Tube lengh	Whole length	Length	Width	Thich
0 ppm (mean)	2x	29-Mar	15.7 ± 0.2[Z]	12.1 ± 0.1	25.0 ± 0.5	33.9 ± 0.3	45.1	34.9	0.3
500 ppm-1	4x	11-Apr	19.6 ± 0.9	15.6 ± 0.5	32.9 ± 1.4	38.6 ± 2.4	63.0	49.0	0.6
500 ppm-2	2x	5-Apr	15.8 ± 0.6	12.1 ± 0.5	29.5 ± 1.2	38.1 ± 0.3	47.5	37.4	0.7
500 ppm-3	2x	8-Apr	15.1 ± 0.3	12.1 ± 0.3	28.3 ± 1.0	37.0 ± 1.3	56.9	42.8	0.6
500 ppm-4	4x	12-Apr	18.6 ± 0.3	13.5 ± 0.7	31.2 ± 0.7	36.5 ± 1.1	64.4	54.8	0.5
500 ppm-5	4x		18.1 ± 0.4	12.7 ± 0.3	29.0 ± 0.9	38.1 ± 2.0	58.8	44.4	0.7
500 ppm-6	2x	8-Apr	14.9 ± 0.5	10.9 ± 1.0	26.7 ± 1.0	34.4 ± 0.9	45.7	35.6	0.5
500 ppm-7	2x + 4x	11-Apr	17.6 ± 1.0	12.8 ± 0.9	31.9 ± 1.3	39.3 ± 1.3	68.1	50.4	0.5
500 ppm-8	4x	13-Apr	16.7 ± 0.3	13.2 ± 0.1	30.9 ± 1.1	39.6 ± 0.5	48.6	35.8	0.4
500 ppm-9	Not determined	Not flowered	—	—	—	—	54.2	49.5	0.8
500 ppm-10	4x + 8x	8-May	17.9 ± 0.8	14.4 ± 0.2	29.5 ± 1.3	35.7 ± 2.1	62.8	53.6	0.9
500 ppm-11	4x	4-May	17.0 ± 1.0	14.1 ± 0.4	29.8 ± 0.5	36.8 ± 0.3	56.0	42.1	0.5
500 ppm (mean)		21-Apr	17.3 ± 0.5	13.3 ± 0.4	30.2 ± 0.6	38.1 ± 0.8	56.4	44.3	0.6

[Z] Mean ± SE (n = 10).

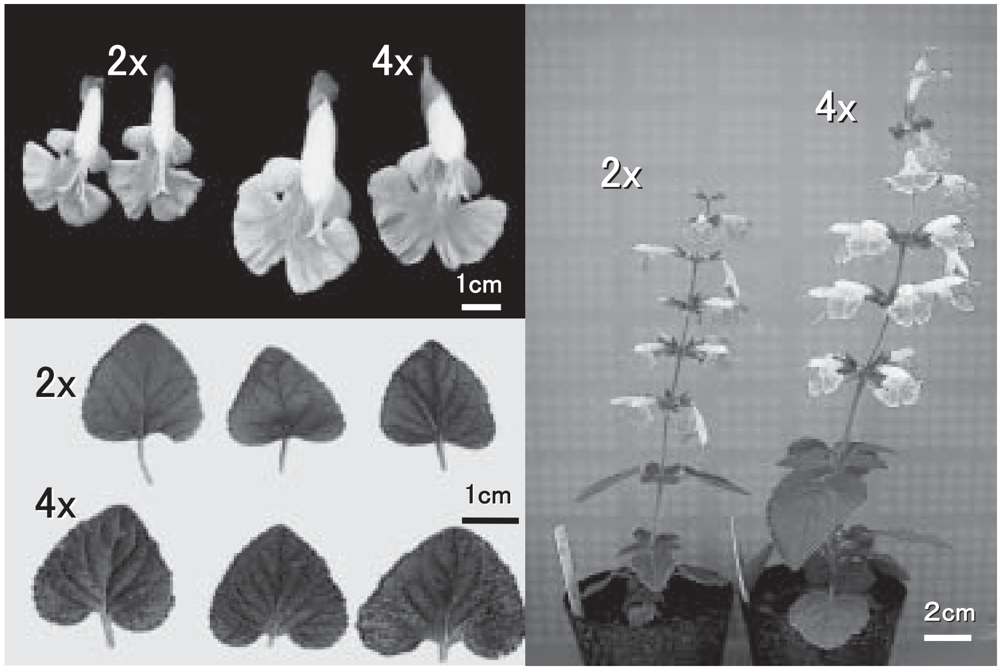

Fig. 3. Morphological differences of flower and leaf between diploid (2x) and tetraploid (4x) plants of *S. coccinea* 'Coral Nymph'.

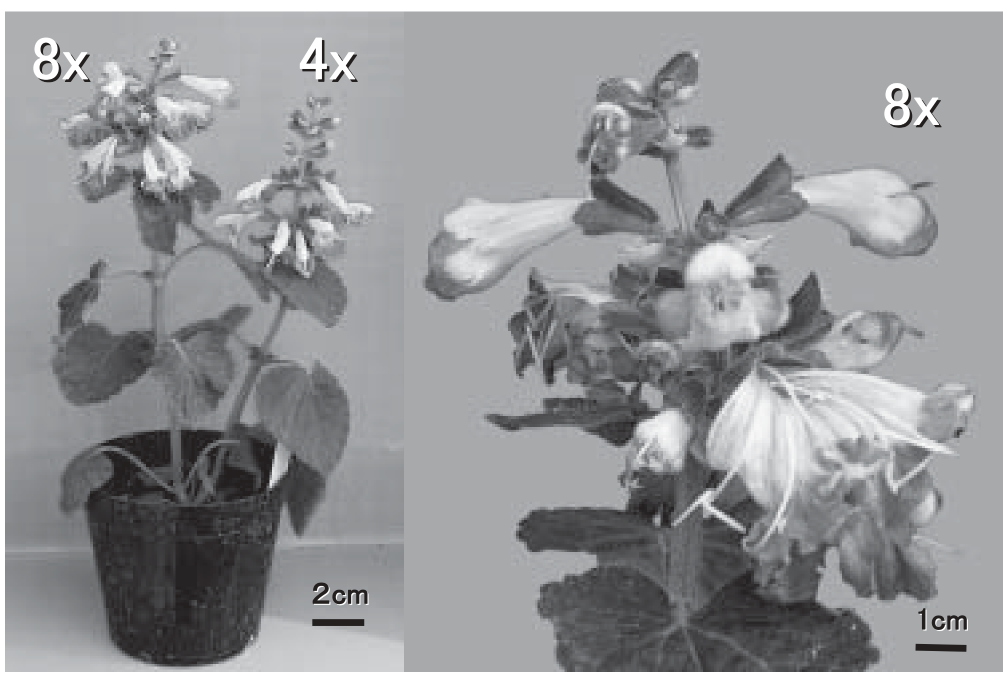

Fig. 4. Flowering of tetraploid (4x) - octoploid (8x) chimerical plants of *S. coccinea* 'Coral Nymph'.

The stomata and pollen grains of tetraploid *Salvia* were also larger than those of diploid plants, as reported in yellow-flowered cyclamen (Takamura and Miyajima, 1996) and *Alocasia* (Thao et al., 2003) (Fig. 5). These characters could be used as indicators of polyploidy in *Salvia* breeding programmes.

Most of the tetraploid *Salvia* plants produced viable pollen grains. Naturally pollinated seeds were obtained from four tetraploid individuals. In total, eighteen seedlings were grown to flowering from these seeds. The flower and leaf morphology of these seedlings closely resembled that of the parents. All 18 individuals were regarded as tetraploid by flow cytometric results which have a peak at about channel 90 of relative fluorescent intensity (Table 2). Alberto et al. (2003) reported that the ploidy level of some native *Salvia* species in Argentina are diploid or tetraploid in the wild; however, there are few studies on the inheritance of

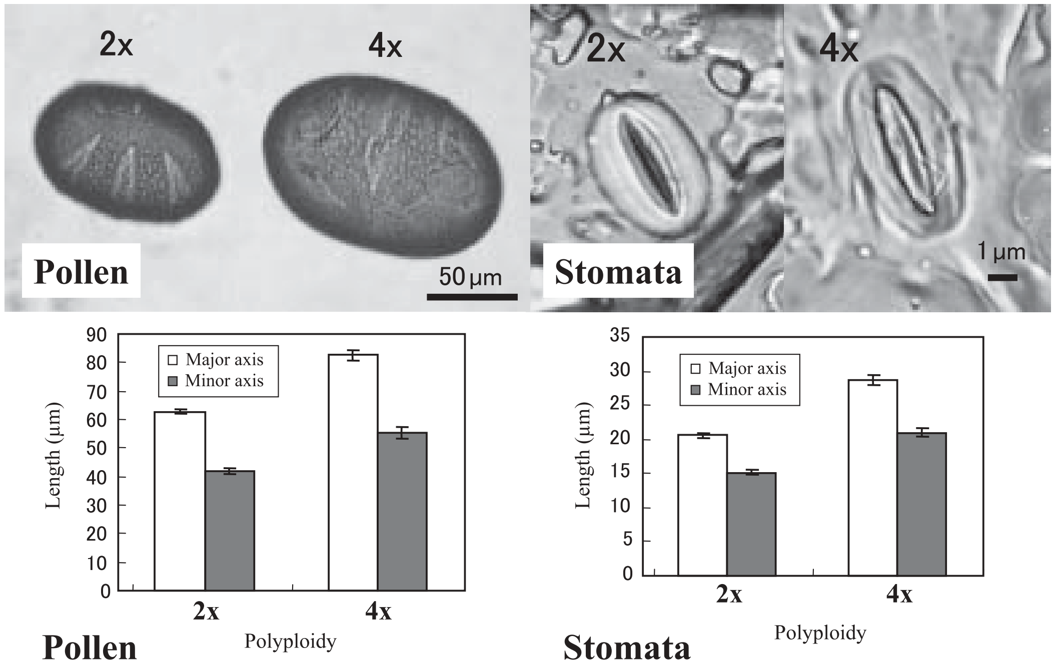

Fig. 5. Morphological differences of pollen and stomata between diploid (2x) and tetraploid (4x) plant of *S. coccinea* 'Coral Nymph'.

Table 2. Ploidy level and characteristics of flower and leaf of the progenies obtained from tetraploid parents of *S. coccinea* 'Coral Nymph'.

Code of tetraploid parents[Z]	Code of progeny derived from the tetraploid	Ploidy level	Flower (mm)				Leaf (mm)		
			Width of lip	Height of lip	Tube length	Whole length	Length	Width	Thich
500 ppm-1 (4x)	1-A	Tetraploid (4x)	17.15 ± 1.35[Y]	13.35 ± 0.37	29.45 ± 0.12	38.35 ± 0.69	45.10	40.10	0.5
	1-B	Tetraploid (4x)	16.43 ± 1.74	12.60 ± 0.72	27.43 ± 1.44	35.50 ± 1.21	60.10	57.60	0.5
	1-C	Tetraploid (4x)	17.63 ± 0.61	14.57 ± 0.43	29.03 ± 0.41	35.60 ± 0.06	65.10	60.80	0.5
	1-D	Tetraploid (4x)	18.10 ± 0.32	13.47 ± 0.44	30.13 ± 0.39	38.77 ± 0.43	58.20	52.50	0.5
	1-E	Tetraploid (4x)	18.20 ± 0.29	13.54 ± 1.30	29.75 ± 3.23	36.05 ± 3.55	72.00	67.30	0.5
	1-F	Tetraploid (4x)	—[X]	—	—	—	—	—	—
	1-G	Tetraploid (4x)	—	—	—	—	—	—	—
	1-H	Tetraploid (4x)	—	—	—	—	—	—	—
	1-I	Tetraploid (4x)	—	—	—	—	—	—	—
	1-J	Tetraploid (4x)	—	—	—	—	—	—	—
	1-K	Tetraploid (4x)	—	—	—	—	—	—	—
500 ppm-4 (4x)	4-A	Tetraploid (4x)	—	—	—	—	—	—	—
500 ppm-5 (4x)	5-A	Tetraploid (4x)	18.25 ± 1.65	13.70 ± 0.00	30.10 ± 0.41	40.40 ± 0.24	63.30	55.20	0.7
500 ppm-10 (4x)	10-A	Tetraploid (4x)	18.43 ± 1.35	12.33 ± 0.80	29.13 ± 0.41	36.60 ± 0.20	58.10	48.50	0.5
	10-B	Tetraploid (4x)	—	—	—	—	—	—	—
	10-C	Tetraploid (4x)	—	—	—	—	—	—	—
	10-D	Tetraploid (4x)	—	—	—	—	—	—	—
	10-E	Tetraploid (4x)	—	—	—	—	—	—	—
500 ppm-11 (4x)	11-A	Tetraploid (4x)	18.43 ± 2.80	13.57 ± 0.47	28.90 ± 2.02	36.43 ± 0.84	—	—	—
	11-B	Tetraploid (4x)	—	—	—	—	—	—	—
	11-C	Tetraploid (4x)	—	—	—	—	—	—	—
	11-D	Tetraploid (4x)	—	—	—	—	—	—	—

[Z] Plant code corresponds to Figure 1.
[Y] Mean ± SE (n = 10).
[X] Data not collected.

tetraploidy in ornamental flowering plants (Blakeslee et al., 1923; Krebs and Hancock, 1981). This study is the first to confirm the inheritance of tetraploidy in cultivated *Salvia*. The stable inheritance of tetraploidy and the associated morphological traits are expected to be ultilized in *Salvia* breeding in the future.

Tetraploidy in *Salvia* was previously studied in a Chinese medicinal species, *S. miltiorrhiza* Bge., with tetraploidy induced by bud culture (Gao et al., 1996). The tetraploid plant of *S. miltiorrhiza* grew vigorously, the leaves were thicker and larger, and the leaf stomata were larger than those of control diploid plants. These morphological variations were also observed in *S. coccinea* 'Coral Nymph' in the present study. The study by Gao et al. (1996) focused on the production of the major medicinal chemical compounds, which was enhanced in tetraploid plants, and flower characteristics were not investigated.

In this study, we induced tetraploidy in *Salvia* cultivar by colchicine treatment of seeds and obtained tetraploids with increased ornamental value on account of their enlarged flower organs. It is expected that this treatment will be applied to other *Salvia* cultivars or species and enable the production of triploid plants by crossing tetraploids and diploids, with the objective of creating new ornamental cultivars and novel morphological traits.

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