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Frequencies of Aneuploid Seedlings Obtained from Aneuploid Apple Accessions (*Malus × domestica*) Pollinated with Diploid 'Hongro'

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To estimate the breeding behavior of aneuploid apple (*Malus* × *domestica*), twelve crosses were undertaken with twelve aneuploid accessions pollinated with diploid 'Hongro' apple. A 2x × 2x cross with 'Hongro' was also carried out as a control experiment for these aneuploid × diploid crosses. The pollinated aneuploid accessions exhibited relatively high fruit set rates. The apple fruit obtained from the pollinated aneuploid accessions contained less seeds per fruit than those from the 2x × 2x cross. The weight of seeds from the fruits of the pollinated aneuploid accessions was not related to the chromosome number of the aneuploid seed parent. Some of the seeds obtained from the pollinated aneuploid accessions failed to grow into seedlings. Metaphase figures of the root tip cells of seedlings derived from the pollinated aneuploid accessions showed that of 155 seedlings examined 115 were diploid, two were triploid and 38 were aneuploid. The chromosome number of the aneuploid seedlings ranged from 35 to 39, except for 48 chromosomes of one seedling. The seedlings with 35 ($2n=2x+1$) chromosomes appeared with the highest frequency of 15/38 (39.5%). The chromosome number of the aneuploid seedlings approached to the diploid ($2n=34$) level, or the number of aneuploid seedlings decreased with increase of chromosome numbers from 35 to 39.

Key words: aneuploid–diploid cross, aneuploid frequency, apple, chromosome number, seed abortion.

INTRODUCTION

The main purpose of an attempt in plant breeding is to organize mating and genetic recombination between wide ranges of genotypes. The emphasis is on the diversity of genes contributing breeding by each genotype rather than on its commercial value (Noiton and Shelbourne, 1992). Aneuploid plants give rise to a high frequency of aneuploid gametes, which in turn yield more aneuploid plants in the following generation. If they were not for selection against aneuploid gametes and sporophytes, the amount of aneuploidy would increase every generation (Doyle, 1986). Based on this theory, organization of crosses between aneuploids and diploids is a way to obtain diverse aneuploid plant progenies. The karyotype of the offspring aids in diagnosis of the contributions made by their female aneuploid parent. On the other hand, generation of more aneuploid progenies with varying chromosome numbers can offer new genetic diversity. For instance in apple plants, 17 types of 2x–1 or 2x+1 aneuploids are possible when the chromosome is in trisomic condition. Further expected respectively are 136 and 680 types of 2x+2 and 2x+3 trisomic apple aneuploids owing to the involvement of excessive two or three chromosomes. Aneuploidy in apples has been previously subjected to trisomic analysis (Diao *et al*., 2009), but the reports on the characterization of genetic diversity of aneuploid apples are a few.

In the previous study, aneuploid apple trees have been successfully obtained through crosses between diploid and triploid plants (Zhang and Park, 2009a). These aneuploid trees grew to reach an adult phase, and accession number was given them. In this communication we report the generation of aneuploid seedlings derived from crosses of twelve aneuploid accessions pollinated with a diploid cultivar. Further examined were the variation in chromosome number and traits in the aneuploid seedlings.

MATERIALS AND METHODS

Plant materials

Twelve aneuploid apple accessions (*Malus* × *domestica*) were chosen as seed parents for this study. They were six–year–old trees obtained from the previous cross experiment with triploid and diploid apple cultivars (Zhang and Park, 2009a). The accession numbers of the twelve aneuploid trees and their parents were listed in Table 1. 'Hongro', a diploid cultivar, was used as a pollen parent. A 'Hongro' × 'Hongro' cross was also carried out as a control experiment to evaluate the results of crosses of aneuploid accessions with diploid 'Hongro'. The experimental orchard was located at Kangwon National University, Chuncheon, Korea.

Crossing of aneuploid accessions with a diploid apple cultivar

Each of the twelve six–year–old aneuploid acces-

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Table 1. Aneuploid apple accessions used for crosses, and their seed and pollen parents

Accession No. of aneuploid apple plant	Chromosome number	Seed parent (Ploidy)	Pollen parent (Ploidy)
SJ277	2n=2x+1=35	Senshu (2x)	Jonagold (3x)
JT4	2n=2x+1=35	Jonagold (3x)	Tsugaru (2x)
JN811	2n=2x+2=36	Jonagold (3x)	NJ.53 (2x)
JF1049	2n=2x+2=36	Jonagold (3x)	Fuji (2x)
JF10	2n=2x+2=36	Jonagold (3x)	Fuji (2x)
JS6	2n=2x+2=36	Jonagold (3x)	Senshu (2x)
JF3942	2n=2x+3=37	Jonagold (3x)	Fuji (2x)
JN6645	2n=2x+3=37	Jonagold (3x)	NJ.53 (2x)
JN4831	2n=2x+6=40	Jonagold (3x)	NJ.53 (2x)
JF1650	2n=2x+11=45	Jonagold (3x)	Fuji (2x)
JF3252	2n=2x+11=45	Jonagold (3x)	Fuji (2x)
JT1035	2n=2x+12=46	Jonagold (3x)	Tsugaru (2x)

sions was pollinated with diploid ‘Hongro’ during May 2008. Pollen used for pollination to aneuploid accessions was collected at the full bloom stage from ‘Hongro’ flowers bagged before the bloom stage. The anthers with pollen were placed in an incubator for 4 h at 30°C until the pollen grains shed. Then, pollen was transferred to vials and placed in a desiccator at 4°C until used. The flowers of aneuploid accessions were emasculated three days before anthesis and bagged to avoid contamination with alien pollen. Arranged pollination was carried out directly onto the stigma by a blush. The pollinated flowers were bagged again to exclude any further cross–pollination. The fruit set rate was recorded in July of the year, and the seeds from the fruits were collected in September 2008. The number of fruits, and seeds in each pollinated accession were recorded for all cross combinations. All seeds derived from each of twelve accessions were weighed individually.

Karyotype analysis

The seeds obtained from twelve aneuploid accessions pollinated with diploid ‘Hongro’ were stratified for three months at 5°C in a refrigerator and individually sown in the seedbed in a greenhouse during January 2009. The seedlings grown for one month after seed sowing were transplanted to small pots.

To examine the frequency of aneuploid seedlings with different chromosome number, karyotypes were examined with root tip cells of these seedlings of about 10 cm height in February 2009, according to the procedure reported by Park *et al.* (1999). Briefly, vigorously growing root tips were collected and pre–treated with 2 mM 8–hydroxy–quinoline at room temperature for 2 h and placed in the refrigerator for 16 to 24 h at 4°C. The material was then fixed in a solution of acetic acid ethanol (1:3 v/v) and kept at –20°C for 24 h. Afterwards the root tips were treated with Flaxzyme (NOVO FERMENT) at 34°C for 2 h and fixed again: chromosome preparations were obtained by the dissociation of the root apical meristems with this enzyme. To observe root tip cell metaphase figure, the treated root tips were taken out, blotted to dry, excised with a fine stainless steel blade and placed on a piece of glass slide using forceps. The root tips trimmed so that they included actively dividing root meristem were treated with one drop of acetic acid alcohol (1:3 v/v) solution and spread the root tip cells with forceps so as to become a single layer of cells. After air–drying, the glass slide was dipped into Giemsa solution, stored in a staining glass jar for five minutes and washed once with distilled water. The chromosomes of metaphase figure of root tip cells were observed with a microscope at 200x and 600x magnifications and photographed using a digital camera (Microscope Camera System, DS–Fit–U2).

RESULTS

Fruit and seed set in aneuploid accessions pollinated with diploid ‘Hongro’

The fruit set rates and seed set rates in aneuploid accessions pollinated with diploid ‘Hongro’ are shown in Table 2. The fruit setting is important to demonstrate the fertility of aneuploid progenies. The fruit set rates in the twelve aneuploid accessions ranged from 40% to 100%. The number of seeds setting in fruits derived from each cross combination, however, could not be compared with each other, since number of fruits obtained varied in each of the aneuploid accessions at harvest. The highest average number of seeds per fruit was 7.0 for JN811 (2n=36) × ‘Hongro’ (2n=34) cross, while the lowest of 0.6 was scored for JT1035 (2n=46) cross. The average number of seeds per fruit was less than 5 for almost all cross combinations except for the JN811 (2n=36) × ‘Hongro’ cross. The average number of seeds per fruit was very low in aneuploid accessions as compared with control cross of ‘Hongro’ (2x) × ‘Hongro’ (2x), in which 9 seeds per fruit were produced.

The seed weight also showed variation in the aneuploid–diploid crosses. The average seed weight in each cross combination ranged from 15.7 mg in JT1035

Table 2. Fruit set and seed set in aneuploid apple accessions pollinated with diploid apple 'Hongro'

Seed parent (Ploidy)	No. of flowers pollinated	No. of fruits obtained (%)	No. of seeds (No. of seeds per fruit)	Average seed weigh (mg)
SJ277 (2x+1)	50	39 (78.0)	77 (2.0)	46.4
JT4 (2x+1)	5	2 (40.0)	4 (2.0)	56.5
JN811 (2x+2)	3	2 (66.7)	14 (7.0)	43.3
JF1049 (2x+2)	35	27 (77.1)	84 (3.1)	34.2
JF10 (2x+2)	60	56 (93.3)	228 (4.1)	33.2
JS6 (2x+2)	J40	34 (85.0)	85 (2.5)	34.6
JF3942 (2x+3)	10	10 (100.0)	28 (2.8)	38.4
JN6645 (2x+3)	20	15 (75.0)	48 (3.2)	23.6
JN4831 (2x+6)	10	9 (90.0)	40 (4.4)	53.7
JF1650 (2x+11)	10	4 (40.0)	9 (2.3)	36.6
JF3252 (2x+11)	38	26 (68.4)	31 (1.2)	43.8
JT1035 (2x+12)	15	11 (73.3)	7 (0.6)	15.7
Hongro (2x)**	70	68 (97.1)	550 (8.1)	66.0

* See Table 1 for aneuploid accessions. **Diploid 'Hongro' was self–pollinated.

(2n=46) × 'Hongro' cross to 56.5 mg in JT4 (2n=35) × 'Hongro' cross. The seeds were lighter than those in 'Hongro' (2x) × 'Hongro' (2x) cross (66.0 mg in average). No relationship was detected between seed weight and number of seeds per fruit. For instance, pollinated JF3252 fruit had 1.2 seeds with 43.8 mg average weight, while pollinated JN811 fruit contained 7.0 seeds with average weight of 43.3 mg.

To compare the frequency distribution of seeds with different seed weight between aneuploid accessions with different chromosome numbers, seeds were pooled for crosses involving the accessions with the same chromosome number (Fig. 1). In SJ277 (2n=35) × 'Hongro' and JT4 (2n=35) × 'Hongro' crosses, a total of 42 seeds appeared in weight range class between 50 mg and 60 mg, and a small number of seeds were weighed from 0 mg to 80 mg. In JN811 (2n=36) × 'Hongro', JF1049 (2n=36) × 'Hongro', JF10 (2n=36) × 'Hongro', and JS6 (2n=36) × 'Hongro' crosses, the number of seeds increased with increase of seed weight between 0 mg to 50 mg classes, whereas number of seed decreased rapidly with increase of seed weight beyond 50 mg classes. Fraction of seeds ranging from 50 mg to 70 mg contained a few seeds. In crosses with aneuploid seed parents with 37 chromosomes (JF3942 and JN6645), number of seeds decreased with increase of weight between 10 mg and 60 mg, and some seeds were weighed less than 10 mg. In JN4831 (2n=40) × 'Hongro' cross, number of seeds was small when their weight was less than 50 mg, whereas it was large when their weight was more than 50 mg. In JF1650 (2n=45) × 'Hongro' and JF3252 (2n=45) × 'Hongro' crosses, most seeds were weighed between 40 mg and 50 mg. Only few seeds were weighed less than 40 mg and 60–70 mg class. Most seeds appeared in the class range of 0–10 mg for the cross T1035 (2n=46) × 'Hongro', and the number of seeds decreased with increase of seed weight up to 50 mg. In the control cross, most seed were weighed between 50 mg and 90 mg. Furthermore, one seed with weight of more than 90 mg was observed. In general, the weight of seeds obtained from the crosses between aneuploid accessions and diploid 'Hongro' was lighter than that obtained from the crosses between diploid 'Hongro'.

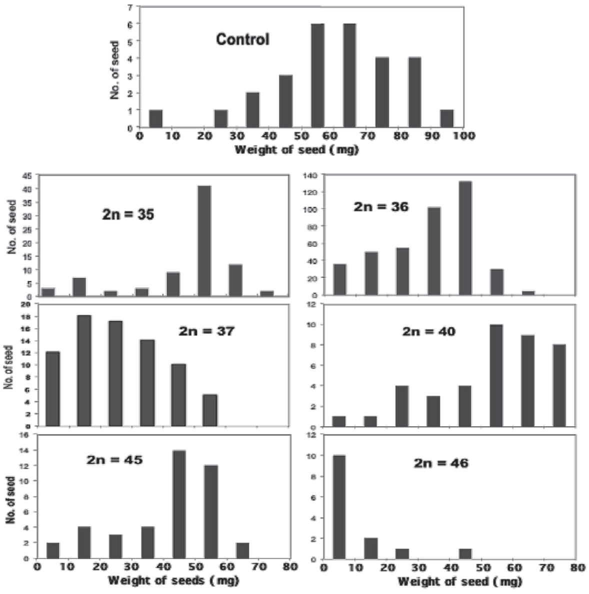

Fig. 1. Frequency distribution of seeds with different seed weight in aneuploid accessions × diploid 'Hongro' crosses. Seeds were pooled for crosses involving the seed parents with same chromosome number, i.e., seed parents were SJ277 and JT4 for 2n=35, JN811, JF1049, Jf10 and JS6 for 2n=36, JF3942 and JN6645 for 2n=37, JN4831 for 2n=40, JF1650 and JF3252 for 2n=45, and JT1035 for 2n=46. A control experiment for these is diploid 'Hongro' × diploid 'Hongro' (upper). See Table 1 for aneuploid accessions.

Aneuploid frequencies in the seedlings of pollinated aneuploid accessions

In the seedlings derived from twelve cross combinations, the metaphase figures of root tip cells demon-

Table 3. Frequency distribution of hybrid seedlings with different chromosome number. The hybrids were obtained from aneuploid accessions pollinated with diploid 'Hongro' apple

Aneuploid accession* (♀)	Aneuploidy	No. of seedlings examined	No. of seedlings with indicated chromosome number																	
			34 (2x)	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51 (3x)
SJ277	2n=2x+1=35	47	40	5													1			1
JT4	2n=2x+1=35	2	2																	
JN811	2n=2x+2=36	10	9		1															
JF1049	2n=2x+2=36	12	7	2	2	1														
JF10	2n=2x+2=36	37	28	3	1		4													1
JS6	2n=2x+2=36	11	9	1	1															
JF3942	2n=2x+3=37	5	2	1	1		1													
JN6645	2n=2x+3=37	20	12	4		3	1													
JN4831	2n=2x+6=40	4	2		1		1													
JF1650	2n=2x+11=45	6	3		1		1	1												
JF3252	2n=2x+11=45	1	1																	
JT1035	2n=2x+12=46	0																		
Total		155	115	16	8	4	8	1	0	0	0	0	0	0	0	0	1	0	0	2

*See Table 1 for aneuploid accessions.

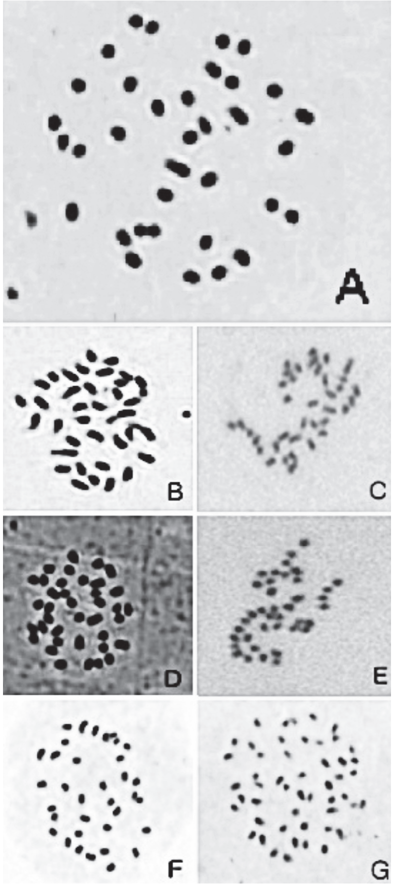

Fig. 2. Metaphase figures of chromosomes of root tip cells of seedlings derived from aneuploid apple accessions pollinated with diploid 'Hongro'. A: 2n=2x+1=35, a seedling from JS6 × 'Hongro'; B: 2n=2x+2=36, from JF1049 × 'Hongro'; C: 2n=2x+3=37, from JF1049 × 'Hongro'; D: 2n=2x+4=38, from JF10 × 'Hongro'; E: 2n=2x+5=39, from JF1650 × 'Hongro'; F: 2n=2x+14=48, from SJ277 × 'Hongro'; G: 2n=3x=51, from SJ3 × 'Hongro'. See Table 1 for aneuploid accessions.

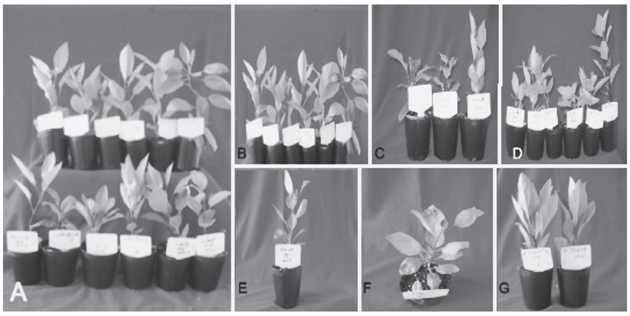

Fig. 3. Morphology of six–month–old seedlings obtained from aneuploid apple accessions pollinated with diploid 'Hongro' apple. A: aneuploid seedlings with 2n=2x+1=35 chromosomes; B: aneuploid seedlings with 2n=2x+2=36 chromosomes; C: aneuploid seedlings with 2n=2x+3=37 chromosomes; D: aneuploid seedlings with 2n=2x+6=40 chromosomes; E: an aneuploid seedling with 2n=2x+11=45 chromosomes; F: an aneuploid seedling with 2n=2x+12=46 chromosomes; G: a triploid seedling with 2n=3x=51 chromosomes. See Table 1 for aneuploid accessions.

strated that the seedlings had chromosome number ranging from diploid (2x=34) to triploid (2x=51) with the highest frequency of trisomics (2n=2x+1=35) (Table 3 and Fig. 2). Of a total of 155 seedlings examined, 117 (75.5%) were euploid and 38 (24.5%) were aneuploid. Of the 117 euploid seedlings, 115 were diploid and two were triploid. As for the aneuploids, their chromosome numbers ranged from 36 to 39, except for one aneuploid seedling with 48 chromosomes. Thus, most of the aneuploid seedlings had chromosome number close to diploid (2n=34). The number of aneuploid seedlings obtained from JF10 (2n=36) × 'Hongro' and JN6645 (2n=37) × 'Hongro' crosses were the highest eight, respectively. As for the JT4 (2n=35) × 'Hongro' and JF3252 (2n=45) × 'Hongro' crosses, no aneuploid seedlings were observed. Since the seeds derived from JT1035 (2n=46) × 'Hongro' cross were very light and a few in number, they did not grow to seedlings. Hence, their chromosome number could not be identified due to lack of normal vigorous roots. Six–month–old aneuploid seedlings are shown in Fig. 3. These seedlings were grown under standard conditions. No characteristic features were found in these aneuploid seedlings so far.

DISCUSSION

The organized aneuploid–diploid crosses and subsequent trait measurement for seeds and seedlings in the present experiment provide a variable conclusion. Although fruit set rates were more than 67% except for 40% in JT4 (2n=35) × 'Hongro' and JF1650 (2n=45) × 'Hongro' crosses, the average number of seeds per fruit was between 0.6 and 7.0. Except for 7.0 seeds per fruit in JN811 (2n=36) × 'Hongro' cross, the number of seeds in the other crosses were less than 5.0. According to this data listed in Table 2, the fruit sett rates were moderate to relatively high, but the fruit of each aneuploid accession had a few seeds. These results agreed well with the study by Zhang and Park (2009b), who found that pear fruit set did not differ when different pollen loads were used; however, the number of perfect seeds per fruit decreased drastically with decrease in pollen load, while the number of dead seeds simultaneously increased. Hence, the fruit set rate is relatively high, but the fruit derived from each cross contained a few seeds. Although each fruit had few seeds, the fruit developed well. These results were confirmed with the findings of Crane and Lawrence (1929), who suggested that in apple only a single seed is often sufficient for the development of the fruit, and even this seed may be imperfect.

In this study, the average seed number of 8 per fruit in diploid × diploid cross was higher than that of aneuploid × diploid crosses under the same cultivation conditions. It was found that the number of seeds per fruit in crosses between diploid and diploid ranged from 7 to 10 in the other studies (Matsumoto *et al.*, 2008). This indicated that the low seed number per fruit in crosses between aneuploid and diploid was related to the aneuploid seed parents themselves. The key reason of low seed number in fruits obtained from the aneuploid and diploid crosses in this study is seemed to have the relationship with the processes of megagametogenesis, pollination and fertilization.

It has been reported by Frank (1956) that seed set trait is not always correlated with pollen fertility in *Clarkia unguiculata*. He has observed one trisomic had only 14 percent stainable pollen but set 50 seeds per capsule. Richard *et al.* (1996) have reported that poor pollination reduce the number of seeds developing in fruit, and influence the cropping behavior of apple trees. The fact that the seeds obtained from aneuploids (2n=2x+1=35) × 'Hongro' (2x) crosses had light weight suggests that the light seed weight trait may be linked to genes related to underdevelopment of the endosperm and/or embryo. In apple, each of dead seeds usually contains a degenerated embryo and/or endosperm, suggesting that crosses between different ploidy often result in abnormal endosperm development and subsequent abnormal embryo development. Some researchers have attributed the ploidy barrier to the imbalance of genomic ratio in endosperm (Ozaki *et al.*, 2004). The failure of endosperm development caused by an unbalanced chromosome number is the most likely explanation for the occurrence of plants with 2x+1, 2x+2 and 2x+3 chromosome numbers in the progenies of crosses (Ozaki *et al.*, 2004).

Although the average seed number per fruit in the pollinated aneuploid accessions was far low compared with the control (diploid × diploid), the average seed weight in the pollinated aneuploid accessions was similar to the control. Jansen and Verhaegh (1993) have reported similar result. According to their suggestion (Jansen and Verhaegh, 1993), this phenomenon is probably the result of the lower seed set in these treatments, which may result in a better supply of nutrients for the remaining seeds. In addition, adverse weather conditions at the time of seed setting may also be responsible for the low seed set (Jansen and Verhaegh, 1993).

The cytogenetics of aneuploidy is a very specialized subject that deals with both theoretical and practical implications. While the doubling of the entire chromosome complement of a given plant usually has little effect on fertility, addition of a single chromosome can result in very drastic changes to the phenotype and lead to sterility. On the other hand, it is possible to replace a chromosome of one species by that of another (a so–called substitution line) and obtain a modified but often viable phenotype. All these manipulations permit us to discover what effect the chromosomes have on the phenotype and to learn more about interactions among chromosomes and the genes they carry (Khush, 1973). Aneuploids generally arise through the fusion of gametes, one or both of which are hypo–or hyperhaploid. Such gametes arise through anomalies in the meiotic division accompanying gametogenesis. The anomaly being most significant for the differentiation of gametes with deviant numbers was late disjunction or non–disjunction of chromosomes during gametogenesis (Koul and Dhar, 1998). The results obtained in this study were those expected. The chromosome number of aneuploid seedlings derived from aneuploids × 2x crosses approached to the diploid parents with the range from 35 to 39. Except for one aneuploid seedling with 48 chromosomes, the aneuploid seedlings with chromosome number between 40 and 50 were not found. Some hypotheses are able to formulate to explain these results. It is possible that, due to a non–random distribution of chromosomes during anaphase I of meiosis, extra chromosomes pass to a single pole, and lead to the production of egg cells with x+1 for aneuploid accessions with 35 (2x+1) chromosomes, x+1 and x+2 for aneuploid accessions with 36 (2x+2) chromosomes, x+1, x+2 and x+3 for aneuploid accessions with 37 (2x+3) chromosomes. Absence of the aneuploid seedlings with x+6 to x+12 chromosomes from the aneuploid seed parents with 37 (2x+6), 45 (2x+11) and 46 (2x+12) chromosomes may be attribute to the failure in megagametogenesis and/or abortion of egg cells, zygotes or embryos. In aneuploid seed parents with chromosome numbers of 35 to 37, numbers of the generated seedlings were more numerous in 35 (2x+1) than in the 36 (2x+2) and 37 (2X+3), probably because they are genetically the least unbalance (Kuspira *et al.*, 1986). These extra chromosomes in addition to the basic diploid number of 34 may represent duplications of part of the basic genome, and are perhaps repeatedly produced by certain structural heterozygotes. These facts and information at hand indicate that in crosses between aneuploid and diploid, aneuploid zygotes or embryos are viable when their chromosome numbers are close to the euploid chromosome number of 34 (2x) and 51 (3x).

In the present aneuploid–diploid cross experiments, the seeds obtained from the JT1035(2n=46) × 'Hongro' cross failed to grow to seedlings (Table 2 and Table 3). This suggests that in cultivated apple there is a high correlation between weight of seeds and the vigor of seedlings. The seeds with light weight germinate poorly with lack of vigor, whereas heavy seeds inevitably result in vigorous seedlings.

Aneuploid, including trisomics, are often morphologically distinct because of the addition of or lack of chromosomes (Ozaki *et al.*, 2004). In this study, however, aneuploid, including trisomics, grew vigorously and showed no abnormal features so far (Fig. 3). This may be due to the character of apple with relatively high patience to aneuploidy, probably because of evolutional process of polyploidization and subsequent diploidization in this genus.

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