Appearance of the Unexpected Triploids in the Hybrid Progeny between Lettuce, *Lactuca sativa* and its Wild Relatives, *L. saligna*

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Summary

Interspecific hybrids between *Lactuca saligna* and *L. sativa* cv. Bronze were obtained only when *L. saligna* was used as a seed parent. The seed set ratio of the F₁ hybrid was very low (12.0%), however, their seed germination rate was 87.5%. In pollen mother cells of the F₁ hybrids, univalent chromosomes were observed at metaphase I and the number of chiasma at diakinesis was fewer than their parental species. At pollen tetrad stage, the number of microspores of F₁ hybrids varied between 2 and 6. In some F₂ hybrids, distorted staining intensities showed a 4:4:1 ratio in all or some of the 5 isozyme loci, suggesting that they were triploids or aneuploids, respectively. The triploids in F₂ progeny were screened by flow cytometric analysis at the 2-week-old seedling stage. Microscopic observation of chromosome revealed that 14 of 103 F₂ plants were found to be triploid, $2n=3x=27$; no aneuploid plants were observed indicating that aneuploids do not survive until the 3- to 4-week-old seedling stage.

Key Words: cytology, flow cytometric analysis, *Lactuca saligna*, lettuce, triploid.

Introduction

Lettuce, *Lactuca sativa* L. that has been cultivated from the time of Egyptian Pharaohs is now one of the most popular salad crops in the world. However, lettuce crops have occasionally been devastated by several diseases. Therefore, the most important and urgent breeding purpose of lettuce is to introduce disease resistances. Some wild lettuce species in the section *Lactuca* have useful genes resistant to *Trichopius ni* Hubener (Whitaker et al., 1974) and downy mildew (Hulbert and Micheltore, 1985; Gustafsson, 1989). Currently, *L. saligna* is one of the most useful wild relatives, but it is only partially cross-compatible with the cultivated lettuce. Hybrids have occasionally been obtained in the cross only when *L. saligna* was used as a seed parent (Lindqvist, 1960; Thompson and Ryder, 1961; De Vries, 1990). Overcoming the sexual barrier between *L. sativa* and *L. saligna* is a prerequisite to improve the cultivated lettuce.

Spontaneous triploid hybrid plants have been reported in intra- and inter-specific crosses in the genus *Citrus* (Esen and Soost, 1971) and *Malus* (Sedyshva, 1995); they were expected to be used to produce seedless cultivars. However, triploid hybrids are difficult to use in the breeding programs of the seed-propagated crops because triploid plants are generally sterile; the contamination of the triploid individuals in the diploid popula-

tion makes the genetic analysis difficult. Therefore, it is desirable to eliminate or distinguish these unexpected triploid plants from normal diploid ones during the early seedling stage in a breeding program.

Isozyme genes can be a useful genetic marker in the breeding programs; for example, the alien chromosome of monosomic addition lines (trisomics) in *Allium* species was identified by isozyme analysis (Shigyo et al., 1998). In our previous study, 5 allozyme gene loci, *Got-1*, *Pdh-1*, *Pdh-2*, *Pgi-1* and *Pgi-2*, were revealed to be interspecific genetic markers between *L. sativa* and its relatives. The genomic components of the hexaploid somatic hybrid between *L. sativa* cv. Bronze and *L. indica* were identified by using these genetic loci (Mizutani and Tanaka, 2003). The abnormal band intensity ratio of 4 : 4 : 1, that is considered to be a reflection of the dosage of alleles indicate that the hexaploid somatic hybrid is composed of 2 *L. sativa* and 4 *L. indica* genomes.

In this study, isozyme analysis and flow cytometric (FCM) analysis to estimate DNA contents of plants and for screening polyploids (Johnston et al., 1999; Zonneveld and Iren, 2000; Elwira and Steen, 1995) were carried out for the detection of triploids, followed by cytogenetic studies of the F₁ hybrids between the 2 species and their F₂ progenies. The erratic meiotic chromosome behaviors of F₁ plants were found to be the cause of the appearance of unexpected triploid plants among their F₂ progenies. Consequently, the ploidy levels of the F₂ progenies were determined by chromosome counting.

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Materials and Methods

Reciprocal crosses between lettuce, *L. sativa* cv. Bronze (Tokita Seed Co., Ltd. Saitama) and its wild species, *L. saligna* were carried out by hand pollination. F₂ progenies were produced through open-pollination of the F₁ hybrids under an insect-protected condition.

Microscopic observations of the pollen mother cells (PMCs) of *L. sativa*, *L. saligna* and their F₁ hybrids revealed that some anomaly existed in the F₁ and F₂ populations. Pollen fertility was estimated on about 500 mature pollen grains by staining with 1.0% aceto-carmine.

For enzyme extraction, 10 mg of expanding cotyledons excised from 1-week-old seedlings were homogenized by using precooled micro-tubes and micropestle with 50 μ l of extraction buffer (10 mM MgCl₂, 1 mM 2-mercaptoethanol, 10 mM KCl and 1 mM EDTA-Na₄ in 50 mM Tris-HCl, at pH 7.6). Enzymes were separated electrophoretically on vertical polyacrilamide slab gels. Enzyme assay for GOT, PGI and 6-PDH was performed according to our pervious report (Mizutani and Tanaka, 2003).

A 500-mg sample of expanding young leaves at 2-week-old seedling stage was prepared for FCM analysis. The sample was chopped in 2 ml of chopping buffer (1% triton-X, 1% 2-mercaptoethanol and 0.5 M Na₂SO₄ in 0.5 M Tris-HCl, at pH 8.0); nuclei were freed from debris by filtration through miracloth into a 15-ml glass centrifuge tube. After centrifugation (2000 $\times$ g, 1 min.), tissue pellets were resuspended with 500 μ l of the chopping buffer and 50 μ l of a propidium iodide solution (1mg $\cdot$ ml⁻¹ PI). FCM analysis was carried out by using EPICS XL SYSTEM II (Vecman Corter Inc., California).

For chromosome counting, root tips of 3- to 4-week-old seedlings of the F₁ and F₂ hybrids and their parents, *L. sativa* and *L. saligna*, were pretreated in 0.2 mM solution of 8-hydroxyquinoline for 4 hr and then fixed for 24 hr in Farmer's solution. After hydrolyzing for 8

min in 1N HCl at 60 $^{\circ}$ C, the root tips were then stained with Schiff's reagent and squashed under a cover slip. At least 10 cells were observed for each seedling.

Results and Discussion

In this study, F₁ hybrid seeds were obtained only when *L. saligna* was used as a seed parent (Table 1). The mean seed set ratio of 4 seed parents of *L. saligna* crossed with *L. sativa* was 12.0%. Nineteen of the 22 F₁ hybrid seeds germinated (87.5 $\pm$ 12.5%) 1 week after sowing; 17 survived (77.6 $\pm$ 15.0%) and bloomed. These results were similar to the conclusion of De Vries (1990) who summarized the cross compatibility between *L. sativa* and its related species.

The chromosome behavior during meiotic division of the F₁ hybrids and their parents (Table 2) show that in spite of the low cross compatibility between the 2 species, meiotic division of the F₁ plants was quite normal. Our data show that almost all PMCs (Fig. 1A, B) have 9 bivalents at metaphase-I. Only 5 (6.2%) of 81 PMCs from 4 F₁ hybrids possessed 2 univalents and 8 bivalents. Thus, the average frequency of bivalents of F₁ hybrids between *L. saligna* and *L. sativa* cv. Bronze was 8.56, whereas those of both parent species were 9.0 (Table 2). Although most of the bivalents observed in the parent species formed ring-shape with 2 chiasmata (Fig. 1A, B), some bivalents in the F₁ hybrids showed x- or rod-shapes with 1 chiasma (Fig. 1C). The mean chiasma frequencies of *L. sativa* and *L. saligna* were 2.2 and 2.1, respectively (Table 2). However, mean chiasma frequency of 4 F₁ hybrids was 1.8, lower than either parent. The chiasma frequency of interspecific hybrids was known to be lower than those of parent plants, depending on the genomic homology of the two parent species (Tashiro, 1984). The low chiasma frequency in the F₁ hybrids observed between *L. saligna* and *L. sativa* suggests that a low homology of genomes exists between them.

Although *L. sativa* and *L. saligna* formed normal pollen tetrads, the F₁ hybrids between them produced abnormal pollen tetrads (Table 3, Fig. 1D). The exis-

Table 1. Germination rate, seed set ratio and survival rate of *L. sativa*, *L. saligna* and their hybrids.

Species (Hybrid)		line	Seed set ratio (%)	No. of seeds sown	% of individuals	
Seed parent	Pollen parent				Germinated	Survived
<i>L. sativa</i>	$\times$ <i>L. sativa</i> (self)		88.9 $\pm$ 0.2 ^y	120	97.5 $\pm$ 1.4 ^y	93.3 $\pm$ 2.4 ^y
	$\times$ <i>L. saligna</i>		0	-	-	-
<i>L. saligna</i>	$\times$ <i>L. saligna</i> (self)		90.8 $\pm$ 1.1 ^y	120	100 ^y	98.3 $\pm$ 2.9 ^y
	$\times$ <i>L. sativa</i>		12.0 $\pm$ 2.3 ^y	22	87.5 $\pm$ 12.5 ^y	77.6 $\pm$ 15.0 ^y
<i>L. saligna</i> $\times$ <i>L. sativa</i>	$\times$ <i>L. saligna</i> $\times$ <i>L. sativa</i> (self)	A	10.3	120	94.0 $\pm$ 6.4 ^z	65.8 $\pm$ 10.9 ^z
		B	14.9	120	91.6 $\pm$ 3.7 ^z	52.5 $\pm$ 11.9 ^z

^y Data show the mean $\pm$ SE of 4 plants.

^z Data show the mean $\pm$ SE of 3 petri dishes each with 40 seeds.

Table 2. Chromosome pairing in pollen mother cells (PMCs) of *L. sativa*, *L. saligna* and their F₁ hybrids at metaphase-I.

Species (Hybrid)	No. of plants observed	No. of PMCs observed	Configuration		% of PMCs with univalents	Frequency of chromosome pairing	Chiasma frequency ^y
			9II	8II+2I			
<i>L. sativa</i>	5	54	54	0	0	9II	2.2 ± 0.1
<i>L. saligna</i>	3	48	48	0	0	9II	2.1 ± 0.2
<i>L. saligna</i> × <i>L. sativa</i>	4	81	77	5	6.2	8.56II+0.88I	1.8 ± 0.1

^yData show the mean ± SE. **Fig. 1.** Cytological observation of *L. sativa*, *L. saligna* and their F₁ hybrids. Metaphase-I (MI) of *L. sativa* (A), *L. saligna* (B), and *L. saligna* × *L. sativa* (C). Abnormal pollen tetrad of F₁ hybrid (D). Somatic chromosomes in root-tip cells of *L. sativa*, 2n = 2x = 18 (E), *L. saligna*, 2n = 2x = 18 (F) and triploid F₂ progeny, 2n = 3x = 27 (G).**Table 3.** Number of microspores in pollen mother cells (PMCs), percentages of abnormal PMCs at pollen tetrad stage in *L. sativa*, *L. saligna* and their F₁ hybrid.

Species (Hybrid)	No. of PMCs observed	No. of microspores in PMCs					% of abnormal PMCs
		2	3	4	5	6	
<i>L. sativa</i>	490	0	0	490	0	0	0
<i>L. saligna</i>	500	0	0	500	0	0	0
<i>L. saligna</i> × <i>L. sativa</i>	560	2	3	546	7	2	2.5

tence of diads in the PMCs indicated the formation of unreduced pollen grains. The pollen fertility of the F₁ hybrids was lower than those of the parents (Table 4). A large variation was also observed in the size of pollen grains of F₁ hybrids (data not shown). F₁ hybrids with low pollen fertility also possessed low seed fertility.

Analysis of 5 loci of 3 dimeric enzymes, *Got-1*, *Pgi-1*, *Pgi-2*, *Pdh-1* and *Pdh-2*, of the F₁ hybrids and the F₂ progenies revealed that the staining intensity ratio of the F₁ hybrids was 1 : 2 : 1, whereas that of the hexaploid somatic hybrids was 4 : 4 : 1. Our previous report indicated that the parent plants were homozygous so that they could be distinguished from each other by the 5 isozyme loci (Mizutani and Tanaka, 2003). We also reported that the F₁ hybrids were heterozygous with

triple-banded phenotypes and that the F₂ progeny segregated into the three phenotypes. Our analyses show that these isozymes could be used for identifying the genome components of the hexaploid somatic hybrid between *L. sativa* and *L. indica* by the intensity of the bands.

In this study, 14 individuals of the F₂ progeny which showed abnormal staining intensities of 4 : 4 : 1 in all of the 5 loci are theoretically considered to be polyploid (triploid), whereas the 16 individuals which showed abnormal staining intensities in some of the 5 loci were aneuploid.

In FCM analysis, the 2C DNA content value of *L. sativa* was 186 ± 3.5 (Fig. 2A), whereas that of *L. saligna* was 156 ± 3.8 (Fig. 2B). In F₁ hybrids between

Table 4. Pollen stainability in *L. sativa*, *L. saligna* and their F₁ hybrids.

Species (Hybrid)	Pollen fertility (%) ^z
<i>L. sativa</i>	90.7 ± 0.5
<i>L. saligna</i>	93.3 ± 1.8
<i>L. saligna</i> × <i>L. sativa</i>	30.9 ± 2.2

^z Data show the mean ± SE.**Table 5.** Ploidy level of F₂ hybrid lines, A and B, between *L. saligna* and *L. sativa*.

Line	Number of plants (%)	
	Diploid	Triploid
A	59 (95.2)	3 (4.8)
B	44 (80.0)	11 (20.0)
Total	103 (87.6)	14 (12.4)

the 2 species, the 2C DNA content were 162.3 ± 9.5 similar to those of *L. saligna* (Fig. 2C). However in the F₂ progeny, the 2C DNA content exhibits 2 peaks (Fig. 2D). We considered the individuals with the DNA contents value of around 250 as triploid (Fig. 2D) which concurs with our data from isozyme analysis.

In the parent plants, the diploid somatic chromosomes were $2n=2x=18$ (Fig. 1E, F) similar in size and morphology (Haque and Godward, 1985). Chromosome numbers of the F₁ hybrids were also $2n=2x=18$ (diploid), but of the 117 individuals of 2 F₂ progeny lines observed (Table 5), 14 were triploid ($2n=3x=27$, Fig. 1G) whereas the rest were diploid ($2n=2x=18$).

Thus, isozyme analysis, FCM analysis and chromosome counting revealed a frequent appearance of unexpected triploid plants in the F₂ progeny between *L. saligna* and *L. sativa*. The frequencies of the triploid individuals were 4.8% and 20.0% in 2 progeny lines A and B, respectively (Table 5). Half of the germinating seedlings were weak from the beginning and died during the early stage of their growth (Table 1). Low survival rates of F₂ progenies (65.8% and 52.5% in line A and B, respectively) might suggest a distortion of genes and/or the existence of aneuploids. Isozyme analysis of the cotyledons indicated the existence of aneuploids, because 16 seedlings showed both distortion in the intensity of triple-banded phenotypes and normal distribution of the intensity on the 5 loci. However, no aneuploids were detected by chromosome counts of the surviving seedlings. The aneuploids might survive at the cotyledonary stage but died prior to chromosome counting.

Generally, unreduced gametes are considered to contribute to the appearance of unexpected polyploid individuals from diploid parents, although the frequency of

Fig. 2 Flow cytometric analysis of *L. sativa*, *L. saligna* and their hybrids. A: *L. sativa*, B: *L. saligna*, C: F₁ hybrid, D: F₂ progeny.

triploid progenies from diploid parents is usually very low (Esen and Soost, 1971). In this study, however, a high percentage of unexpected triploids in the F₂ progeny between *L. saligna* and *L. sativa* indicate that unreduced gametes might have resulted from abnormal meiotic division, such as the univalent at the metaphase I and the existence of diads at the pollen tetrad stage in PMCs. The low survival rates of many haploid gametes and diploid seedlings may have contributed to the increase in unreduced gametes and triploid seedlings. The low pollen fertility (30.9%, Table 4), low seed set ratio (10.3% and 14.9%, Table 1) of F₁, and low survival rate of F₂ seedlings (65.8 and 52.5%, Table 1) support the hypothesis that unreduced gametes contribute to the occurrence of the triploid individuals. Our microscopic observations of PMCs do not help to explain the unexpected appearance of triploids because the frequency of diads in PMCs was quite low (0.4%) compared with the frequency of triploids (4.8 to 20%). However, the survival rate of the unreduced gametes might be higher than that of the genetically distorted haploid gametes of the interspecific F₁ hybrids.

In our previous study, isozyme analysis revealed the genomic components of the hexaploid somatic hybrid between *L. sativa* and *L. indica* (Mizutani and Tanaka, 2003). Isshiki et al. (1995) screened triploid individuals of *Colocasia esculentum* by isozyme analysis. In conclusion, our analysis of 5 loci, *Got-1*, *Pgi-1*, *Pgi-2*, *Pdh-1* and *Pdh-2*, in F₂ progeny between *L. saligna* and *L. sativa* indicates that both triploids and aneuploids existed at the early stage of the seedlings, but chromosome

counts detected only triploids.

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栽培種レタス *Lactuca sativa* とその近縁野生種 *L. saligna* 間の雑種後代における3倍体の出現

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摘 要

レタス栽培品種ブロンズとその近縁野生種 *L. saligna* 間の雑種は、*L. saligna* を種子親に用いた時のみ得られた。その雑種個体 (F_1) の種子結実率は12.0%と極めて低かったが、得られた種子の発芽率は87.5%と高く、それぞれの親植物の自殖で得られた種子の発芽率と大きな差は認められなかった。花粉母細胞の第一分裂中期において、それぞれの親植物は全て2価染色体を形成したのに対し、雑種個体 (F_1) では低頻度ではあるが1価染色体の形成もみられ、キアズマ形成頻度は親植物と比較して低かった。花粉4分子期で親植物は正常な4つの小孢子だけを形成したのに対し、雑種個体 (F_1) では2から6の小孢子を形成したものもみられた。雑種後代 (F_2) の5つのアイソザイム遺伝子座の表現型には、バン

ドの濃度の歪みが観察された。これらの全ての遺伝子座の表現型で歪みを示した個体は3倍体であり、5つのうちのいくつかで歪んでいた個体は異数体であると判断された。これらの個体は、フローサイトメトリック分析において、相対DNA量は2倍体の約1.5倍であり、体細胞染色体の観察結果から、3倍体 ($2n=27$) であることが明らかになった。*L. saligna* と栽培種レタスとの交雑で得られた103の雑種後代 (F_2) において、14個体で3倍体を得られたが、播種後2週間目の実生におけるアイソザイム分析によって存在が示された異数体は、観察できなかった。このことは、染色体を観察した播種後3~4週間目まで、異数体が生存できなかったことによるものと考えられた。