Regeneration of double haploid plants from unpollinated ovary cultures of watermelon

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Abstract

Background: Watermelon (*Citrullus lanatus*), a major fresh fruit, is planted worldwide. Because double haploid plants may be used as parents to shorten watermelon breeding cycle, the present study optimized conditions for regenerating haploid plants from ovaries without pollination.

Results: The results revealed that, the 10% sodium hypochlorite sterilized for 10 min is best for ovary enlargement. In addition, a dark culture period of 14 days promoted the ovary enlargement. The MS medium containing 0.5 mg/L NAA, 1.0 mg/L 6-BA and 0.5mg/L KT promoted the embryoid differentiation. The M2 medium containing 0.02 mg/L TDZ, 0.5 mg/L NAA, 0.5 mg/L 6-BA could be used for producing complete plants. The different genotypes affected the embryoid induction. The present study obtained regenerated plants that were established in field. Flow cytometry analyses revealed that the regenerated plants were haploid, diploid or tetraploid plant. The seedlings which were treated with culture medium can increase the chance of chromosome doubling. The SSR marker analyses showed that the diploid and tetraploid plants were homozygous at all six loci tested, indicating that these regenerated plants were double- or tetra-haploid plants.

Conclusions: Haploid and homozygous diploid can be obtained through the culture of unpollinated ovary of watermelon, which is an effective way to innovate watermelon germplasm. The present study provides homozygous plants for future watermelon breeding.

Background

Watermelon (*Citrullus lanatus)* belongs to the family Cucurbitaceae. The annual production of watermelon is about 90 million tons worldwide, and thus making it the most important fresh fruit. The center of origin of Citrullus is in southern Africa (Erickson et al. 2005). The watermelon fruits contribute to the diets of consumers throughout the world. Watermelon also comprised with important nutritional materials, such as sugars, lycopene and amino acids(Collins et al. 2007; Penelope et al. 2006; Toshio et al. 2005). The conventional breeding of watermelon using sexual hybridization is slow because many generations are required to make the breeding parents homozygous, thus it could not meet the needs of the modern market. Using homozygous double haploid plants as parents could reduce the number of generations required, thus speed up breeding programmes.

Gynogenesis, also known as plant regeneration from unfertilized female gametophytes, has been widely used to induce haploid embryos and plants via ovule or ovary cultures *in vitro.* Haploid production through in vitro biotechnological techniques allows breeders and researchers to access new methods to screen for better appearance, good tastes and resistance to diseases more quickly and efficiently. Up to now, haploid breeding has been used on several species of Cucurbitaceae family(Gemes-Juhasz et al. 2002; Min et al. 2016; Rakha et al. 2012), Cucurbita family(Kurtar et al. 2018; Wei et al. 2012), as well as other family. For example, a cucumber cultivar bred with haploid plants was released in production (Przyborowski 1996), and a brief report on Cucurbitaceae species haploid induction based on

biotechnology approaches was conducted by Ge´mes-Juha´sz and Jaksˇe (Juhász and Jakše 2005). Furthermore, many studies have paid attention to haploid production in cucumbers and other cucurbits, and provided a comprehensive review report on haploid production of Cucurbitaceae family(Gałazka and Niemirowiczszczytt 2013). The development of *in vitro* techniques for watermelon haploid production will provide progression of watermelon breeding and cultivation, which represents an especially notable achievement in the fields of biotechnology.

Even though a number of species has been successfully obtained by haploid cultivation by biotechnology techniques, the regenerated plants induction of watermelon from haploid still encounter much difficulty, and now the method still did not allowed in the breeding. It has been reported that many factors influence the induction, including maternal genotypes, physiology status donor plants, developmental stage of embryo sac, medium component and culture conditions. In the present study, haploid plants were mainly obtained through the callus pathway re-differentiation and haploids are not easy to survive and are difficult to use. Therefore, the purpose of the experiment is double haploid. We will obtain double haploid not only through the callus pathway re-differentiation but also direct embryoid. We discussed the following aspects: (1) the effects of sterilization, culture condition and hormones on ovule enlargement. (2) the effects of culture media and donor genotype on embryoid induction.(3) The regeneration plant ploidy and homozygosity. Further, the important conclusion was regarding to obtain optimal medium and culture condition for double haploid breeding of watermelon.

Results

Effects of sodium hypochlorite with different concentrations on ovule enlargement

To determine the sterile time of sodium hypochlorite on the rate of ovule enlargement, the sections of ovary were sterile for 10 or 15 min. As is presented in Table 6. Results revealed that when the sterile time was 10 min, the ratio of ovule enlargement was increased along with the increase of sodium hypochlorite concentration, and the ratio should no significant difference when the concentration was 8% and 10%. When the sterile time was 15 min, the ratio of ovule enlargement and sodium hypochlorite concentration showed negative correlation. Additionally, when the concentration of hypochlorite is 10%, and the sterile time is 10 min, the ratio of ovule enlargement showed highest (62.12%), while when the concentration of sodium hypochlorite is 10%, and the sterile time is 15 min, the ratio of ovule enlargement showed lowest (30.48%). The results indicated that when the sodium hypochlorite concentration is low, the ratio of ovule enlargement and the sterile time should positive correlation in an appropriate range. But the longer sterile time often induced lower ratio of ovule enlargement.

Effects of dark culture on ovule enlargement

To further identify the role of dark culture on ovule enlargement, the sections of ovary were cultured under different dark time. As is presented in Fig. 1, comparing with the control (no dark culture), dark culture for 7 days and 14 days significantly increased the ratio of ovule enlargement. The ratio of ovule enlargement in 7 days and 14 days showed no significant difference. The results indicated that a certain time of dark culture can promote ovule enlargement.

Effects of differentiation medium on embryoid differentiation

To explore the effects of differentiation medium on embryoid induction, 8 kinds of medium was used. As is presented in Table 7, the highest callus percentage was in DM1 medium, the highest browning mortality was in DM7 medium, the highest embryo rate was in DM5 medium.

Effects of induction medium on embryoid induction

To explore the effects of medium kinds on embryoid, the ovary sections of four watermelon cultivars were planted in 7 kinds of induction medium. The ovary sections could grow into regenerated plants and bud in five kinds of induction medium (EI1, EI2, EI4, EI5, EI6), among which, EI6 showed best effect. The ovary in EI3 and EI0 showed no regenerated plant (Fig.2, Fig.3).

Effects of genotypes on embryoid induction

To further explore whether genotype affects the embryoid, the samples sections of T02, T03 and T04were planted in EI5 and EI6 medium. As is presented in Table 8, T04 showed highest ratio (1.67%) of embryoid induction, while T02 showed no embryoid in EI5 medium. T03 showed higher ratio (1.67%) of embryoid induction, while T02 and T04 showed lower (0.83%) in EI6 medium.

The ploidy identification of regenerated plants

In the present study, the regenerated plants were obtained. To identify the ploidy of regenerated plants, the flow cytometer was employed. As is presented in Fig. 4a, the main peak fluorescence intensity of the control diploid plants was at 100.The peak fluorescence intensity of one sample was 50 (Fig. 4b), indicating the sample is haploid. The peak fluorescence intensity of the others were between 50 and 100, indicating they were haploid or the chimera of haploid and diploid (Fig. 4c).The peak fluorescence intensity of one sample was 100 (Fig. 4d), indicating the sample is diploid.The peak fluorescence intensity of one sample was 200 (Fig. 4e), indicating the sample is tetraploid..

Identification of homozygosity of the regenerated plants

A total of 83 core SSR markers that distributed in the 11 chromosomes were applied to identify the homozygosity of the diploid regenerated plants. All primers could produce clear bands. The homozygosity could be identified by six pairs of primers (the detailed primer sequences were presented in the Table 9). The results revealed that the diploid regenerated plant was homozygous (Fig. 5).

Discussion

The traditional ovary section sterilization of Cucurbitaceae plants often based on the 75% ethyl alcohol and Sodium hypochlorite, however, the appropriate sterile time and concentration of Sodium hypochlorite on different plants are vary, which could affect both the sterilization effect and the ovule differentiation. Increasingly studies have released to explore the appropriate concentration and sterile time of Sodium hypochlorite on different species. For example, 10% of Sodium hypochlorite sterile for 15 min or 10 min was used on cucumber (Cao et al. 2011; Koseki et al. 2004), 10% of Sodium hypochlorite sterile for 10 min was also used on pumpkin(Chen and Leisner 1985). However, the appropriate concentration and time of Sodium hypochlorite on watermelon ovule induction were still unclear. In our present study, we found that the high concentration with long time of Sodium hypochlorite sterilization significantly decreased the ratio of ovule enlargement, while low concentration with long time of Sodium hypochlorite sterilization promoted the ration of ovule enlargement. The results might due to that Sodium hypochlorite plays a dual role on ovule, it not only used as a disinfectant, but also suppressed the activity of ovary.

Mounting studies have reported that light is necessary on ovule induction (Campion and Alloni 1990; Chandrasekera et al. 2017; George et al. 2008; Yang and Zhou 1982). Much research has demonstrated that high temperature under dark affects the ovule or ovary regeneration in Cucurbitaceae plants (Golabadi et al. 2017; Tantasawat et al. 2015a). However, little study has performed to explore the effects of dark time on ovule enlargement induction. The previous study has identified that dark culture could decrease the production of callus and promoted gynogenesis in cucumber ovary culture (Gemes-Juhasz et al. 2002). A certain time of dark culture combined with constant temperature and light showed best effect on bread wheat ovary induction(Castillo et al. 2015). Our present study found that dark culture for 14 days showed best effect on ovule enlargement, which might indicate that dark culture could decrease the production of callus, and further promote the ovule enlargement.

Culture medium is a principal factor in controlling the gynogenesis *in vitro,* and culture media component formulation likewise contributes to the progress of gynogenic methods. The modified medium is the changeable of organic nitrogen and carbohydrates.The preliminary study has reported that the microelements, macroelements and organic elements affect the embryoid induction of cucumber, and vitamins and glycine were considered to promote the ovule enlargement(Malik et al. 2011; Min et al. 2016; Moqbeli et al. 2013; Rakha et al. 2012; Zhan et al. 2009). In the present study, we first screened the

appropriate medium for ovule enlargement of watermelon, the results revealed that M2 medium, which brimmed with organic matter, was appropriate for ovule enlargement. The result can be triggered to gynogenesis cultivation *in vitro.*

The hormones of TDZ, NAA, BA and KT are widely used in gynogenesis in vitro. TDZ, an active growth regulator, is commonly applied in induction and regeneration media. NAA, BA and KT are the key hormones for unpollinated ovule and ovary culture. The previous study has reported that the TDZ, KT showed important effect on ovule enlargement in cucumber (Dong et al. 2016), while the levels of NAA and 6-BA determined the embryoids differentiated into regenerative plants (Wei et al. 2015). Further, induction medium without TDZ or at excessive TDZ concentration often lead to low outcomes of embryos in vitro(Diao et al. 2009; Malik et al. 2011; Moqbeli et al. 2013). In the present study, the concentrations of TDZ, NAA, BA and KT affect ovule enlargement, among which, TDZ and BA showed best effect on ovule enlargement. The results indicated that the appropriate concentration of hormones is needed on ovule enlargement of watermelon.

Much research has identified that donor genotype is the main factor in affecting gynogenesis, such as rice(Gueye and Ndir 2010; Li et al. 1999) and cucumber (Tantasawat et al. 2015b). The donor plant genotype affected the haploid production was first defined in cucumber and melon in family Cucurbitaceae (Sauton 1988b) then the other cucumber species (Claveria et al. 2005; Lei et al. 2004; Lotfi et al. 1999; Przyborowski and Nlemirowicz-Szgzytt 2010; Raweerotwiboon), melon (Cuny et al. 1993; Sauton 1988a; Sestili and Ficcadenti 1996), summer squash(Baktemur et al. 2014; Kurtar et al. 2002) were also observed with this phenomenon by the reports of various scholars. While whether donor genotype affect the ovule enlargement on watermelon was still unclear. In the present study, we found that different samples in same culture media showed different ratio of ovule enlargement and embryoid induction. Additionally, the surrounding environment, such as the embryo exact month, the culture season, as well as the culture area, has been also identified to affect the outcome of gynogenesis. For example, the embryos available time for excision has been proved to be greater in summer rather than in spring and winter (Lim and Earle 2008; Przyborowski and Nlemirowicz-Szgzytt 2010).

In addition, the seedlings which were treated with culture medium can increase the chance of doubling. The medium can double the plant, but the reason is not clear.The efficiency of doubled haploid production Wheat × Maize Cross for the seedlings treated with culture medium and transplanted into pot was 67.6% and 8.6%, respectively (Chen et al. 2013).Most of seedlings transplanted into pot had no tiller and grew weakly; this is the reason for low frequency of survival plant and very low frequency of doubling after colchicine treatment.

Conclusion

The present study revealed that, the 10% sodium hypochlorite sterilized for 10 min is best for ovary enlargement. The dark culture promoted the ovary enlargement. The MS medium contains of 0.5 mg/L NAA, 1.0 mg/L 6-BA and 0.5mg/L KT promoted the embryoid differentiation. The M2 medium contains

0.02 mg/L TDZ, 0.5 mg/L NAA, 0.5 mg/L 6-BA could produce complete plants. The present study obtained regeneration plants which are tetraploid, haploid and diploid plant, respectively.The seedlings which were treated with culture medium can increase the chance of doubling.The diploid plants were homozygous double haploid plants.

Methods

The plant materials used in the present study were four F1 cultivars of watermelon. Three cultivars, named as T01, T02 and T03, were generated in Zhengzhou Fruit Research Institute, Chinese Academy of Agriculture Science. The other cultivar, T04, was obtained from Yangling Agricultural High-tech Development Joint Stock Co., Ltd. The details of the four cultivars were presented in Table 1.

Explant collection

The ovary samples were collected when the second female flower was bloomed.

The female flowers were bagged one day before they were bloomed, the robust ovaries free of diseases and pests were obtained immediately on the day. They were collected at 7:00–8:00 am.

Explant pretreatment

The collected ovaries were cleared by running water for 30 minute, sterilized using 75% ethanol for 30 second, and then peeled to remove their skin. Skinless ovarieswere sliced into 1 millimeter segments that were disinfected with sodium hypochlorite at different concentrations and. washed with sterile water for 3 times. Finally, the ovary segments were dried using filter papers and then planted in plant tissue culture medium.

Sodium hypochlorite on ovule enlargement

The medium in the present study is M2 (Table2.) + 1.0 mg/L 6-BA+0.5 mg/L NAA + 0.05 mg/L TDZ + 600 mg/L Casein acid hydrolysate + 30g/L saccharose + 6 g/L agar. T03 was used to assess the effects of the concentration (5%, 8%, 10%) and sterile time (10min, 15min) of Sodium hypochlorite on ovule enlargement (Table 3).

Effects of dark culture time on ovule enlargement

The T04 was used to assess the effects of dark culture time (dark culture at 25 °C for 0 or 7 or 14 days) on ovule enlargement.

The culture condition

The culture medium pH = 5.82–5.86. The culture condition: dark culture at 35°C for 5 days, the lighting time was 16 h/day, the light intensity was 3000 Lx. All experiments were repeated for three independent times.

Differentiation medium on induced differentiation of enlargement ovule

The enlargement ovules (enlargement on the induction medium) are detached from ovary sections and transferred to differentiation medium.

The detailed components of differentiation medium were presented in Table 4. 30g/L saccharose and 6 g/L agal were added in the medium. After 30 days, to assess the embryos, callus and browning status.

Effects of medium on embryoid induction

The basic medium was M2 medium. The induction medium was M2 medium combined with 30g/L saccharose, 6 g/L agar, four types of hormones (TDZ, NAA, 6-BA, KT) and casein hydrolysate (Table 5). All medium PH: 5.82–5.86. The samples of T02, T03 and T04 were planted in the 7 types of medium to observe the embryoid growth status.

Effects of donor genotype on embryoid induction

The ovary segments of T02, T03, and T04 were cultured on EI5 and EI6 induction medium (Table 5) containing 30g/L sucrose and 6 g/L agar. The samples were cultured in darkness at 35 °C for 5 days and at 25 °C for 10 days before they were transferred to light conditions with a photoperiod of 16 h and light intensity of 3000 Lx.

Identification the ploidy of regenerated plants

The ploidy of plants regenerated from the ovary cultures was determined by comparing with the DNA contents of the diploid donor plants using a flow cytometer (Germany PARTEC PA-Ⅱ). Briefly:

1. The young leaves of the regenerated plants were chopped and soaked with 400 μL cell lysis buffer (Partec HR-A)
2. The samples were incubated with 30 μL extracting solution, and 1.6 mL DAPI staining solution (Partec HR-B) for 2 min, and then tested on the flow cytometer.

3. The ploidy of the samples was determined by comparing the relative DNA contents of the samples and the diploid control.

Confirmation of homozygosity of regenerated plants using SSR markers

(1) DNA extraction

Genomic DNA was extracted from young leaves of regenerated plants, T04 and its parents using a improved CTAB method(Aubakirova et al. 2014). DNA quality and concentration were analyzed using 0.8 % agarose gels (Invitrogen, China) and a NanoDrop 2000 (Thermo Scientific, USA). DNA was stored at –80 °C.

(2) PCR amplification and polyacrylamide gel electrophoresis (PAGE)

PCR reactions were conducted in a 20 μL volume containing 2 μL genomic DNA (30–50 ng/μL), 16 μL of Mix (included in buffer, dNTP, Taq polymerase) and 2 μL primers. The PCR reactions were performed with the following program: initial denaturing for 5 min at 95 °C, then 35 cycles of 30 s denaturing at 95 °C, 30 s annealing at 56 °C, 1 min extension at 72 °C. An additional 10 min extension at 72 °C was allowed at the end of the program. After the reaction stopped, the PCR products were stored at 4 °C. The primers used in the present study were 6 of primer pairs (Table 9) of the 83 core SSR markers distributed in the 11 chromosomes of watermelon (Aubakirova et al. 2014).

PCR products(0.8μL) were loaded on a polyacrylamide gel(Liu et al. 2015).PAGE was run at 220 V for approximately 90min and visualized using silver staining.

Statistical analysis

These variables were square root transformed before performing analysis of variance using the SPSS software version 16.0 and Excel 2017. The data were subjected to analysis of variance (ANOVA), and mean values were separated based on Duncan's multiple range test. The detailed calculate method of several parameters were as follows:

The rate of ovule enlargement = the number of ovary segments with ovule enlargement /total segments ×100%

The rate of callus induction = the number of ovules with callus/ number of enlarged ovules ×100%

The rate of embryo formation = the number of embryoid / number of enlarged ovules × 100%

Abbreviations

MS Murashige and Skoog

NAA Naphthaleneacetic Acid

6-BA 6-Benzylaminopurine

KT 6-Furfurylaminopurine

TDZ Thidiazuron

SSR Sequence-tagged microsatellites

Declarations

*Ethics approval and consent to participate:*Not applicable

*Consent for publication:*Not applicable

*Availability of data and materials:*All data generated or analysed during this study are included in this published article.

*Competing interests:*The authors declare that they have no competing interests.

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Authors' contributions: Y. Z.,D. S.,Y. D.and J. L. designed the technical route of the article.

D. S.,Y. D.,W. L., G. A. and P. G. planted test materials and took test samples.

Y. Z. and W. S. carried on the experiment operation.

Y. Z. wrote the paper.

Y. Z. and J. L. analyzed the results and revised the paper.

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Tables

Table 1 Fruit characteristics of watermelon cultivars tested

Cultivar code	Cultivar Name	Fruit shape	Fruit size（kg）	Fruit development (d)	Fruit color
T01	Zhengkang NO.2	oval	6-9	26	red flesh
T02	Zhengkangjufeng	oval	8-12	33	red flesh
T03	Zhongke NO.2	round	5-8	28	red flesh
T04	Xinong NO.8	oval	6-9	35	red flesh

Table 2 Composition of MS and M2 (mg/L)

Composition	MS	M2
(NH4)2SO4	0	500
NH4NO3	1650	0
KNO3	1900	2000
CaCl2·2H2O	440	165
MgSO4·7H2O	370	75
KH2PO4	170	420
MnSO4·4H2O	22.3	15
ZnSO4·7H2O	8.6	10
H3BO3	6.2	7.2
KI	0.83	1.5
Na2MoO4·2H2O	0.25	0
CuSO4·5H2O	0.025	0
CoCl2·6H20	0.025	0
FeSO4·7H2O	27.8	27.8
Na2EDTA·2H2O	37.3	37.3
Inositol	100	150
Niacin	0.5	5
Vitamin B1	0.1	1
Vitamin B6	0.5	2
Glycine	2.0	5

Table 3 Treatment combinations of different concentration and sterilization time

Treatment	Concentration of sodium hypochlorite solution (%)	Sterilizing time (min)
1	5	10
2	8	10
3	10	10
4	5	15
5	8	15
6	10	15

Table 4 Composition of differentiation media*

Differentiation Medium code	NAA (mg/L)	BA (mg/L)	KT (mg/L)	GA3 (mg/L)	Adenine (mg/L)	Triacontanol (mg/L)
DM1	0.1	0.5	0	0	0	0
DM2	0.5	0.5	0	0	0	0
DM3	0.2	1.0	0	0	0	0
DM4	0.5	1.0	0	0	0	0
DM5	0.5	1.0	0.5	0	0	0
DM6	0.5	1.0	1.0	0	0	0
DM7	0	4.0	0	5.0	30	0
DM8	0	0.2	0	0	0	2.0

*The basal medium for these differentiation media was MS medium.

Table 5 Composition of embryo induction media*

Medium code	TDZ(mg/L)	NAA(mg/L)	BA(mg/L)	KT(mg/L)	casein hydrolyzed(mg/L)
EI0	0	0	0	0	0
EI1	0.02	0.5	0.5	0.5	0
EI2	0.02	0.5	0.5	0.5	600
EI3	0.04	1.25	1.5	0	0
EI4	0.01	0.25	0.25	0.25	0
EI5	0.05	0.5	1.0	0	600
EI6	0.02	0.5	0.5	0	0

*The basal medium for embryo induction media was M2 medium.

Table 6 Effects of sterilization treatments on ovule enlargement *in vitro*

Treatment	Concentration of sodium hydrochloride (%)	Sterilization time (min)	Ovule enlargement rate (%)*
1	5	10	48.87±10.33ab
2	8	10	58.57±2.86a
3	10	10	62.12±5.55a
4	5	15	57.89±7.00ab
5	8	15	40.00±6.55bc
6	10	15	30.48±4.59c

* The values in the table are averages of three replicates. The letters in the same column represent the difference significant between the values at the 0.05 probability level.

Table 7 Effects of different differentiation media on ovule differentiation

Medium number	callus percentage (%)	browning mortality (%)	embryo rate (%)
DM1	63.33±10.93bc	36.67cd	0.00b
DM2	75.00±13.23ab	25.00de	0.00b
DM3	46.67±5.00de	53.33abc	0.00b
DM4	86.67±7.26a	13.33e	0.00b
DM5	55.00±7.64cd	45.00bc	1.11a
DM6	43.33±13.02de	56.67ab	0.00b
DM7	28.89±12.95f	71.11a	0.00b
DM8	31.67±6.67ef	68.33a	0.00b

Note: The values in the table are averages of three replicates. The letters in the same column represent the difference significant between the values at the 0.05 probability level.

Table 8 Effect of genotypes on embryo induction rate

code	Medium type	Inoculated ovaries Number	Number of slices with embryo	Embryo induction rate (%)
T02	EI5	120	0	0b
T03	EI5	120	1	0.83a
T04	EI5	120	2	1.67a
T02	EI6	120	1	0.83a
T03	EI6	120	2	1.67a
T04	EI6	120	1	0.83a

Note: The letters in the same column represent the difference significant between the values at the 0.05 probability level.

Table 9 Information about the SSR primer used in detection of homozygous diploid

Marker name	Chromosomes	Sequence	
		Sense primer 5’ to 3’	Antisense primer 5’ to 3’
BVWS02102	Chromosomes1	TAAGAGAGGCCGCACTGAAT	ATCCCTAAGCTTTGCCACAC
BVWS00751	Chromosomes2	CTCCTTTTTATGCGGACCAA	TTTTTGAGCATTTGAGGAATGA
BVWS02351	Chromosomes6	ATCCAAACCCGTGTGAGAAG	AACCAAACACACGTGAAGCA
BVWS00304	Chromosomes7	TGACCTTTTTGTTGGGTAATTTTG	TTCCAATCCTATCCCCACTAAATCT
BVWS02066	Chromosomes9	TCACGTGAAGTTGAAATGGG	AAAAGTGGTGGGTGCAGAAG
BVWS01709	Chromosomes11	TGCTCTCGCTCTCATTCTGA	ACCTTCTCTTTTCCCGCAAT

Figures

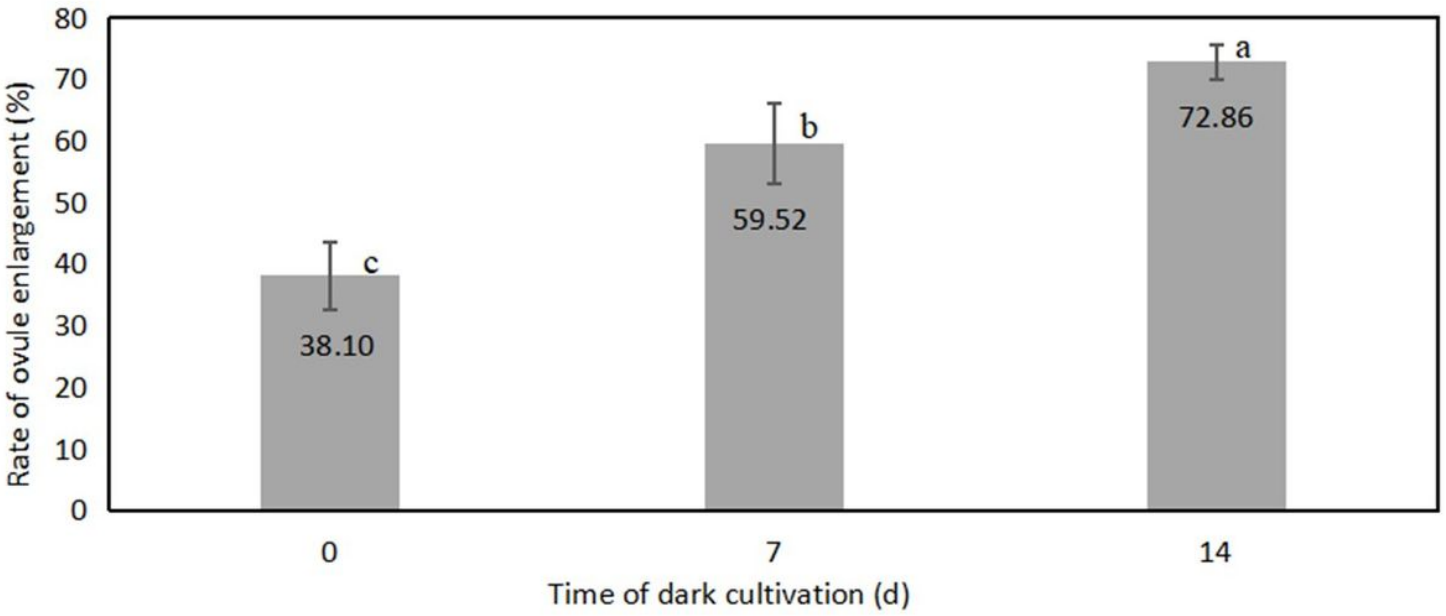

Figure 1

Effects of different dark cultivation on ovule enlargement.The letters in the same column represent the difference significant between the values at the 0.05 probability level.

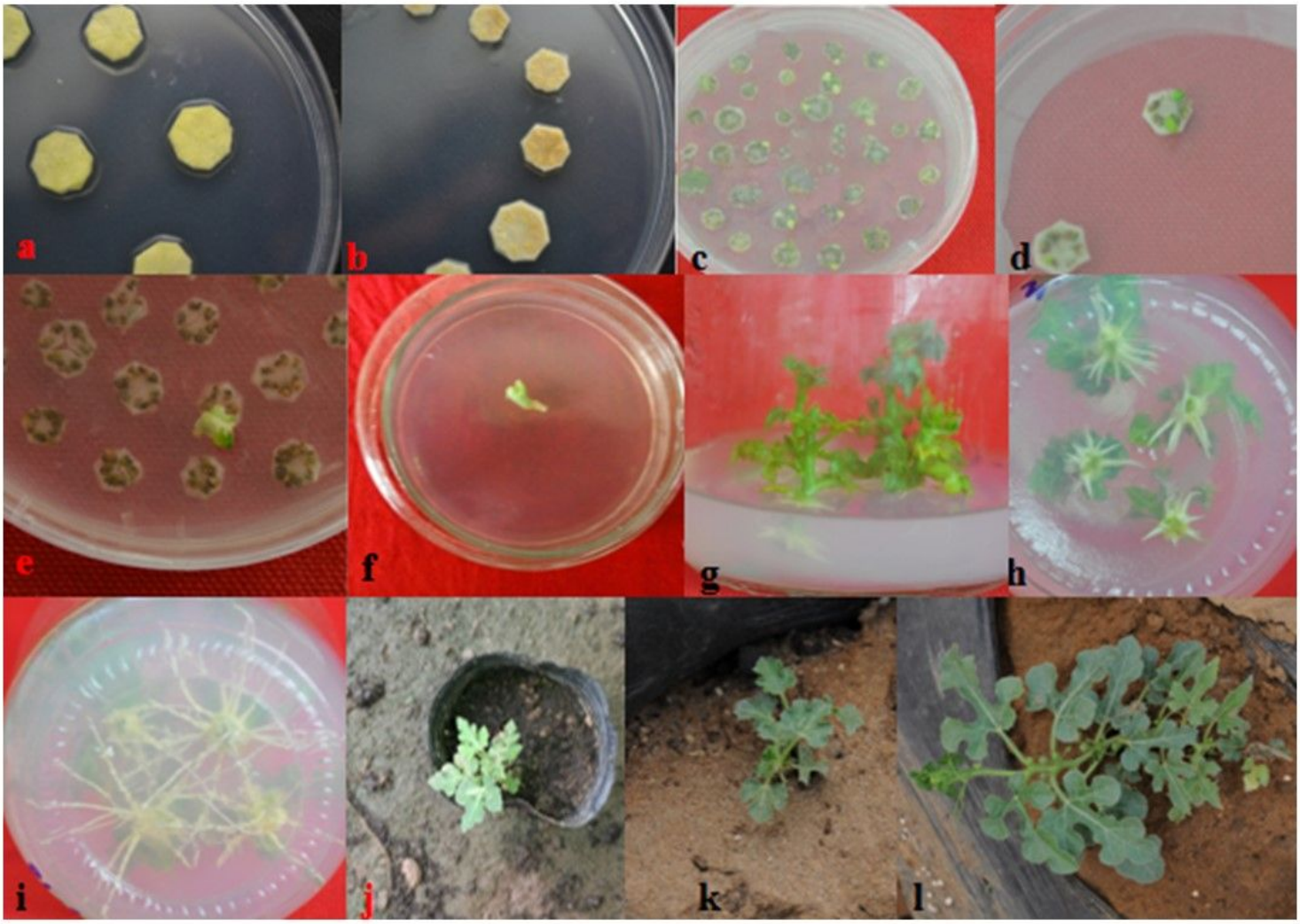

Figure 2

Different developmental stages of ovary cultures and regenerated plants.a Unpollinated ovaries at 0 day on tissue culture medium. b Ovary cultures at 3 days on medium and treated with high temperature. c Ovary cultures showing enlargement of ovules at 20 days on medium in dark for. d light turns Green and enlarged ovules at 25 days on medium under light. e, f directly induced plants at 50 days. g regenerated plants treated with strong seedlings. h regenerated plants on rooting medium for 7 days. i regenerated plants on rooting medium for 12 days. j a regenerated plant after acclimatization. k, l Regenerated plants were transplanted in a field. Note: The Petri dish used is a 90 mm culture dish. The bottle used is 60mm in diameter and 80mm in height.

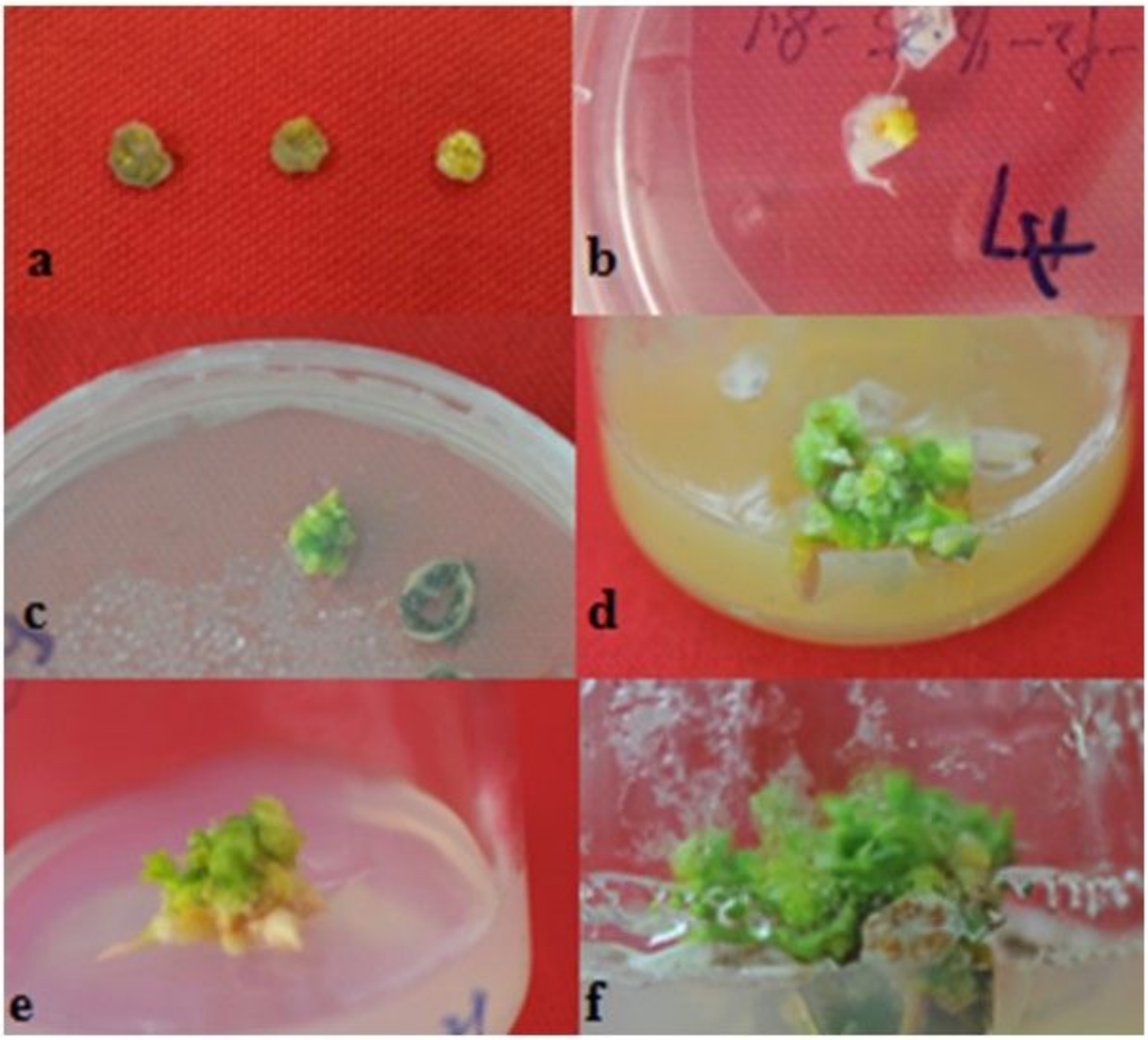

Figure 3

Other developmental pathways.a 3 different developmental situations, left this condition is directly induced into embryoid later, middle this condition is browning later, right this condition is induced embryogenic callus later. b culture of embryogenic callus on 25 days, c culture of embryogenic callus on 35 days. d, e cultivation of plants differentiated by embryogenic callus on 50 days. f regenerated plants.

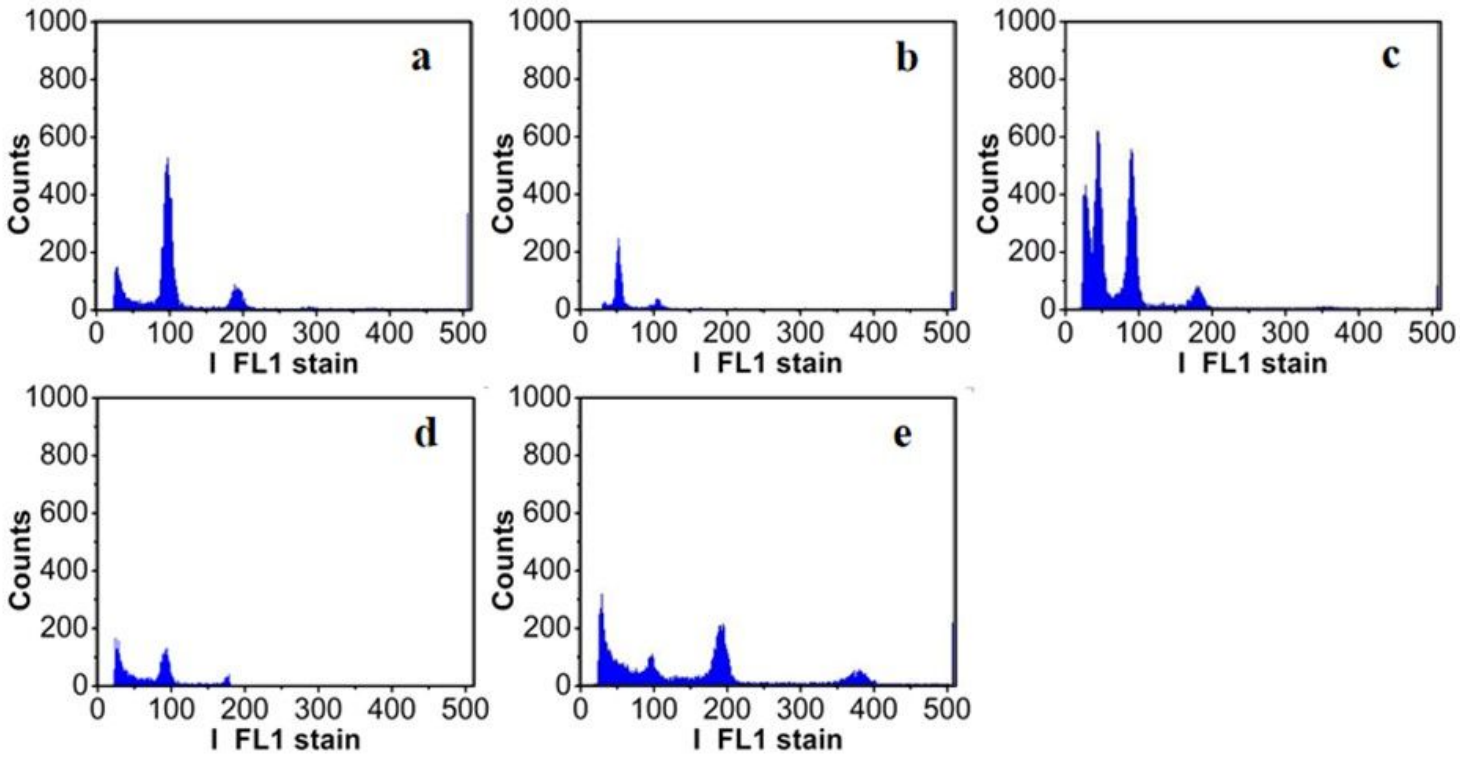

Figure 4

Flow cytometry histograms of watermelon plants showing different ploidy levels. a donor plant (2n). b regenerated haploid plant(n). c regenerated plant with mixed ploidy levels (n and 2n).d regenerated diploid plant(2n).e regenerated tetraploid plant (4n).

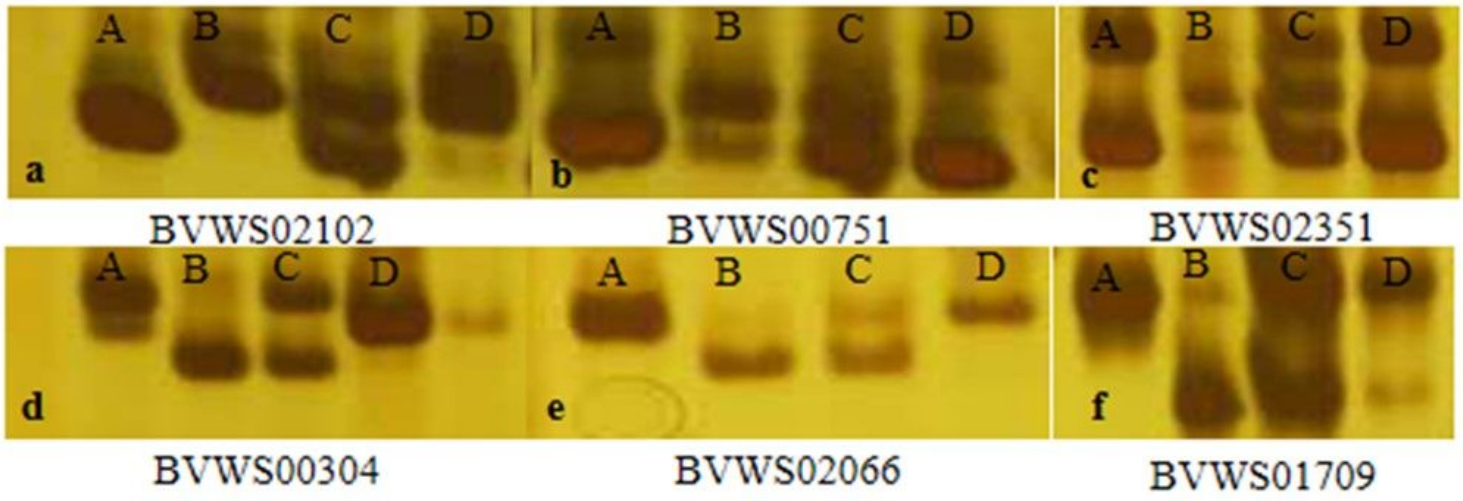

Figure 5

The results of SSR analysis. a-f polyacrylamide gel electrophoresis of microsatellites BVWS02102, BVWS00751,BVWS02351,BVWS00304,BVWS02066 and BVWS01709 showing the homozygous state of ovary culture-plants from the parents, F1 hybrids and the plants regenerated from unfertilized ovary of the

F1 hybrids population respectively. A and B: parents. C: F1 hybrids. D: plants regenerated from unfertilized ovary of the F1 hybrids