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Haploid induction and plant production in bottle gourd by pollination with gamma irradiated pollen

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Abstract

Bottle gourd [*Lagenaria siceraria* (Molina) Standley] has been used exclusively as rootstocks for Cucurbit crops. Haploid technique increases the selection efficiency for developing resistant bottle gourd varieties. This work focused on haploid induction in bottle gourd through in situ parthenogenesis by pollination with gamma-irradiated pollen. Pollination were carried out for six genotypes of bottle gourd with 50, 75, 100, 125 and 150 Gray (Gy) doses of gamma rays. Production of *in vitro* haploid plants was influenced by irradiation dose, genotype and embyo stage. Seeds at 17 to 21 days after pollination was found to be optimum for embryo rescue. Pollination with irradiated pollen at 100 to 125 Gy was effective for haploid induction. The ploidy level of the 11 parthenogenetic plantlets was confirmed by flow cytometry and 36.3% haploid and 9% triploid plantlets were obtained. This work provides valuable germplasm resources for bottle gourd genetic analysis and breeding programs.

Key Message

Parthenogenesis in bottle gourd

Introduction

Bottle gourd [*Lagenaria siceraria* (Molina) Standley] belongs to the *Lagenaria* genus of the Cucurbitacerae family. It is a multipurpose crop species cultivated for food, medicine and domestic purposes such as making utensils, musical instruments, fishing floats and for decoration (Jeffery 1976; Roopan et al. 2016). Becides, bottle gourd has been used worldwide as rootstocks for other cucurbit crops to cope with soil-borne disease (Lee and Oda 2003). It has been documented that bottle gourd rootstocks enhanced disease resistance to *Fusarium* wilt (Zhang et al. 2021) and powdery mildew (Kousik et al. 2018); mitigated abiotic stresses, such as heavy metal, drought and salinity (Nawaz et al. 2018; Niu et al. 2018); and improved the nutrient uptake, plant growth and yield (Zhong et al. 2019). To develop resistant bottle gourd varieties, it is necessary to develop inbred lines which usually takes about 8 to 10 years by conventional breeding programme to overcome the problem of heterozygosity (Guler et al. 2017). However, producing haploid plants is of outstanding advancement which could generate pure lines in a single generation and thus improve the selection efficiency.

Haploids are known to be useful lines in plant breeding. Doubling the chromosome number of haploid plants produced homozygous lines which are valuable lines in genetic analysis and breeding programs (Kurtar and Balkaya 2010). Moreover, haploids can also be used for production of seedless triploids through somatic diploid-haploid hybridization (Luro et al. 2004) and to facilitate assembly large genome sequences in structural genomics (Aleza et al. 2009). The common techniques for haploids production were androgenesis (*in vitro* anther microspore culture), gynogenesis *(in vitro* ovule-ovary culture) and in situ parthenogenesis (*in vitro* rescue of parthenogenetic embryos pollinated with irradiated pollen) (Dal et al. 2016). Irradiated pollen is a widely used technique for in situ parthenogenetic haploids induction. The irradiated pollen is genetically inactive but germinable which can stimulate the division of the egg cell and induce parthenogenesis (Cuny 1992; Kurtar and Balkaya 2010). Since the first application in petunia (Raquin 1985), the irradiated pollen technique has been successfully used in many crops such as mandarin (Froelicher et al. 2007), winter squash (Kurtar and Balkaya 2010), bottle gourd (Guler et al. 2007) and melon (Dal et al. 2016; Hooghvorst et al. 2020). Among the Cucurbit crops, *in situ* haploids by irradiated pollen have been achieved in watermelon (Gürsöz et al. 1991; Sari et al. 1994), melon (Sauton and Dumas de Vaulx 1987; Sari et al. 1992; Lotfi et al. 2003; Hooghvorst et al. 2020), cucumber (Sauton 1989; Lotfi et al. 1999; Taner et al. 2000; Dolcet-Sanjuan et al. 2006), squash (Kurtar and Balkaya, 2010) and pumpkin (Kurtar et al. 2009). However, there was very few studies on in situ parthenogenesis induced by irradiated pollen in bottle gourd (Guler et al. 2007). An optimization in the irradiated pollen technique in bottle gourd is needed to increase the

efficiency of plant breeding. The aim of this work was to evaluate the parthenogenesis capacity of six bottle gourd genotypes and induce haploid plants through the gamma irradiated pollen technique.

Materials And Methods

Plant Materials

Six bottle gourd genotypes were used as plant material (Fig. A.1). Three genotypes were inbred lines (BG-1, BG-5, BG-6) and the other three were commercial varieties (BG-2, BG-3, BG-4). Bottle gourd seeds were grown in plastic house conditions with a temperature of 28 ^{O}C, humidity of 75% and a 16h/8 h day/night photoperiod at the Institute of Vegetable Crops, Jiangsu Academy of Agricultural Sciences in Nanjing, P.R. China.

Pollination with irradiated pollen

Male flowers were collected on the day before anthesis, excised with forceps to remove the petals and sepals. The treated anthers were placed in 90 mm petri dishes and kept in ice boxes until irradiation. The anthers were irradiated by a Co^{60} source at 50, 75, 100, 125 and 150 Gray (Gy) doses of gamma rays at Nanjing University of Aeronautics and Astronautics, Nanjing, P.R. China. The female flowers opened on the next day were isolated by holding the petals with aluminum clips to avoid any pollen contamination. In the next morning, the freshly opened female flowers were pollinated with the irradiated anthers and closed immediately with aluminum clips to avoid external pollination. Every two to three anthers were used to smear the stigma of one female flower and fruits were harvest on 15 to 21 days after pollination (DAP) and the fruit setting rate was calculated.

In vitro embryo culture

Bottle gourd fruits were washed with running tap water and dried on filter pater. The fruits were then flame-sterilized with 75% alcohol followed by disinfection with UV light for 30 min in a laminar flow cabin. Thereafter, the fruits were cut into two pieces. The seeds were extracted individually with sterilized forceps and scalpels under a Stereo Microscope (NSZ-608T, China) in a laminar flow cabin. The embryos were cultured on solid MS (Murashige and Skoog 1962) or E20A medium (Sauton and Dumas de Vaulx 1987) at 25 ± 2°C with 16h/8 h day/night photoperiod (2500–3000 lx). Types of embryos were classified and record. After 4 to 5 weeks of culture, the regenerated plantlets (7 to 8 cm) were acclimatized in a growth chamber at 25 ± 2°C under 16h/8 h day/night photoperiod (2500–3000 lx) with 75% humidity for 10 days. They were then transferred into plastic pot filled with seedling substrate (Pindstrup, Denmark) and managed in a greenhouse with a temperature of 28 ^{O}C, humidity of 75% and a 16h/8 h day/night photoperiod. Picture of the whole process were given in Fig. A.2.

Ploidy level evaluation

The ploidy level of plantlets was determined by three different methods: chromosome counting in the root tip cells, chloroplast number of the guard cells and flow cytometer. For chromosome counting method, root tips from 15-day-old seedlings were washed with sterilized water followed by treatment with 2 mM 8-hydroxyquinoline solution for 3 h at 25°C in dark. The treated root tips were fixed in Carnoy's fixative solution (acetic acid:ethanol = 1:3) for 24 h at 4°C. Thereafter, root tips were cut into pieces of 1 to 3 mm in length and digested with enzymolysis mixture (4% cellulose R-10, 2% pectinase and 2% pectolyase Y-23 in 0.01 M citrate buffer (pH = 5)) for 50 to 70 min at 37°C. The root tips were then mashed and the slides were flame-dried with one drop of Carnoy's fixative solution. Samples were counterstained with DAPI (Vector Laboratories). Images were captured by Olympus BX51 microscope (Olympus, Japan) and processed with Adobe Photoshop CS6 (Adobe System).

For observation of the chloroplast number of the guard cells, hypodermis of leaves was striped carefully with forceps, placed on microslides with one drop of 1% I2/KI solution (Lugol's iodine) and covered with micro coverslips. Chloroplast numbers of the guard cells were observed using a light microscope (Olympus, Japan) under 40×10 magnification. Five visual areas were counted for each sample.

For flow cytometer method, samples were prepared according to the instruction of the CyStain UV Precise P Kit (Sysmex Partec, Germany). In brief, a piece of 0.5 square centimeter leaves was chopped in 0.5 ml Nuclei Extraction Buffer for 60 s followed by incubation for 30 s to 5 min. Then the sample suspension was filtered through a 50 um CellTrics filter. The filtered nuclear suspension was mixed with 2.0 ml Staining Buffer for 30 to 60 s and then was analyzed by a CyFlow Space Flow Cytometer (Sysmex Partec, Germany) with a 365 nm laser. A diploid bottle gourd was used as the internal control.

Results

Effect of different irradiation doses on fruit set

The anthers of bottle gourd were irradiated with 50, 75, 100, 125 and 150 Gy gamma rays. The effect of different irradiation doses on different bottle gourd genotypes was presented in Table 1. A total of 492 female flowers was pollinated. No parthenogenetic bottle gourd fruits were obtained by pollination with irradiated pollen with 150 Gy in bottle gourd genotype BG-3. Otherwise, all irradiation doses were successful in parthenogenetic bottle gourd fruits development. Fruit set ratio (FSR) varied in different genotypes and irradiation doses. The highest FSR (78.6%) was observed with 50 Gy in BG-5 followed by BG-1 (71.4%), whereas the lowest FSR was 20% in BG-1, BG-2 and BG-5 with 150Gy.

Table 1
Effect of different irradiation doses on fruit set of bottle gourd genotypes

Genotype	Gamma ray dose(Gy)	Number of pollinating female flowers	Number of fruit set	Fruit set rate(%)	Genotype	Gamma ray dose(Gy)	Number of pollinating female flowers	Number of fruit set	Fruit set rate(%)
BG-1	50	21	15	71.4	BG-4	50	18	9	50.0
	75	21	13	61.9		75	18	8	44.4
	100	15	7	46.7		100	15	5	33.3
	125	21	11	52.4		125	18	7	38.9
	150	15	3	20.0		150	15	4	26.7
BG-2	50	15	8	53.3	BG-5	50	14	11	78.6
	75	15	6	40.0		75	14	8	57.1
	100	15	7	46.7		100	13	5	38.5
	125	15	5	33.3		125	8	3	37.5
	150	10	2	20.0		150	5	1	20.0
BG-3	50	16	9	56.3	BG-6	50	25	15	60.0
	75	16	6	37.5		75	25	9	36.0
	100	11	3	27.3		100	25	6	24.0
	125	13	3	23.1		125	25	6	24.0
	150	10	0	0		150	25	7	28.0

In vitro embryo culture and plant regeneration

A total of 40 immature fruits of bottle gourd were harvested at 15, 17, 19 and 21 DAP to assess the most suitable stage for successful embryo rescue. We found that seeds obtained from immature fruits pollinated within 15 DAP were not well developed and no embryoids were observed. By contrast, immature embryos died in fruits pollinated more than 21 DAP. Those cotyledon-shaped embryos from 21 DAP fruits were determined as diploid plants. Seeds from fruits harvested between 17 and 21 DAP were suitable for embryo rescue. They have relatively hard shell and embryoids with various shapes (Fig. 1) could be observed and used for embryo culture.

Effects of different irradiation doses and genotypes on embryo induction and plantlet regeneration was presented in Table 2. It was observed that embryo induction was correlated with both irradiation doses and genotypes. In general, the number of induced embryos was significantly decreased with the increasing concentration of irradiation doses. Meanwhile, the embryo induction varied among six genotypes. Embryos were induced at all irradiated doses in BG-4 with the highest of 70 embryos at 50 Gy irradiation doses. In BG-2 and BG-3, the highest embryo induction was observed at 125 Gy irradiation doses. One embryo was obtained in BG-6 at 150 Gy whereas no embryos were found in other five genotypes. It was also found that almost all of the induced embryos could be converted into plantlets. The maximum conversion ratio was recorded in BG-4 (21.88%) while the minimum was recorded in BG-2 (0.31%). In overall, a total of 391 plantlets were regenerated from 408 embryos.

Table 2
Effects of genotype and irradiation doses combinations on haploid production of bottle gourd

Genotype	Gamma ray dose	Number of cultured seeds	Number of induced embryos	Number of plants regeneration	Rate of plants regeneration	Number of haploid plants	Rate of haploid regeneration
BG-1	50	320	47	44	13.75	0	0
	75	320	17	15	4.69	0	0
	100	320	1	0	0	0	0
	125	320	0	0	0	0	0
	150	320	0	0	0	0	0
BG-2	50	320	4	4	1.25	0	0
	75	320	5	5	1.56	0	0
	100	320	3	1	0.31	0	0
	125	320	10	10	3.13	1	0.31
	150	320	0	0	0	0	0
BG-3	50	320	45	45	14.06	0	0
	75	320	29	28	8.75	0	0
	100	320	3	3	0.94	1	0.31
	125	320	43	43	13.44	0	0
	150	-	0	0	0	0	0
BG-4	50	320	70	70	21.88	0	0
	75	320	31	31	9.69	0	0
	100	320	33	33	10.31	0	0
	125	320	19	17	5.31	0	0
	150	320	14	13	4.06	0	0
BG-5	50	280	15	14	5.00	0	0
	75	280	9	7	2.50	0	0
	100	280	3	2	0.71	0	0
	125	280	1	1	0.36	1	0.36
	150	320	0	0	0	0	0
BG-6	50	240	3	3	1.25	0	0
	75	240	0	0	0	0	0
	100	240	1	1	0.42	0	0
	125	240	1	0	0	0	0

Note: Rate of plants regeneration = mean No. of plants regeneration/ No. of cultured seeds×100%; Rate of haploid regeneration = mean No. of haploid plants/ No. of cultured seeds×100%

Genotype	Gamma ray dose	Number of cultured seeds	Number of induced embryos	Number of plants regeneration	Rate of plants regeneration	Number of haploid plants	Rate of haploid regeneration
	150	240	1	1	0.42	1	0.42
Note: Rate of plants regeneration = mean No. of plants regeneration/ No. of cultured seeds×100%; Rate of haploid regeneration = mean No. of haploid plants/ No. of cultured seeds×100%							

Ploidy level determination

All 391 plants were determined for their haploid status using chromosome counting and 380 turned to be diploids. Ploidy level of the other 11 plantlets was further determined by flow cytometry (Fig. 2). Eleven chromosomes were visible in haploid plantlets. Plants obtained from pollination with irradiated pollen under 100 Gy gamma rays were all diploid, except for one triploid was produced from 50 Gy gamma ray treated pollen of BG-5. One haploid plant was obtained from pollination with irradiated pollen by 100 Gy gamma rays in BG-3. Two haploid plants were obtained from BG-2 and BG-5 pollinated with 125 Gy gamma ray treated pollen. Overall, four of the tested plants (36.3%) were haploid and one plant (9%) was triploid. Haploid plants generally exhibited weak vigor when compared with diploid plants (Fig. 3). Their leaves were smaller and dark green. Petals, stamens, anthers and other reproductive organs were significantly smaller. Besides, no pollen or a small amount of pollen and early flowering were found in haploid plants in comparison to diploid plants. The best dose for haploid production in bottle gourd was 100 to 125 Gy gamma ray.

Discussion

The early findings on haploid induction through irradiated pollen confirmed that the optimum dose of irradiated pollen for parthenogenesis induction varies among different plants or species. Lower irradiation doses between 25 to 50 Gy were found to be the best for haploid induction in squash (Kurtar et al. 2002). Haploids were successfully induced with doses of 200 to 300 Gy in melon (Lim and Earle 2008; Gonzalo et al. 2011; Godbole and Murthy 2012; Hooghvorst et al. 2020), watermelon (Gürsöz et al. 1991) and cucumber (Sauton 1989; Lotfi et al. 1999). Doses of 300 to 400 Gy were optimum for mandarin (Froelicher et al. 2007) and citrus (Kundu et al. 2017). Relatively higher dose of 1500 Gy was effective for haploid induction in kiwifruit (Chalak and Legave 1997). That the parthenogenetic haploid may arise with pollen grains irradiated with high irradiation doses was known as “Hertwig Effect” found in animals (Pandey and Phung 1982). The popular explanation for the diversified doses among different species was the negative correlation between radioresistance and pollen diameter (Guler et al. 2007). Successful parthenogenetic haploid induction in squash (180 um diameter) with lower doses (25 to 50 Gy) gave support for this explanation (Kurtar et al. 2002). However, a contrary result was found in *Lagenaria genus* (60 to 80 um diameter), successful haploids were induced with low doses of 50 to 75 Gy (Teppner 2004; Guler et al. 2017). The present work confirmed those findings in *Lagenaria genus*, parthenogenetic haploid plants with eleven chromosomes was obtained at lower irradiation doses 100 to 125 Gy.

Genotypes were one of the crucial effectors for a successful parthenogenesis induction. Melon genotype “Piel de Sapo” was found with lower parthenogenetic ability than other genotypes like *chinensis*, *cantalupensis* and *inodorus* (Hooghvorst et al. 2020; Lotfi et al. 2003; Lim and Earle 2008; Gonzalo et al. 2011; Dal et al. 2016). In this work, frequency of fruit set decreased with the increasing dose of irradiation. Higher fruit set ratio was observed in bottle gourd at lower irradiation doses, however, no haploid plantlets were obtained in six tested bottle gourd genotypes pollinated with irradiated pollen at lower irradiation doses up to 100 Gy (Table 1, 2). A reasonable explanation for this tendency is that lower irradiation doses partially inactivate the pollen grains, which turns to their normal germination and fertilization of the egg cells, and formation of diploid embryos (Cuny 1992; Kundu et al. 2017; Hooghvorst et al. 2020). Besides, our results showed that not all six tested bottle gourd genotypes could regenerate haploid plantlets irrespective of irradiation doses. Haploid plants were only obtained in BG-2, BG-3, BG-5 and BG-6 though with low conversion rate into plantlets (Table 3). This might be due to the differences in pollen sensitivity among different genotypes and its viability along with the irradiation exposure.

Pumpkin (Kurtar et al. 2009), winter squash (Kurtar et al. 2002) and loquat (Blasco et al. 2016) were found more sensitive to gamma-ray dose, their pollen germination was significantly affected. While in mandarin (Froelicher et al. 2007) and kiwifruit (Chalak and Legave 1997; Musial and Przywara 1998), effect of gamma ray irradiation is limited.

The efficiency of parthenogenetic embryo production was low as a total of four haploid plantlets was obtained in this work. For the maximum production of haploid plantlets from embryos pollinated with irradiated pollens, days after fecundation when the fertilized embryos were processed is essential. Previous report in bottle gourd (Guler et al. 2017) used fruits at 17 to 21 days after pollination for embryo detection, therefore, fruits at the same growth period were prior to detected. A parallel experiment was conducted to detect those fruits harvested before 17 or later than 21 days after pollination, while either no embryoids or haploid embryos were obtained. Based on this, seeds should be opened for embryo extracted in limited time period, to ensure embryos from each genotype pollinated with vary irradiation doses could be excised, we detected the same seed numbers (320 seeds) for each irradiation dose per genotype. Seeds were inspected one by one which process is laborious and time-consuming. Besides, the smooth surface of immature seeds also makes embryos excision difficult. The efficiency and effectiveness of three different methodologies for parthenogenetic embryos detection were evaluated. X-ray radiography of seeds (Claveria et al. 2005; Dal et al. 2016; Hooghvorst et al. 2020) were found the most efficient method, followed by the most commonly applied one-by-one method (Godbole and Murthy 2012; Guler et al. 2017; Kundu et al. 2017). Because of the higher contamination, the seed culture in liquid medium was the least acceptable method (Lotfi et al. 2003; Kurtar and Balkaya 2010; Hooghvorst et al. 2020). These alternative methods gave inspiration for improvement of embryo excision and haploid induction in our future research.

One unexpected triploid plantlet was found in this work. Triploids were commonly occurred naturally in *Citrus* genus, which was interpreted as the fertilization of an unreduced female gamete by a haploid pollen through a second division restitution mechanism (Luro et al. 2004; Froelicher et al. 2007). The profiles of triploid tested with molecular markers on *Curtis* (Morovec and Bohanec, 2013) are incompatible with the above generally admitted hypothesis for *Citrus*, in which the authors proposed that the triploids were the result of *in vitro* regeneration of fertilized polar nuclei from endospermal cells (Morovec and Bohanec 2013; Gilles et al. 2017). Considering the most of the triploid *Citrus* occurred spontaneously in nature, the unexpected triploid plantlet obtained in this work could be explained by the hypothesis arise by Morovec and Bohanec (2013) for *Curtis*.

Conclusion

We obtained haploid plantlets through in situ parthenogenesis in bottle gourd. The induction condition is pollination with gamma-irradiated pollen at 100–150 Gy followed by *in vitro* embryo culture at 17 to 21 DAP. These haploid plants will be valuable for genetic analysis and bottle gourd breeding programs.

Declarations

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Authors Contribution Statement

Zhao Q and Zhang M have contributed equally to this work.

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Competing Interests

There have no competing interests.

Author Contributions

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Zhao Q, Zhang M, Qi Han and Yang XP. The draft of the manuscript was written by Zhang M and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Figures

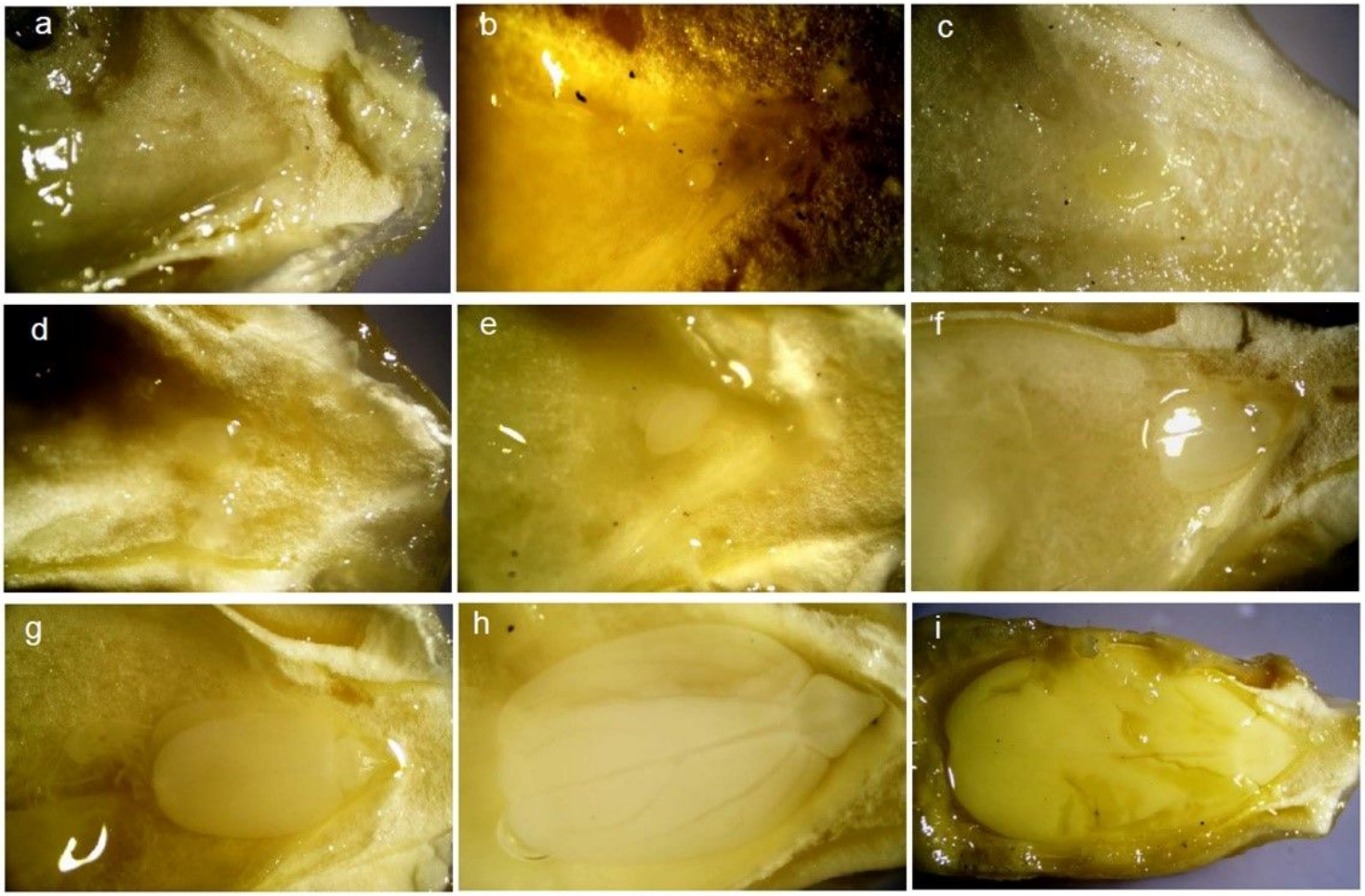


Figure 1

Different shapes of embryo in bottle gourd pollinated with irradiated pollen.

a no embryo; **b** point; **c** globular; **d** heart; **e–h** cotyledon; **i** diploid

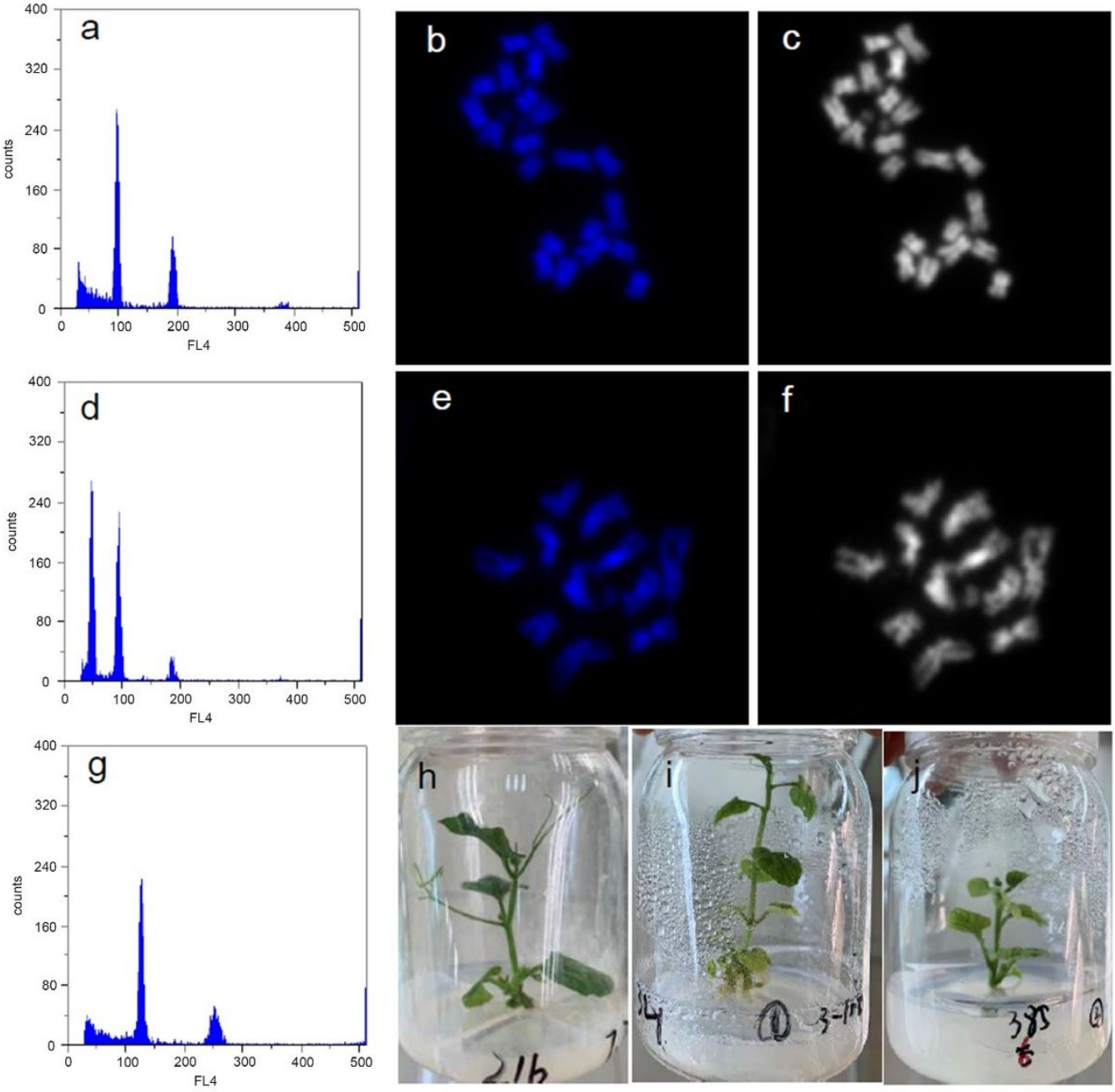


Figure 2

Ploidy level identification of regenerated plantsin BG-2 genotype

a Diploid control (Flow cytometry); b, c Diploid control 2n=22 (chromosome count); d Haploid (Flow cytometry); e, f Haploid n=11 (chromosome count); g Triploid (Flow cytometry); h, i Regenerated plants of diploid, haploid and triploid

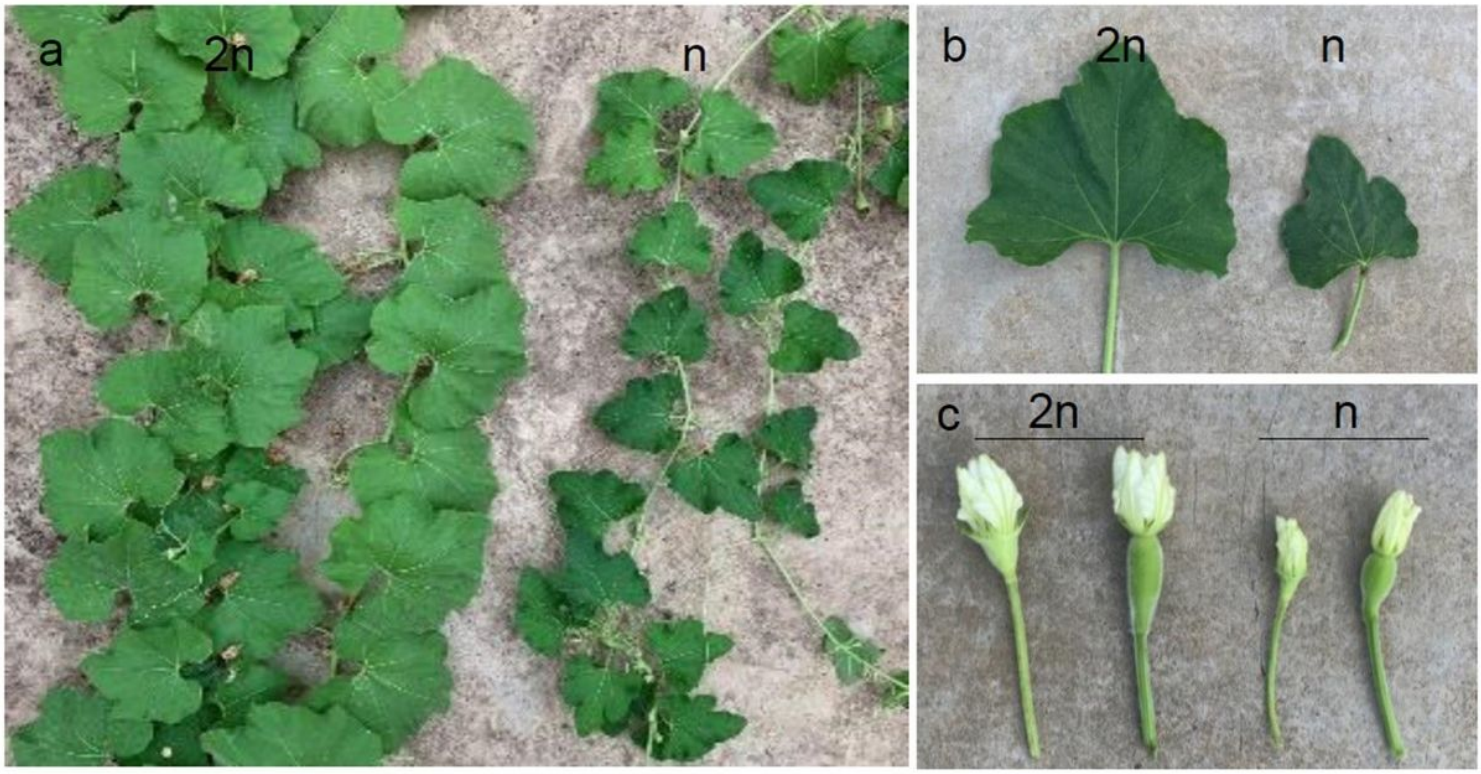


Figure 3

The morphological characteristics of haploid and diploid plants

a Diploid plants (left) and haploid plants (right) during flowering period; b Leaves of diploid (left) and haploid (right) plants; c Female and male flowers of diploid (left) and haploid (right) plants.

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